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Replicating Vaccines

A New Generation

Philip R. Dormitzer
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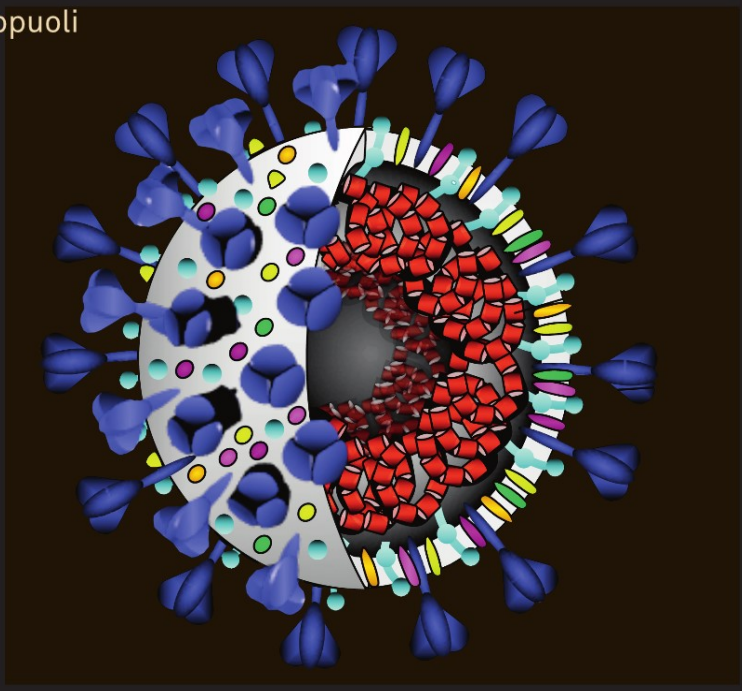

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Preface

Live vaccines were the first vaccines to be used in prevention of infectious diseases, and they are among the most successful vaccines in the more than 200-year history of modern vaccination. To just mention a few of the most prominent examples: live vaccines against smallpox (vaccinia virus, from which the term vaccine is derived), yellow fever (17D strain), polio (Sabin), and tuberculosis (Bacillus Calmette-Guérin – BCG) have literally changed the course of history. These vaccines cause mild or subclinical infections, which closely mimic natural infection by the wild-type pathogens. In many cases, this kind of vaccination elicits long-lasting and comprehensive immune responses without a need for booster immunizations or the inclusion of immune-stimulatory substances (adjuvants). However, live vaccines can often also be burdened by the threat of causing vaccine-induced disease, which may particularly affect immunocompromised individuals or result from spontaneous genetic reversions to a more virulent phenotype. These risks, even if very small, are considered increasingly unacceptable nowadays within a society that expects preventive medicine to be essentially risk-free.

Safety considerations together with an exploding increase of scientific capabilities for recombinant expression and characterization of proteins have shifted the field of vaccine science towards the development of subunit vaccines during the past decades. This development is accompanied by a rapid growth of our understanding of the innate and adaptive immune response and has led to new types of immune-stimulatory substances. These substances could overcome the limitations of subunit vaccines by shaping and enhancing the immune response. The capability to sequence entire genomes has produced the field of reverse vaccinology, which allows identification of new protein subunit vaccine components. The tremendous advances in understanding protein structure have recently created the new area of structural vaccinology, which introduces the concept of rationally designed vaccine antigens.

Do these advances in protein vaccines and adjuvant design mean that the area of replicating vaccines is coming to an end? We do not think so, and this book provides ample evidence to support this conclusion.

Essentially, the same technological advances that are propelling the development of new recombinant and subviral vaccines are also guiding the rational design of a new generation of replicating vaccines, which will combine the intrinsic immunological strengths of this type of vaccine with a flawless safety profile. Molecular biology and immunology provide a deep understanding of pathogenic determinants and pathogen–host interactions as well as the ability to specifically modify these factors. Historically, live vaccines were either derived from apathogenic natural strains or attenuated by methods of serial laboratory passages in various host cells, leading to an undirected process of genetic adaptations. The molecular mechanisms of attenuation were mostly unknown at the time these vaccines were first widely used. In fact, in many cases, the basis for attenuation of currently used live attenuated vaccines still is not fully understood. However, we now witness a quantum leap of technological capacities to specifically modify the genetic make-up of viruses and bacteria. This ability enables the generation of rationally designed live vaccines and, beyond that, the development of completely new types of replicating vaccines, such as vectored vaccines, single-round infectious vaccines, or replicon vaccines. These approaches are linked by the fact that microbial genome amplification and protein expression take place in the vaccine, but the production and spread of infectious progeny as well as the vaccines' interaction with the host defense system are specifically modified to achieve a maximum of vaccine safety and immunogenicity.

This book's intention is to span and illustrate with specific examples a large spectrum of replicating vaccines. We do not attempt to cover the entire field of new approaches. A complete enumeration would be an almost impossible goal, given the enormous wealth of creativity that shapes the development of new replicating vaccines. However, we do intend to provide the reader with a range of typical examples to paint a comprehensive picture of the existing and arising technologies. The topics included range from established or recently introduced live vaccines to novel exploratory approaches, including vectored and replicon based vaccines. In this context, we chose to apply the term "replicating" more broadly than has been done by most authors. Traditionally, "replicating" is considered synonymous with "infectious", describing a microorganism capable of infecting, multiplying, and spreading in a host. Thus, replicating, infectious, and live vaccines were clearly separated from nonreplicating vaccines such as inactivated whole virus, subunit, or subviral particle vaccines. However, an entire class of new approaches, including self-replicating nucleic acids (replicons), single-round infectious particles, or conditionally replicating agents, does not fully fit either of these two traditional definitions. These novel, rationally designed agents can undergo limited or partial processes of the microbial replication cycle, but they either do not spread to new cells or spread restricted by certain growth conditions or for only a very few replication cycles. However, all of these new approaches share the property of genome replication and protein expression in the host. Growing evidence suggests that the immune response elicited by such vaccines closely mimics that of more typical, classic live vaccines. For these reasons, we extend the meaning of "replicating vaccine" to also include vaccines that undergo partial, limited, or defective

pathogen replication cycles, and we have included such vaccines within the scope of this book, "A new generation of replicating vaccines". These new types of replicating vaccines promise to carry the successful concept of live vaccines into a new era by combining the immunological strengths of live vaccines with the safety of noninfectious protein vaccines.

The book is structured into four sections, each devoted to another group or aspect of replicating vaccines. Part I provides an overview of existing and recently introduced live vaccines, highlighting their strengths as well as some limitations and concerns. These articles illustrate both the tremendous potential for live vaccine approaches as well as the existing need for improvement with some of these vaccines.

Part II is devoted to the rational design and genetic modifications of microorganisms to generate attenuated vaccine strains. The capability to genetically manipulate bacterial and viral genomes has recently increased by technological leaps in DNA sequencing and synthesis capacities and the establishment of reverse genetics.

Part III summarizes implications of our increased understanding of host–pathogen interactions on the development of live vaccines. This includes the molecular analysis of host tropism and innate immune mechanisms. Insights into how microorganisms interact with cellular components and counteract the host cell defense mechanisms have resulted in a multitude of new approaches for attenuated strain development. These approaches include the directed alteration of host tropism, the generation of increased vulnerability to the host defense system, and the generation of microorganisms that are readily propagated in the laboratory but cause only abortive infections upon inoculation into the vaccine. Part IV highlights some of the above mentioned new types of replicating vaccines that carry the concept of live vaccines a step further. These vaccines include single round infectious particles (pseudo-infectious), vectored vaccines, replicons, and chimeric live vaccine strains.

The next decade will see some members of the new generation of replicating vaccines progress through clinical trials, achieve licensure, and benefit human health. As with all new technologies, there will be many challenges to be addressed, including issues of production, stability, safety, and efficacy. It will be exciting to watch and participate in these new developments, which ultimately will fulfill the promise of creating a safe and friendly life insurance for the twenty-first century.

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Part I
Today's Live Attenuated Vaccines

Live Vaccines and Their Role in Modern Vaccinology

Gordon Dougan, David Goulding, and Lindsay J. Hall

Abstract Since the invention of vaccination by Jenner live vaccines have been key components of immunization programs. However, in the modern era the justification for the role of live vaccines is worth re-evaluating. Here we discuss, using specific examples, about how the use and development of live vaccines will be managed in the genomics era.

1 Introduction

The use of living microorganisms as a basis for immunization has been central to the development of vaccines. Indeed, the very first recognized vaccine against smallpox, developed by Edward Jenner, was based on a live inoculum containing poxviruses. Live vaccines offer a potential advantage in that they can theoretically stimulate the immune system in a manner that more closely mimics that encountered during infection where immunity is acquired more "naturally" (Fig. 1). This is in contrast to the use of the inactivated or purified components of the infecting agent. Of course, this simplistic view does not completely hold up to the rigors of modern scientific appraisal but there are kernels of truth in this hypothesis. Indeed a significant proportion of vaccines registered for use in humans over the years are "live". However, it would be fair to say that in the modern world legislators and many scientists would prefer to replace all live vaccines with nonliving antigens. Thus, the approach is gradually falling out of favor as we learn more about how to stimulate appropriate immunity using better and more defined vaccine formulations. The trends working against the live vaccine approach include the requirement for a better

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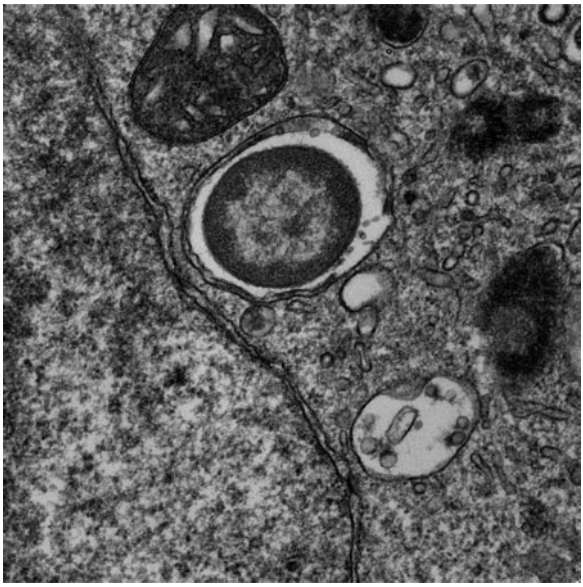

Fig. 1 A transmission electron micrograph showing attenuated salmonella living inside a human cell

definition of vaccine composition and higher safety standards. Both of these factors work against a live approach as antigen definition is significantly more challenging in a live entity and it is difficult to convince legislators and scientists alike that anything living does not have the capacity to evolve a more virulent phenotype, especially as so many modern vaccines harbor some element in an immunocompromised state. Nevertheless, many licensed vaccines are based on live antigens, including rubella and Sabin polio, and appraisal of the field is still warranted.

2 Live Vaccines, a Brief History

Originally, the microorganisms on which live vaccines were developed originated from two sources. One approach was to identify a related microbe in a host other than humans and "adapt" this to humans for vaccination purposes, sometimes using a passage through an intermediary host. The theory behind this approach is that many pathogens exhibit a phenomenon known as host restriction or adaptation, in that they are more virulent in one host species than another. In the case of Jenner's smallpox vaccine he reputedly exploited material harboring a Poxvirus adapted to cattle to immunize humans against smallpox. Indeed, he was able to demonstrate immunity to smallpox using a direct challenge [1]. The smallpox vaccine in use today is based on Vaccinia virus, which we know is related to Variola, the cause of smallpox, but is genetically very distinct. Modern molecular studies have analyzed

the differences in the genetic makeup of Vaccinia versus Variola viruses and they are indeed significant and there are, thus, many reasons why Vaccinia is attenuated in humans [2, 3]. Indeed, the Vaccinia virus used for vaccination may have acquired further attenuating mutations as it has been passaged over the years and here lies another potential problem with live vaccines, genetic drift.

The second approach is to passage a microbe that has a virulent form in humans over a long time period in an alternative host or even through some sort of in vitro system. In fact, a combination of both approaches is often employed. Indeed BCG, the current tuberculosis vaccine based on virulent *Mycobacterium bovis*, was obtained using extensive in vitro passage through laboratory media. BCG is known to differ genetically from parental *M. bovis*, although the direct ancestral *M. bovis* was lost. Genome analysis on BCG has identified a series of potentially attenuating lesions in the *M. bovis* genome. Further, it has been shown that different vaccine lots of BCG stored in different companies or geographical locations have accumulated different sets of genetic lesions, illustrating the perils of genetic drift. Indeed, this drift may in part explain differences in efficacy observed with BCG-based vaccination programs over the years [4–6].

Although the above approaches were extremely productive over the past century or so for providing a source of vaccines the approach is now obsolete and more rigorous genetic and quality control approaches will be required to generate any future live vaccines suitable for licensing. The modern science of genomics will demand a full genetic validation and a rigorous seed lot system for any vaccine for use in humans and it is from this perspective that we will continue this review.

3 A Brief Summary of the Modern Perspective

Before continuing it is worth providing a brief summary of the state of play in the live vaccine field. At the present time we have several live vaccines that are still extensively used as well as others that have a significant “track record” in humans, including several based on different poxviruses. Thus, the existing live vaccines are an accepted class that, because of a long safety record, do not have to fit into the potential requirements for any completely new live vaccine. BCG will serve as an example of such a vaccine. Even though these vaccines are likely to be used long into the future they could be subjected to a more rigorous quality regimen. For example, it is possible that genome sequencing or functional genomic studies could be exploited to improve the reproducibility of the manufacture of these vaccines at different sites and by different companies. Such an approach could be undertaken in an attempt to tackle issues such as genetic drift. Vaccine production lots could be sequenced to identify mutant lots, although such an approach would be resisted by some manufactures as a “can of worms” that could potentially be opened!

It is difficult to imagine that any new live vaccine would escape such rigorous analysis in terms of quality. Using modern approaches it will be possible to validate the genetic and biochemical make up of live vaccine lots at a level that was

previously unimaginable. Routine whole genome sequencing could be relatively easily applied to vaccine lots and even functional genetic studies such as RNA sequencing and proteomics could be applied. Further, longer term studies at a whole genome level on genetic stability (down to the accumulation of SNPs, indels and rearrangements) could be requested. What we are alluding to here, is a better validation of the genetic composition of any live vaccines. Thus, live vaccine design in the future will move towards a more rational approach.

4 Rational Attenuation

As illustrated above, scientists previously relied on natural selection to drive the generation of attenuated microbes suitable for use as live vaccines. This natural selection could have occurred truly in the wild as pathogens adapted to a lifestyle in different host species. Alternatively natural selection could have been artificially driven by scientists forcing a pathogen to adapt to a novel environment such as a laboratory growth medium. We know from modern genome studies that such adaptations can be rapid and almost continuous as different genetic and selective forces are brought to bear on genomes. For example, a range of different genetic changes accumulate as even laboratory adapted *Escherichia coli* K12 are passaged on laboratory media [7, 8]. We know from studies on the vaccine strains of polio that simple SNPs were responsible for both attenuation and the pattern of reversion to virulence as the virus was reintroduced into the human host during live vaccination [9, 10]. We also know that some viruses, particularly those with RNA-based genomes, exhibit a significant level of natural genetic flux. This flux presents challenges to defining a consensus genome and here quasi-populations have to be taken into account. Thus, taking all these factors into consideration, we can retrospectively monitor the stability of mutations as well as explore the potential value of any mutation as attenuators.

As we continue to build up our understanding of the molecular basis of virulence we increase our ability to select mutations as candidates for creating attenuated forms of any pathogen. We can explore the role that individual genes or even nucleotides play during the infection process and hence have an opportunity to interfere with infection at particular stages of the process. Obviously, this will have a knock on effect for immunity as we can explore the ongoing immune response during infection and try to identify key points at which immunity is stimulated prior to the onset of clinical disease. Pathogens will try to modulate immunity to gain an advantage in the host and we can explore methods whereby we can hijack these characteristics and modify them to self defeat the pathogen.

All of these approaches can be summarized by the term “rational attenuation”. By this, we can consider introducing defined mutations into the genome of a pathogen that generate a rationally attenuated microbe that is highly immunogenic, induces protective immunity but does so without endangering or incapacitating the recipient vaccinee. This term is useful as a definition but is not accepted by all

workers in the field as the term rational is open to philosophical scrutiny. Nevertheless, we will use it to describe modern approaches to attenuation.

5 Creating a Rationally Attenuated Vaccine

Any new live vaccine will most likely have to be fully characterized in terms of genomic architecture (at the genome sequence level) and the genetic basis for attenuation will have to be fully defined. Hence, in these cases rational attenuation will involve the generation of defined attenuating mutations on a fully sequenced genetic background. It may be conceptually difficult to achieve this goal starting with an avirulent form of microbe, such as a commensal as they are already attenuated. However, methods to demonstrate safety and a consistent level of attenuation/immunity will be required. Commensals, by their own definition, are bacteria that colonize an individual without normally causing disease. Working with these vaccine vehicles would be expected to circumvent some of the safety and environmental issues associated with wide scale immunization regimens based on genetically engineered attenuated pathogens. Some commensals, however, do have the capacity to cause disease, particularly if their host is compromised in some way [11]. Indeed, commensals which are not pathogenic in humans can harbor genes encoding potentially toxic proteins, as disease manifestation often involves the coordinated interaction of many genes, and toxins alone are not sufficient for the expression of full virulence. The use of molecular approaches such as whole genome sequencing and comparative genomics is therefore of value to identify any potential "pathogenic" loci such as those encoding toxins, which can then be removed by reverse genetics prior to their use as vaccines. In contrast, by starting with a fully virulent host microbe it should be possible to demonstrate a clear degree of attenuation using model systems but safety studies in humans will always be required.

There are now a significant number of different candidate vaccines that have been created and tested based on some form of rational attenuation. Some of these were created before whole genome sequencing became routine, but for viruses in particular, this approach has been in place for some time. There are many specific examples of such live vaccines presented in more detail throughout this volume so it is not appropriate to go through multiple examples here. The authors, instead, focus on their area of particular expertise.

6 Creating Rationally Attenuated Live Enteric Bacterial Vaccines

There has been an historical interest in the generation of attenuated enteric bacterial strains that can form the basis of live oral vaccines. This is in part driven by the observation that full protection against many mucosal pathogens in the intestine (and potentially at other body surfaces) requires some form of mucosal immunity,

often coupled with a systemic immune response [12]. Again, this is not universally accepted but it is certainly a factor that has driven the field. Enteric pathogens can target different sites in the intestine and can exhibit differences in their abilities to invade tissues. For example *Vibrio cholerae* primarily targets the small intestine whereas Shigella species target the colon. *V. cholerae* is hardly invasive at all whereas pathogens such as *Salmonella typhi*, the cause of typhoid, are highly invasive and cause primarily systemic diseases. Nevertheless, many attempts have been made to create attenuated vaccine strains with differing degrees of genetic definition.

7 Typhoid as an Example

The only licensed live oral typhoid vaccine is based on a *S. typhi* strain known as Ty21a, which was created empirically using chemical mutagenesis. Although the genetic basis of attenuation was thought by some to be a mutation in the *galE* gene it was subsequently shown that *galE* mutants of *S. typhi* can retain some significant degree of virulence and can cause typhoid [13–15]. These observations stimulated a search for a rational approach to genetic attenuation in *S. typhi* and a myriad of candidate attenuating genes have been identified leading to candidate vaccines [16–19]. One of the first rationally attenuated *S. typhi* was a strain harboring mutations in both the chorismate (aro) and purine (pur) pathways. The chorismate pathway is the only biosynthetic route by which bacteria can synthesize the aromatic ring whereas the purine pathway is required for nucleic acid biosynthesis. As the availability of aromatic compounds and purines is limiting in the human host these mutants starve in vivo and are consequently attenuated. However, *S. typhi aro pur* double mutants were found to be over attenuated and consequently poorly immunogenic in humans, properties that were reproduced in mice [20]. In contrast, *S. typhi* harboring two defined deletion mutations in the *aro* pathway were subsequently shown to be less attenuated and more immunogenic in humans [21]. Unfortunately, they were also somewhat reactogenic and viable bacteria were found in the blood of volunteers. Hence, further mutations were required to create an immunogenic but less aggressive strain.

A *S. typhi* strain known as M-01ZH09 has recently completed phase II studies as a single-dose live oral typhoid vaccine and is being prepared for an efficacy study in a typhoid endemic region. M-01ZH09 harbors mutations in the chorismate pathway (two independent *aro* mutations) and in a gene *ssaV* involved in adaptation to survival in macrophages (a gene in the Salmonella Pathogenicity Island SPI2). The SPI2 mutation (*ssaV*) was added because this mutation destroyed the ability of bacteria to survive in macrophages/blood and this addition rationally removed the vaccinaemial phenotype [17, 22]. Hence, attenuation is driven by starvation for essential nutrients and by a missing step in the pathogenic process (survival in macrophages) [18]. M-01ZH09 was created after a series of experiments in mice, in other animals and in human volunteers [17, 18, 23]. Hence, in this case a rational

approach benefited live vaccine design. A critically important feature of any live vaccine is that excessive replication and potential virulence is not present when the immunizing strain enters an immunocompromised, or indeed any form of compromised host. Hence, it would be desirable to build in mutations, which are attenuating even in the absence of a vigorous immune response. Significantly, the SPI2 mutations described above for *S. typhi* are attenuated even in severely compromised hosts such as those defective in an interferon response [18].

Similar rational attenuation approaches have been exploited with other enteric pathogens. *V. cholerae* strains lacking active cholera toxin have been created and these have shown some promise as live oral cholera vaccines [24, 25]. However, issues such as poor immunogenicity in the field have compromised the wide scale use of these vaccines, at least to date. Many laboratories have tried to create Shigella (dysentery) vaccines based on attenuated Shigella species. Although this work has been in progress in one form or another for over 50 years no vaccine of this type has been licensed. This is, in part, because it has proved impossible to design a Shigella vaccine which is both immunogenic and nonreactogenic [26]. At the moment all immunogenic vaccine candidates have proved to be too reactogenic in early human studies [27]. Thus, we need to dissect immunogenicity away from reactogenicity to move this stalled field forwards.

8 Mice Are Not Men

Moving vaccination regimens between species presents problems at the best of times but there are particular problems in the case of live vaccines. Many pathogens and even microbes in general exhibit some degree of host adaptation or even restriction. Hence live vaccines worked up in a particular model species may struggle when moved into the human target. Of course these problems are not restricted to live vaccines, a good illustration is the relative failure of DNA vaccination in humans, but it is real. Most candidate vaccines are developed to a significant degree in mice before being transferred to humans either directly or through an intermediate clinical test species such as nonhuman primates. A way around this problem is to use a surrogate vaccine species adapted to the nonhuman to develop the vaccine and then transfer the knowledge gained to the target human adapted species. A good example of this approach is with Salmonella vaccines where much of the vaccine development is performed using the surrogate species *S. typhimurium* before transferring the developed approach (including selection of attenuating mutations) into the human restricted *S. typhi*. With viruses comparators would be cattle versus human RSV, cattle versus human rotaviruses and SIV/HIV based vaccines. Surrogate approaches are fraught with potential problems but in general they are the only obvious route available where a good immunological correlate of protective immunity is not available. Host restriction barriers are generally multi-factorial and can involve immunomodulation, potentially compromising immunological readouts [28, 29]. Another property of any live

vaccine that is difficult to predict between species is reactogenicity. The level of attenuation can differ significantly between compromised hosts and unlike humans, animals cannot articulate their feelings. Careful monitoring of animals, including gait, temperature and physiology can help predict where potential reactogenicity problems might exist.

9 The Delivery of Antigens by Live Vectors

Live vaccines were originally designed to elicit protection against one species. This could be the same pathogen in terms of species, as with live polio vaccine or it could be an immunologically related pathogen as with BCG and smallpox vaccine. However, through genetic engineering it is possible to consider any live vaccine as a potential delivery vehicle for any antigen from any pathogen. This is the live vector concept. Antigens from pathogenic viruses, bacteria and even helminths have been expressed in heterologous live vaccine vehicles. The heterologous antigen can be delivered as an expressed antigen or even in the form of DNA/RNA designed to be expressed in the host [30–33]. Immunogenicity can primarily be targeted at the heterologous antigen and not the vehicle. Alternatively, when utilizing a live vector the aim can be to induce immunity against both the delivery vehicle and the targeted heterologous antigen. This is particularly attractive as you can obtain a "2-for-1" vaccine against disease.

Live vaccine delivery vehicles have been built on derivatives of viruses, bacteria or even parasites [34–36]. Many are designed to undergo a limited period of replication within the host but others are essentially nonreplicative and serve as a means to target antigen to the correct tissue or intracellular target, optimal, for example, for inducing cytotoxic T cell responses [37–39]. A further consideration for heterologous antigen delivery is the mode of expression of the heterologous antigen. Expressing a foreign antigen can impact on the competitiveness or fitness state of any replicating entity and it is important that this does not unduly impact on immunogenicity. This issue has been extensively investigated in live bacterial vaccine delivery systems. Here factors such as gene stability (chromosomal versus plasmid location), timing of expression and the eventual location of the expressed antigen (inside or outside the cell) have been considered (Fig. 2). One approach has been to exploit the use of promoters that only become significantly activated once the vaccine vehicle has entered the host (so-called in vivo inducible). This can potentially enhance stable expression and immunogenicity of foreign antigens, while reducing the metabolic load during vaccine preparation. Different delivery vehicles may require distinct forms of "fine tuning" in order to yield optimal immunogenicity [31, 40]. A good example is the use of the anaerobically induced *nirB* promoter in *S. typhi* [41].

One of the potential advantages of exploiting a live vehicle for antigen delivery is their ability to potentially induce both local and cellular immune responses in the host. Many vehicles can stimulate IgA production when delivered mucosally and

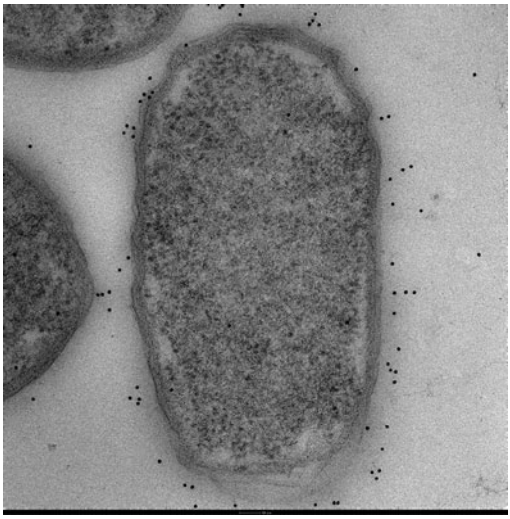

Fig. 2 A *Salmonella typhimurium* expressing the Vi capsular antigen of *S. typhi* as a clearly visible example of a bacterial vector engineered to express a heterologous antigen. The Vi antigen is detected by immunogold labeling of anti-Vi antibodies

some can also promote potent cytotoxic responses to carried antigens. These are attractive features and ones which have encouraged the more recent work on this approach to vaccination. Live vehicles have also been exploited in conjunction with other vaccination regimens, including DNA vaccination, in so called prime-boost approaches. Prime boosting has proved to be particularly useful for generating effective immune responses against challenging pathogens such as HIV, *M. tuberculosis* and malaria [33, 42–45]. One of the aims of using a prime boost approach is to obtain a mixed immunological response in the vaccine, including potentially humoral and cellular immunity or simply to bias immunity to a Th1 rather than a Th2 response [46]. An alternative approach has been to incorporate the expression of host immunological effectors or regulators such as cytokines from the vaccine vehicle, although there are specific safety issues associated with the delivery of immunologically active self antigens [47–49]. Nevertheless, this approach continues to receive attention especially within the field of cancer immunotherapy.

10 Conclusions

Live vaccine development continues to be an area of significant investigation both in the academic and industrial vaccine communities. Immunization with live vaccines can potentially stimulate key components of the cellular immune response. Safety remains an issue for serious consideration and a holistic genomic approach may be needed for the future quality control of live vaccines.

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Live Attenuated Vaccines: Influenza, Rotavirus and Varicella Zoster Virus

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Abstract Since vaccinia virus was first used to protect against smallpox in the eighteenth century, live attenuated vaccines have proved to be highly effective in reducing the morbidity and mortality caused by many human viral pathogens. Contemporary live viral vaccines are designed using several different strategies to achieve attenuation. These basic principles and approaches are illustrated by vaccines to prevent rotavirus, influenza and varicella-zoster virus infections that are described in this chapter. As shown from the experience with these three vaccines, contemporary live attenuated viral vaccines have had a major impact on disease caused by these ubiquitous human pathogens.

1 Introduction

The value of live viral vaccines was established historically by the recognition that inoculation with vaccinia virus protected against smallpox. In this case, a closely related but much less virulent pathogen of cattle elicited protective immunity in people. Contemporary live viral vaccines are designed using several different strategies to achieve attenuation. These basic principles and approaches are illustrated by vaccines to prevent rotavirus, influenza and varicella zoster virus (VZV) infections that are described in this chapter. In the case of VZV, influenza and one of the current rotavirus vaccines, attenuation is accomplished through laboratory

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manipulations of a naturally occurring "wild type" parental strain recovered from an infected individual. For one of the rotavirus vaccines, a "Jennerian" approach, similar to that used for smallpox, has been used. In all cases the goal of attenuation is to preserve sufficient replicative capacity so that the vaccine virus will induce a robust and broad adaptive immune response similar to the natural infection, but not the illness expected, after inoculation of the wild type virus. For this purpose, the attenuated virus must retain infectivity at the site of inoculation, whether the vaccine is given by oral or intranasal mucosal inoculation, as is the case for the live attenuated rotavirus and influenza virus vaccines, or by subcutaneous injection of VZV vaccines. Some live attenuated viral vaccines are associated with mild symptoms, such as fever or rash in some recipients but the tropisms of the parent virus that typically damage host cells are disrupted. VZV vaccines are derived from a clinical isolate that was attenuated by the traditional approach of sequential passage in human and nonhuman cells and by adapting the virus to grow at low temperature. The rotavirus vaccine make by GSK was also attenuated by multiple passage of wild type human rotavirus in cell culture. The other rotavirus vaccine and the influenza vaccine's attenuation relies on the inherent capacity of these viruses to undergo reassortment. This strategy can be adapted to achieve recombinants that have genes from a related, nonhuman virus as in the case of rotavirus or in which genes from virulent strains are inserted into a "backbone" consisting of genes that have acquired attenuating mutations by cold adaptation or other methods, as was done to create live attenuated influenza vaccines. The ability of the vaccine virus to replicate in the human host becomes restricted as a consequence of these laboratory manipulations. However, it is critical that live attenuated vaccine viruses retain their genetic stability, to assure both that adaptive immunity comparable to that elicited by the wild type virus is maintained and that replication in the host does not produce a reversion to the virulence of the wild type virus. Since live attenuated viral vaccine strains may be transmissible, genetic stability must also be retained despite replication in secondary contacts. Once attenuation has been achieved, the development of live attenuated viral vaccines requires defining the optimal infectious dose and dosage regimen to elicit adaptive immunity against the pathogen. Finally, their potential to provide protection against infection must be confirmed in large-scale efficacy trials. As shown from the experience with rotavirus, influenza and VZV vaccines, live attenuated viral vaccines have major benefits for reducing the morbidity and mortality caused by these ubiquitous human pathogens.

2 Influenza

2.1 Introduction

Influenza is the major cause of epidemic and pandemic severe respiratory disease in people of all ages in all areas of the world. Influenza is also an important natural pathogen of other animal species, including birds, horses and pigs. Natural infection

with wild-type influenza virus elicits a long-lasting immune response that protects the individual from influenza illness following re-exposure to the same, or very similar, strain of influenza but not from influenza strains that are antigenically distinct from the infecting strain. As influenza virus evolves it undergoes genetic changes in all its genes, including those encoding the major antigens on the virion surface [the hemagglutinin (HA) and neuraminidase (NA) glycoproteins], which are targets of protective immunity. Because the virus can undergo antigenic drift and shift, it may cause multiple symptomatic infections throughout a lifetime. Inactivated influenza vaccines were first put into use over 50 years ago for military personnel and have been in general use for more than 30 years. Although inactivated vaccines are generally safe and effective, there is room for improvement, especially in very young children and elderly adults and in situations where the vaccine strain is "antigenically mismatched" with the circulating strain. In order to address some of the deficiencies of the inactivated influenza vaccine, live attenuated influenza vaccines (LAIV) have been developed.

2.2 Virology, Epidemiology and Pathogenesis

The influenza viruses are members of the Orthomyxoviridiae family, characterized by a negative sense, single-stranded, eight segment RNA genome surrounded by a lipid membrane. The two major surface glycoproteins, the HA and NA, are inserted into the outer lipid membrane and determine the serologic classification of the specific viral strain. Three genera of influenza virus (A, B, and C) circulate in humans but types A and B cause most of the morbidity and mortality and these two types are those that are currently incorporated into the various licensed vaccines. Influenza naturally infects humans as well as several other animal species including avian species including poultry, pigs and horses. These animal hosts often serve as a reservoir for the evolution of new pandemic strains via gene reassortment interactions.

Worldwide, influenza viruses cause considerable morbidity and mortality every year, with an estimated 35,000 deaths in adults >50 years old and an average of 114,000 excess hospitalizations each year in the United States [1]. The seasonal winter outbreaks of influenza result from antigenic drifts that occur every few years in each of the three (or four) major influenza viruses, the two A strains (H3N2, H1N1), and one or more B strains, that are now circulating and the introduction of new susceptibles into the population. Influenza viruses are spread by inhalation of viral particles aerosolized by coughing and sneezing [2]. The pathogenesis of influenza virus infection begins with infection of the respiratory mucosal epithelium. Influenza is an acute febrile illness associated with myalgias, headache, cough, rhinitis, and otitis media, which is usually self-limited but can progress to pneumonia. Generally, the risk of influenza morbidity and mortality is highest in persons >65 years old, young children under 5 years, and persons with chronic cardiac or pulmonary disease or immunocompromising conditions. As was seen in

the recent pandemic of variant H1N1 virus, pandemic strains can occasionally cause increased morbidity or mortality in healthy young adults as opposed to the elderly. Because first encounters with influenza often cause lower respiratory tract infection, the hospitalization rate for influenza is 100 per 100,000 children aged 0–4 years [1] High infection rates in children of school age also facilitate influenza spread. Influenza pandemics result from antigenic shifts associated with reassortment events or emergence of new strains from avian reservoirs, as occurred in 1918, 1957, 1968 and 2009. Under these conditions, an influenza virus with an HA and/or NA that had not previously infected humans and that can infect, cause disease and be transmitted efficiently, is introduced into a large naïve population. At any given time, the potential risks of a new pandemic are virtually impossible to estimate accurately but such a pandemic constitutes a major public health emergency as occurred with the recent emergence of the novel variant H1N1 strain in 2009.

2.3 Immunology

Immune responses to a wild-type influenza infection are robust and leave the individual with a strong immunological memory that prevents the same or an antigenically similar variant from causing disease for decades. The response can be measured in many different compartments, including IgG and IgA antibodies in the serum, secretory IgA in the nasal secretions, and T, B and NK cells in the peripheral blood as well as various lymphoid tissues, especially those in the respiratory tract. Functional antibodies that neutralize the virus or prevent it from binding its cognate receptor are designated hemagglutination inhibiting (HAI) antibodies and can be found in the serum and occasionally in nasal secretions. Cellular immune responses and additional antibody responses target a variety of regions on the viral HA and NA surface glycoproteins and other proteins encoded by the virus, particularly M, NP, and NS. The quantity of HAI and the amount of neutralizing antibody in serum have been correlated with the extent of protection from disease; some evidence indicates that the serum titer of antibodies to NA is also correlated with protection. Despite the presence of these multiple components of immunological memory and effector function and substantial information correlating some of these responses with protection, the fundamental role each has in preventing illness following re-exposure to influenza remains to be elucidated.

The immune response to inactivated influenza vaccine has been extensively studied [2]. The immune response to vaccination with LAIV has been studied in several different settings and the immune response is qualitatively similar but quantitatively less than that elicited by natural infection. After LAIV immunization, mucosal IgA, serum HAI, and neutralizing antibodies and cellular T and B cell responses are observed. Lower responses are not surprising given that the vaccine stimulates immunity by replication in the upper respiratory tract, the site of replication of the wild-type virus. The level of replication of the LAIV strain is significantly reduced compared to that of wild-type virus. Despite evidence for

vaccine-induced immunity in both local and systemic compartments, the specific functional role of any particular immune response and validated correlates of LAIV-induced protection from influenza disease in vaccinated individuals have not been defined.

LAIV elicits the most robust immune response in young children, particularly those that are seronegative for influenza at the time of vaccination [3–5]. Seroconversion rates, measured by the presence of HAI antibody, are as high as 80–90% in young children after two doses of vaccine. Seroconversion rates are lower for children or adults that have preexisting antibody at the time of vaccination. The presence of antibody at the time of immunization may limit the extent of replication of the vaccine in the upper airways, evidenced by lower rates of shedding, and may mask the boosting of the immune response using relatively crude measures of immunogenicity such as HAI antibody in the serum. In children, LAIV induces nasal secretion of IgA and production of circulating IgG and IgA antibody secreting cells (ASC) 7–10 days after immunization [6, 7]. Children 6–36 months of age have measurable IFNγ-secreting cells in their PBMCs by 13 days after LAIV; these responses were not evident in children vaccinated intranasally with heat-inactivated LAIV or intramuscularly with inactivated vaccine [8]. In a study of children aged 5–9 years, blood was collected at 10 and 28 days post vaccination and stimulated with the A/H3N2 strain ex vivo. Both CD4 and CD8 influenza-specific T cell frequencies were increased in these children compared to their prevaccination values. These increases were greater than those observed for children vaccinated with the seasonal trivalent inactivated influenza vaccine (TIV) in the same study and the $CD8^+$ T cells induced by LAIV underwent a number of specific phenotypic changes [9–11].

Vaccine studies often rely on correlate markers to demonstrate that the vaccine will perform as expected under the conditions being studied. A robust correlate of protection is an immunological marker that when present coincides with protection from disease upon subsequent exposure to the wild-type virus and the lack of which correlates with susceptibility to illness. Due to high rates of efficacy demonstrated for LAIV combined with the difficulty in using traditional serum-based influenza assays to measure an immune response in adults, these markers have been difficult to identify for LAIV. Virtually all adults have had multiple encounters with wild-type influenza and/or have been vaccinated for influenza. Thus, most adults have readily measurable levels of influenza antibody in their serum prior to vaccination. In contrast to studies in young seronegative children, vaccination of adults with LAIV does not usually produce a measurable increase in serum HAI antibody titers. Recent studies on T cell immunity following vaccination showed similar results [9, 11]. The levels of prevaccination influenza-specific CD4 and CD8 cells increase with age of the subjects and adults have significantly higher baseline quantities than children [11]. The level of prevaccination influenza specific CD4 T cells seems to be a critical determinant of whether or not vaccinees experience a subsequent rise in either CD4 or CD8 T cells. In contrast to the T cell and HAI responses, adults generally have increased influenza-specific antibody-secreting B cells in the blood 7–10 days post LAIV vaccination. Although only 16% of adults have a serological

response measured by a fourfold or greater increase of HAI antibody following immunization, approximately 80% of the subjects have a measurable increase in the number of influenza-specific IgG-secreting antibody-secreting cells in the peripheral blood. This was true for individuals who had been vaccinated in the prior year as well as those who were not [6, 7]. These data demonstrated that LAIV elicits a readily detectable B cell response in most adults, which is consistent with the clinical experience that LAIV is highly efficacious in an adult population aged 18–49 [12, 13].

2.4 *Vaccine Development, Composition, and Mechanism of Attenuation*

Development of live, attenuated vaccines based on the cold-adapted (*ca*), attenuated *ca* A/Ann Arbor/6/60 and *ca* B/Ann Arbor/1/66 backbones has spanned several decades. The vaccine contains three vaccine strains, two attenuated influenza A strains and one attenuated influenza B strain. These vaccine strains are genetic reassortants each harboring two gene segments from the currently circulating wild-type virus conferring the appropriate antigens (e.g., A/H3N2, A/H1N1, or B) and six gene segments of live, attenuated influenza A or influenza B donor virus or master donor virus (MDV). The resulting 6:2 genetic reassortant combines the attenuation inherent to the MDVs with the antigens needed to elicit a neutralizing immune response that should prevent disease caused by currently circulating strains of influenza. LAIV is used for active immunization of subjects from ages 2 through 49 and is currently manufactured in specific pathogen-free embryonated chicken eggs. The three constituent attenuated influenza A and B strains are blended and filled into sprayer devices used to deliver the vaccine liquid into the nasal passages.

In the 1960s, investigators set out to attenuate influenza virus for vaccine use through a process designated as cold-adaptation. Forcing the virus to replicate efficiently at lower than normal temperatures resulted in changes to its genetic makeup making it less fit to replicate at normal and elevated body temperatures, thereby attenuating the strain. Biological characterization of *ca* A/Ann Arbor/6/60 and *ca* B/Ann Arbor/1/66 demonstrated that the resulting viruses are cold adapted, as defined by ability to replicate to titers at 25°C that were similar to titers obtained at 33°C. The strains are also temperature sensitive (*ts*), as defined by replication of the virus at 39°C that was debilitated compared to its replication at 33°C [14]. The spectrum of temperatures at which the *ca* virus replicated well was lower than the wild-type viruses that caused disease. Further characterization of *ca* A and B/Ann Arbor strains in the highly susceptible ferret model demonstrated that these strains were attenuated (*att*) compared to wild-type influenza viruses and were unable to replicate in the lung tissues of ferrets or elicit signs of influenza-like illness (ILI) [15]. These two strains provide the genetic background of all LAIV strains, imparting their *ca*, *ts*, and *att* properties to the vaccine.

Sequence analysis and comparison of the genomes of *ca* A/Ann Arbor/6/60 and *ca* B/Ann Arbor/1/66 to their respective parental strains confirmed that a number of changes had accumulated during cold passage. The introduction of reverse genetics enabled biological traits to be associated with specific nucleotides without having to account for potential problems caused by constellation effects. Five nucleotide positions distributed between the PB1, PB2, and NP gene segments of A/Ann Arbor/6/60 controlled both the *ts* and *att* properties [16]. Studies with B/Ann Arbor/1/66 revealed that three positions (two in PA and one in NP) control the *ts* phenotype, an additional two nucleotides in M control the *att* phenotype, and another subset of three changes in PA and PB2 are responsible for the *ca* phenotype [17, 18]. When the minimal set of mutations are made in divergent influenza strains the biological traits transferred; thus, the fundamental mechanisms restricting the replication of these vaccine strains at elevated temperatures are a result of the complex genetic signatures that affect multiple points of the replication cycle to provide a robust and stable set of attenuating changes to the viruses.

Following intranasal administration, the vaccine virus infects and replicates in epithelial cells of the upper respiratory tract resulting in an immune response. Characterizing the genetic stability of the vaccine in humans was important for understanding the properties of the vaccine. In a study of genetic stability, 98 children, 9–36 months of age, were vaccinated with LAIV and nasal swab samples were taken at frequent and regular intervals. Of the children in the study, 86% shed at least one of the three strains in the vaccine. The *ca* and *ts* phenotypes were preserved in all the shed viruses tested [19]. Of the isolates, 54 were chosen at random and their genomes sequenced in their entirety and compared to the sequences of the strains used to vaccinate the children. These analyses revealed that some genetic changes occurred in a majority of shed isolates and in some cases the mutations were shared by multiple isolates [20]. Interestingly, in most cases, the change(s) evident in the virus that was shed were representative of changes that existed in the bulk vaccine material. Despite the presence of these mutations, all isolates invariably retained their characteristic biological attenuated properties.

A corollary concern associated with genetic stability and vaccine shedding is the potential for person-to-person transmission of the virus. The study of the genetic stability of the vaccine in children was also designed to assess the probability of transmission of the vaccine virus. In addition to the 98 children vaccinated with LAIV, 97 children attending the same day care center received placebo. Nasal swabs were obtained at frequent intervals from each child and the presence of vaccine virus was assessed. Vaccine virus was recovered from 80% of the vaccinated children and from only one placebo recipient [19]. The influenza B vaccine virus recovered from the placebo recipient was shed on only 1 day. The transmitted vaccine isolate was shown to retain its characteristic *ca* and *ts* properties and exhibited the attenuation phenotype in ferrets and the placebo child from whom LAIV was recovered had no symptoms of influenza. These results indicated a 0.58% probability of vaccine transmission occurring from a single contact of a vaccinated young child with an unvaccinated young child [19].

The likelihood of transmission from a vaccinated adult is expected to be substantially lower because LAIV shedding is much less than in children.

2.5 Safety and Efficacy

Vaccines derived from *ca* A/Ann Arbor/6/60 and *ca* B/Ann Arbor/1/66 have been extensively characterized in clinical studies. Prior to the mid 1990s, monovalent and bivalent forms of these vaccines were evaluated in over 15,000 subjects in a number of different clinical studies, many sponsored by the NIH [21]. Studies of commercially produced, frozen and refrigerator stable, trivalent formulations of LAIV (Flumist®, MedImmune) have been conducted in a wide range of settings in individuals from 6 months to over 80 years of age. These studies have been done both before and after licensure.

LAIV has reproducibly prevented ILI caused by all three currently circulating influenza types. A meta-analysis of placebo-controlled studies showed that the mean efficacy of two doses in previously unvaccinated young children was 77%, with efficacies of 85%, 76%, and 73% against A/H1N1, A/H3N2, and B, respectively. The mean efficacy of one dose in previously vaccinated children was 87% [8, 22–30]. A single dose of vaccine, while not optimal, has been shown to provide a high degree of clinical efficacy among previously unvaccinated young children [26, 31].

Three studies were conducted in which LAIV and TIV were compared. The largest of these included over 8,000 children. LAIV was shown to reduce the burden of illness by nearly 55% compared to TIV. Of note, the A/H3N2 strains circulating in this study were antigenically mismatched to the two vaccines and the children vaccinated with LAIV had 79% fewer cases of modified ILI compared to the TIV group [24]. In two other studies, one conducted in children with recurrent respiratory illness and the other in older children with asthma, LAIV was also shown to be more efficacious than TIV [22, 27]. Immunity elicited by LAIV may provide for a larger margin of error for antigenic mismatching than occurs after inactivated vaccine administration. LAIV has been shown to provide protection against significantly antigenically drifted variants in several clinical settings. In 1997–1998, children were immunized with a trivalent blend of LAIV containing the A/Wuhan/359/95 (H3N2) strain. The H3N2 virus that subsequently circulated was A/Sydney/05/97, which has an H3 that is quite distinct from the antigen contained in the vaccine. Despite this mismatch, the vaccine conferred efficacy greater than 85% against the A/Sydney/05/97 virus [24]. During the same season, LAIV was also shown to protect adults against the drifted H3 strain [12]. In a direct comparison study of LAIV and TIV in children, LAIV reduced modified ILI caused by an antigenically drifted A/H3N2 strain by 79% compared to TIV [23].

Two placebo controlled field studies in adults have been reported using either effectiveness endpoints [12] or culture confirmed prevention of ILI in adults 60 years or older [32]. In a series of field studies in young adults, TIV was more

efficacious than LAIV; however, both groups suffered less illness than observed in the placebo group. In a study conducted in the 2007–2008 influenza season with 1,952 subjects, the inactivated vaccine was shown to have an efficacy of 72% compared to a placebo and LAIV had an efficacy of 29% compared to a placebo [33–35]. These two vaccines have also been studied in military personnel. In a retrospective cohort analysis, LAIV was more effective than TIV at preventing influenza illness in recruits and TIV was slightly more effective in nonrecruits [36]. In an analysis of more than a million nonrecruits, TIV was more effective at lowering health care encounters for pneumonia and influenza than LAIV and the latter was shown effective in only one of the three seasons analyzed. However, LAIV was effective in the subset of vaccine-naïve service members at levels similar to TIV [37]. The less robust results of these studies in adults compared to studies in children, where LAIV has appeared to be more efficacious than TIV, may reflect the interaction of LAIV with the already flu-experienced immune system of the adult host. Presumably the higher level of preexisting immunity in adults is more restrictive for immune responses to the replication dependent LAIV than for the parenterally administered, non replicating TIV.

Safety is obviously a critical issue when evaluating a live viral vaccine. In controlled studies, the most common adverse events in children 2 years of age or older given LAIV were runny nose or nasal congestion, low-grade fever, decreased activity and decreased appetite. In the youngest children, who received two doses of vaccine, no significant differences were observed following the second dose. In adults, the most common adverse events were runny nose/nasal congestion, cough, and sore throat, which were all short lived. In a large safety database study using the Northern California Kaiser Hospital system, a 3.5-fold increase in asthma events was noted within 42 days of vaccination in the prespecified age stratum of 18–35 months [38]. The observation was further investigated in the large efficacy study of LAIV and TIV in young children. In the age stratum less than 24 months of age (6–23 months), 3.2% of children in the LAIV group had medically attended wheezing events within 42 days of vaccination compared to 2.0% in the TIV group. This difference was significant. There was no significant difference in rates after 42 days in this age group or in the children 24 months of age or older [1, 23]. These finding led to the licensure of LAIV for children over the age of 2 years.

2.6 Issues for the Future

The utilization of the live virus vaccine technology continues to be refined and improved. Recent studies in children should encourage greater use of this vaccine in this highly susceptible and vulnerable population. The current manufacturing methods used to make LAIV, like those used to produce the inactivated vaccine, are based on production technologies that are over 50 years old. More modern production methods, including manufacturing in cell culture substrates, are needed

and are being developed. In addition, the generation of the 6:2 reassortant viruses used to initiate vaccine seed strain production is being refined and integrated with the use of reverse genetics technology. Finally, the attributes that make this vaccine effective in young children are being further explored and LAIV strategies are being developed for pandemic solutions where the advantages over inactivated TIV for producing large amounts of vaccine are substantial.

3 Rotavirus

3.1 Introduction

Rotaviruses are the most frequent cause of severe diarrheal disease in young children worldwide and are also ubiquitous enteric pathogens of many other mammalian and avian species. By 5 years of age, 1 in 50 children worldwide will have been hospitalized and 1 in 205 will have died from rotavirus-associated causes. Virtually all of these deaths occur in children living in developing countries. The worldwide morbidity and mortality associated with rotavirus makes it one of major vaccine-preventable causes of infant mortality. Natural infection with wild-type rotavirus elicits immunity that efficiently protects from subsequent severe illness, irrespective of the rotavirus serotype. In the past decade, two live attenuated, orally administered rotavirus vaccines have been developed and introduced in many countries; both have proven safe and effective. Several third generation candidates are in late-stage development. In the USA, the introduction of one of the currently licensed rotavirus vaccines has been associated with a remarkable decline in rotavirus illness. However, the overall impact of the licensed rotavirus vaccines on disease in extremely poor countries, where they are needed the most, remains to be determined.

3.2 Virology, Epidemiology and Pathogenesis

Although human rotaviruses (RVs) were discovered in the intestines of children with diarrhea only 36 years ago [39], substantial progress has been made in our understanding of their role in human disease. Rotaviruses are the most important cause of severe watery diarrhea in all regions of the developed and less developed world. During diarrheal illness, rotaviruses are shed in the stool in great quantity (in amounts as high as $10^{10–11}$ particles per gram of stool), which allowed the rapid development of sensitive and specific antigen detection diagnostics. Based on results from these simple diagnostics tests, it soon became clear that RVs were the cause of approximately 20–30% of severe diarrheal disease requiring hospitalization in children under the age of 5, worldwide [40]. Recent estimates indicate that

there are over 114 million rotavirus diarrheal episodes annually; these lead to approximately 24 million clinic visits, 2.4 million hospitalizations (40% of all diarrheal hospitalizations), and over 500,000 deaths in children under 5 years of age [41].

The burden of disease from rotavirus infection is not restricted to the less developed world. Studies from Europe indicate that approximately half of severe gastroenteritis in children less than 5 years of age is caused by rotavirus. In studies from the US, 50% of children hospitalized or treated in the emergency department for gastroenteritis were infected with rotavirus [42]. These data lead to the estimate that one of every 150 children under 3 years of age will be hospitalized and 1 of 11 will be seen as an outpatient in an emergency department for treatment of rotavirus disease. In the US, rotavirus is estimated to cause 20–40 deaths, 55,000–70,000 hospitalizations, and 410,000 physician visits annually [43]. The overall costs of rotavirus disease in the US are thought to exceed a billion dollars annually.

Rotavirus disease occurs with high frequency around the world, in both temperate and tropical climates and in both developed and less developed countries. The large quantity of virus that is shed probably explains why improvements in hygiene in the developed world have not reduced the incidence of infection. In temperate climates, prior to the introduction of vaccines (see below), rotavirus disease occurred seasonally in the cooler dryer months of the year [44]. In the US, waves of rotavirus infection tend to start in the southwest in the fall and end in the northeast in the spring, whereas in Europe infections tend to spread from south to north over generally the same time frame [45]. Of note, a recent study predicts that wide-spread vaccination will alter this seasonal trend [46]. The seasonality of rotavirus infections fluctuates far less in tropical climates but the highest numbers of infections occur in the coolest and driest months of the year [47].

Rotaviruses, like other members of the Reoviridae family, have a double stranded (11 segment) RNA genome and icosahedral symmetry. The viral serotype is determined by its two surface proteins, VP4 (P type) and VP7 (G type) [48]. Both of these proteins are the targets of neutralizing and protective antibodies. Due to its segmented RNA genome, the genes encoding VP4 and VP7 segregate relatively independently. At least eleven distinct human VP4 P types and ten VP7 G types have been isolated [49]. However, only a small number of P and G type combinations are encountered with any significant frequency in people and just four combinations, P(8)G1, P(8)G2, P(8)G3, and P(4)G2, account for over 90% of all isolates. Serotypic diversity does change over time and based on geography, especially in the less developed world. In the last decade, isolation of P(8)G9 and viruses has been more frequent. The relationship between serotypic diversity and protective immunity is still not well understood but it seems clear that a significant level of heterotypic immunity is produced following an initial rotavirus infection. Because of this immunity, severe episodes of illness after the primary infection are relatively uncommon [50].

From studies of animals and experimental infection of adult volunteers, the incubation period for rotavirus is usually less than 48 h. It was thought that in immunocompetent children, rotavirus infection was restricted to the mature enterocytes

on the tips of the small intestinal villi. However, recent studies in humans and animals indicate that this paradigm is not correct; most rotavirus infections are associated with some level of viremia and systemic replication [51]. The clinical relevance of the findings of extraintestinal spread and replication of rotavirus is still unclear and the great bulk of rotavirus replication clearly occurs in the mature villus tip cells of the small bowel. The pathologic changes in the intestines of children infected with rotavirus include shortening and atrophy of the villi, mononuclear infiltration in the lamina propria and distended cisternae of the endoplasmic reticulum. A direct relationship between the extent of enteric histopathologic changes and disease severity has not been demonstrated. In a mouse model, rotavirus disease is associated with very modest histopathology.

3.3 Immunology

Studies of natural rotavirus infection in man and animals demonstrated the existence of acquired immunity both to recurrent disease and, to a lesser extent, reinfection following primary infection [50]. Passive transfer studies of monoclonal antibodies in mice demonstrated that neutralizing antibody to either VP4 or VP7 could transfer either homotypic or heterotypic protection, depending on the antibody specificity in vitro [40]. Interestingly, other studies in mice have shown that non-neutralizing IgA antibodies to the antigenically conserved VP6 protein can also mediate protection, apparently via an antiviral effect occurring during transcytosis [52]. This novel intracellular neutralization event could help explain the well-documented clinical observation of heterotypic immunity following primary infection with a single rotavirus serotype. Several studies in the mouse model indicated that that B cells were the critical determinant of protection from reinfection after infection whereas $CD8^+$ T cells were responsible for restricting the course of viral shedding during primary infection [53]. $CD4^+$ T cells aid $CD8^+$ T cells and B cells and apparently can mediate active protection via an IFNγ-dependent pathway after immunization with recombinant VP6.

Rotavirus-specific fecal IgA responses occur in the majority of children after a symptomatic infection. They peak from 1 to 4 weeks after infection and then decline rapidly. It is reasonable to hypothesize that the transient presence of mucosal immunity after primary infection contributes to the absence of sterilizing immunity to reinfection. Secondary and subsequent rotavirus infections tend to boost the fecal IgA response and in many children eventually induce sustained, protective fecal anti-rotavirus IgA levels [54]. Studies of human neutralizing antibody responses against rotavirus have shown that upon first exposure to rotavirus, children develop higher homotypic than heterotypic antibody levels [53], although both types of response are usually present. Studies in animal models and humans indicate that the presence of intestinal antibodies is probably the primary protective effector mechanism against rotavirus. Protective humoral immunity in several, but not all, animal models is associated with the presence of neutralizing antibodies

directed at VP4 and/or VP7. In studies performed in day care centers and orphanages where antibodies to rotavirus were measured very shortly before a rotavirus outbreak, intestinal and/or serum antibody levels correlated with protection against rotavirus reinfection [55]. Levels of rotavirus-specific antibodies (stool IgA in particular) were correlated with protection in some but not all studies involving naturally infected as well as vaccinated children. In vaccine studies, some investigators [56] found a correlation between the presence of neutralizing antibodies and protection; however, the percentage of children with detectable serotype specific neutralizing antibody titers is always significantly less than the percentage of children protected by vaccination [57]. Thus, although serotype specific neutralizing antibodies seem to play a role in protection, it seems likely that heterotypic antibodies or against other proteins or other mechanisms also play a role in immunity.

Recent studies have also drawn attention to the possible importance of the innate immune response and interferon in regulating rotavirus immunity. In the gnotobiotic porcine model, probiotic treatment with *Lactobacillus acidophilus* significantly enhanced both B and T cell responses to attenuated live virus infection [58]. It has been shown that levels of type I and II IFN are elevated in rotavirus-infected children and animals [59, 60]. Both type I and II interferon are able to limit rotavirus infection in vitro and, in early studies, IFNα administration successfully alleviated RV diarrhea in cattle and pigs. The IFN-regulatory factor 3 (IRF3) interacts with the RV protein NSP1, clearly linking RV infection to innate immunity [61]. NSP1 also inhibits activation of NFκB by a novel mechanism involving targeted degradation of an F-box protein of the E3 ligase complex [62, 63]. Studies in vivo demonstrated that the systemic virulence of selected strains of rotavirus was enhanced and a lethal biliary and pancreatic disease induced when interferon signaling was abrogated during rotavirus infection [64]. Hence, innate immunity plays a critical role in modulating rotavirus infection in vitro and in animal model systems but the role in humans remains largely unexplored.

3.4 *Vaccines Development, Composition and Mechanism of Attenuation*

Two live attenuated, orally administered rotaviral vaccines are currently licensed and in use in many countries around the world [48, 65–67]. Both vaccines have been shown to be safe and effective as well as cost-effective in developed and developing countries. The aim of anti-rotavirus vaccination strategies is to reproduce the level of immunity induced following natural infection. Natural infection, either symptomatic or asymptomatic, efficiently prevents subsequent severe rotavirus disease but does not necessarily prevent reinfection or mild illness. Based on the observation that animal rotaviruses appear to be substantially restricted for growth, pathogenicity, and transmission in heterologous hosts such as humans (host range restriction), the initial strategy for rotavirus vaccine development was a modified Jennerian approach using either live simian/human or bovine/human rotavirus

reassortants as vaccines. In this approach, an animal origin rotavirus that is restricted for growth in humans is reassorted with a human rotavirus and reassortants are isolated with genomes that are primarily animal in origin but which contain genes encoding VP4 or VP7 from the human parent. Such reassortants are expected to induce protective immunity to human rotaviruses because of the presence of human surface proteins (VP4 or VP7) but not to cause severe disease because most of their genome was derived from the animal rotavirus parent which is restricted for growth in the human host. Of note, these vaccines are fully replication competent in cell culture and they are derived from potentially virulent animal strains but they have undergone considerable cell culture passage which, in itself, could also be a cause of attenuation. The first modified Jennerian vaccine of this type was a quadrivalent rhesus vaccine (Rotashield™, Wyeth/Lederle) consisting of four monoreassortants between human G types 1 through 4 and the simian RRV strain [68]. The vaccine contained components for expression of these G types based on the assumption that immunity to the four most common human rotavirus types would be needed to induce a high level of efficacy. This vaccine was shown to be highly immunogenic and efficacious in multiple phase III studies in the USA, Finland and Venezuela and was licensed for use in the United States. Of note, the level of efficacy substantially exceeded the level expected based on the type-specific neutralizing antibody response induced by the immunization and this finding later was repeated with the bovine rotavirus based modified Jennerian vaccine. Shedding studies of the RRV-based vaccine indicated that it was shed in moderate quantity and was able to transmit with reasonable efficiency to unvaccinated children in the environment. The consequences of this shedding and transmission were never evaluated but it did not appear that the virus gained virulence during passage in humans. Unfortunately, after licensure and administration to almost one million American children, the RRV-based vaccine was withdrawn from the market because of its strong temporal association of the first dose of vaccine (which was licensed to be given orally at 2, 4, and 6 months of age) with intussusception in older (over 3 months of age) children receiving their first dose. Interestingly, the impact of this vaccine on the total attributable risk of intussusception, especially if initially administered to children only at 2 months of age or below, remains unknown but was likely to be very small. At the time the vaccine was withdrawn from manufacture, it was undergoing efficacy evaluation in less developed countries which were never completed. However, immunogenicity studies carried out in Bangladesh indicated that the RRV-based vaccine appears to have been more immunogenic in this setting than either of the two currently licensed second generation vaccines.

Subsequently, a second generation pentavalent modified Jennerian vaccine called RotaTeq™ (Merck), based on mono or di-reassortants derived from the mixed infection of a bovine rotavirus (WC3) and human rotavirus strains which provided human G1, G2, G3, G4 (VP7), and P[8] (VP4) was introduced [65]. This vaccine contained five (rather than four) separate reassortants based on the theoretical consideration that immunity to all five serotypic species would be most efficient at inducing protective immunity. The basis of attenuation for this vaccine, like the

preceding Rotashield™ product, is presumed to be the inherent host range restriction linked to its primarily bovine-origin genome. The specific genetic and mechanistic basis for host range restriction of the two reassortant-based vaccines is not well understood, although several studies indicate that this restriction is linked, at least in part, to rotaviral protein NSP1 and host specific inhibition of the innate immune system [69]. Studies of viral shedding have demonstrated that, in general, the bovine-based reassortant vaccine is highly restricted for replication in humans and is shed in a very limited fashion compared to wild-type human rotavirus or to the RRV-based vaccine although no head-to-head comparison was ever done. Nevertheless, recent studies have demonstrated that severely immunodeficient children can become chronically and symptomatically infected with this vaccine [70]. As with the RRV vaccine, this vaccine is administered orally in a three dose regimen.

The other currently licensed vaccine (Rotarix™, GSK) is a more traditionally constructed, live attenuated vaccine. A virulent G1P human rotavirus strain (89-12) was multiple passaged in monkey kidney cell culture in order to acquire a suitable level of attenuation to reduce virulence [66]. The rationale underlying the development of this monovalent vaccine was that a single natural rotavirus infection, either symptomatic or asymptomatic, very effectively provides protective immunity to subsequent severe disease, irrespective of serotype. Therefore, it seemed logical that a single attenuated human rotavirus strain might do the same. As with the Merck vaccine, the molecular basis for attenuation of this vaccine candidate is unknown although presumably, in this case, point mutations introduced during multiple passage in cell culture are responsible for the attenuation. Since the actual molecular basis for attenuation of the two currently licensed vaccines remains unknown, it is difficult to comment definitively on their level of genetic stability except to say that, to date, evidence for reversion to virulence has not been found. This vaccine is also administered orally, but in this case only two doses of vaccine are given.

3.5 Safety and Efficacy

The two second generation vaccines have been evaluated for safety and efficacy in studies representing a wide variety of socioeconomic conditions in several countries. In very large field studies both were shown to be safe and effective. Protection rates in developed or moderately developed countries provided by both vaccines are very similar; rates vary from 70–80% against any rotavirus disease to 90–100% against severe gastroenteritis. Interestingly, to date, no appreciable advantage of the multivalent over the monovalent vaccine has been observed. Large pre- and post-licensure studies have shown that these vaccines are not associated with intussusception if the first dose is administered to children under the age of 3 months [71]. The effectiveness of the pentavalent Merck vaccine in preventing rotavirus-associated gastroenteritis and hospitalizations was demonstrated in the United States and the monovalent vaccine effectively prevented rotavirus-associated

deaths in Mexico [72–74]. Since successful efficacy and/or immunogenicity trials in a variety of countries around the world have been completed, a general WHO global recommendation for the use of these vaccines was issued in June 2009 [75]. Of note is the fact that recent studies of the two new vaccines in very poor areas of Africa and Asia indicate that these vaccines are less effective (49.5–76.9% protection rates against severe disease) in these countries than has been observed in developed countries [76]. However, they are still very cost-effective in terms of number of severe rotavirus-induced diarrheas prevented: Overall in African trials, Rotarix prevented 3 out of 5 episodes of severe rotavirus-induced disease per 100 vaccinated children [75].

3.6 Issues for the Future

Although two safe and effective live attenuated rotavirus vaccines are now available, several important practical issues are not yet resolved. Exactly how important the moderate decrease in efficacy of these vaccines in very poor countries is and whether it can be circumvented in some straightforward way is not known. Second, the two current vaccines cost too much to be affordable without substantial subsidies and cheaper vaccine products will be required to serve the global needs in the long term. The two vaccines are currently highly effective but rotaviruses certainly have the ability to evolve serotypically and it remains to be seen if new strains of human rotavirus will emerge that are less effectively countered by immunity elicited by the current vaccines. Finally, as with all vaccines, rare and unexpected adverse events are always possible and continued vigilance regarding safety, especially in very immunosuppressed children is necessary.

Several third generation rotavirus vaccines are in various stages of development. A pentavalent live attenuated reassortant vaccine based on another bovine strain (UK) is currently undergoing evaluation in several less developed countries including China, India and Brazil. This vaccine has been shown to be highly efficacious in phase two trials in Finland. Monovalent human rotavirus vaccines derived from naturally attenuated strains recovered from human infants in India and Australia are in various stages of evaluation. The Indian strain (116E) appears to be highly immunogenic in preliminary studies [77]. Several groups have proposed that some form of parenterally administered inactivated vaccine might be safer vis-a-vis the risk of intussusception. Such a vaccine might be more immunogenic and hence more effective in some less developed regions where efficacy of the live virus vaccines seems to be restricted [76]. No data from human studies is currently available to evaluate the utility of this strategy. Finally, the quadravalent rhesus rotavirus-based vaccine is currently undergoing a re-evaluation in a phase three efficacy trial in Africa based on the early data indicating that this vaccine appeared to be more immunogenic than the current two commercial vaccines and hence, might be substantially more efficacious in a less developed setting. The results of this interesting trial should be available in the next year or two.

4 Varicella Zoster Virus

4.1 Introduction

VZV is a human alphaherpesvirus, most closely related to herpes simplex viruses (HSV) 1 and 2. VZV has the typical morphology of herpes virus particles and while its double stranded DNA genome is the smallest of the human herpesviruses, at least 70 gene products are encoded [78]. VZV causes varicella (chickenpox) as the primary infection and establishes life-long persistence in sensory ganglia; reactivation from latency produces the clinical syndrome referred to as zoster (shingles) [79, 80].

4.2 Epidemiology

The molecular epidemiology of VZV has been investigated extensively based on the detection of single nucleotide polymorphisms in selected VZV open reading frames and by whole VZV genome sequencing of more than 20 isolates [81, 82]. VZV circulates as five distinct clades that exhibit varying predominance in different geographical areas but overall, the genetic diversity of the virus is limited. While clades 1 and 2 are the most divergent, genome sequences were 99.83% identical, with only188 site differences [83]. Sequences of nine clade 1 viruses showed 99.9% identity compared with Dumas strain, which is the first VZV genome that was sequenced [84]. Clade 1 viruses are most common in Europe and North America, clade 2 viruses are predominant in Asia and clade 5 is most prevalent in Africa. The Oka virus used to derive VZV vaccines is a clade 2 strain. As expected, immigration has redistributed European, African and Asian clades.

VZV skin lesions contain high concentrations of infectious virus during both primary and recurrent infections, which results in a highly successful strategy for persistence in the human population. Whereas the prevalence of other human herpesviruses has declined in developed countries, varicella epidemics continue to produce high infection rates. Episodes of zoster in older individuals provide a constant mechanism for reintroducing the virus, causing varicella in naïve individuals who are in close contact and who then spread the virus to other susceptibles. In temperate climates, VZV is acquired almost universally during childhood; attack rates are substantially lower in tropical areas. Before varicella vaccine was introduced, the incidence of varicella in the United States was ~four million cases per year, reflecting the number of children in the annual birth cohort. Secondary bacterial infections and VZV encephalitis were the most common morbidities; hospitalization rates were estimated to be 2–5 per 1,000 cases and approximately 100 fatal cases were reported annually [85]. The estimated incidence of herpes zoster is >1 million cases per year in the United States and complications, especially post-herpetic neuralgia, are frequent in older individuals [80]. While the

morbidity caused by VZV in healthy children and adults is significant, illness associated with this ubiquitous pathogen can be much more severe in immunocompromised patients. Children who are immunodeficient because of underlying disease or immunosuppressive therapies may develop progressive varicella; the risk of VZV reactivation is much higher in immunocompromised children and adults, and whether or not it is manifest as cutaneous zoster, reactivation may cause life-threatening disseminated VZV infection.

4.3 Pathogenesis of Primary and Recurrent VZV Infection

As defined clinically, the events in primary VZV infection include respiratory inoculation, viremia and the appearance of vesicular skin lesions. Studies of VZV pathogenesis using the severe combined immunodeficiency (SCID) mouse model show that VZV exhibits a marked tropism for T cells in human thymus/liver xenografts in vivo; VZV is also highly infectious for human tonsil T cells, particularly those in the subpopulation of activated, memory CD4 T cells, in vitro [86, 87]. VZV is readily transferred into skin xenografts when infected tonsil T cells are injected into the circulation of SCID mice. Infected T cells exit capillaries and initiate replication in epidermal cells, which progresses over a 10–21 day period until the lesion reaches the skin surface; cell–cell spread of VZV in skin is modulated by a potent innate response of the epidermal cells surrounding the newly forming lesion [88]. Induction of the IFN pathway and upregulation of NFkB signaling are prominent in adjacent cells. These observations suggest a model of primary VZV pathogenesis in which the virus infects respiratory epithelial cells, enters T cells in tonsils and other lymphoid tissues of the Waldeyer's ring and initiates a T cell-associated viremia which transports the virus to skin sites of replication; after a period of subclinical lesion formation during the incubation period, the characteristic varicella exanthem appears.

In the course of primary infection, VZV gains access to cranial nerve and dorsal root ganglia and as suggested by recent evidence, to autonomic enteric ganglia as well [89]. Access is presumed to occur via retrograde transfer along neuronal axons from skin lesions, T cell viremia or both. VZV-infected T cells transport the virus into DRG xenografts in the SCID mouse model [90]. Persistent infection is established in neurons for the life of the host. Abortive replication limited to ganglia or to subclinical skin replication may occur; however, when VZV reactivation causes zoster, VZ virions are presumed to move by anterograde axonal transport to the skin dermatome where vesicular lesions appear. In contrast to HSV, VZV reactivation can destroy neurons and satellite cells in the affected ganglia [80]; cell–cell spread with fusion of neurons and surrounding satellite cells is observed in VZV-infected DRG xenografts in the SCID model [91]. Recurrent VZV may also lead to viremia. Migrating T cells may become infected during trafficking through skin or ganglion sites of VZV replication, allowing viral transport to lungs, liver, brain and other organs in immunocompromised patients.

4.4 Host Response

In addition to innate cellular responses, NK cells appear to be critical since NK cell deficiencies are associated with severe, often fatal primary VZV infection. In the healthy host, VZV-specific immunity emerges in parallel with the appearance of skin lesions during primary VZV infection; both VZV antibodies and VZV-specific CD4 and CD8 T cells are induced [92]. However, cell-mediated immunity is necessary to resolve varicella, as shown by the risk of progressive infection in immunocompromised children who developed VZV IgG and IgM antibodies but failed to mount a VZV-specific T cell response [93]. Adaptive CD4 and CD8 T cells and IgG antibodies that recognize various VZV proteins persist but their peptide specificity is not well-characterized. VZV antibodies that bind envelope glycoproteins exhibit neutralizing activity. The extent of both antibody and T cell mediated memory immunity to VZV may be determined by the initial clonal expansion or by secondary stimulation from varicella exposures or subclinical reactivations, or by all of these mechanisms. Circulating VZV memory T cell frequencies are ~0.1–0.2% in immune adults [94].

While reported, symptomatic second episodes of varicella are rare, even among severely immunodeficient patients. A clinical history of varicella is often unreliable; individuals with apparent second cases had no evidence of prior infection when specimens obtained before the episode were available [95]. Protection from varicella illness appears to be induced regardless of the clade that caused the initial infection, which can be explained by the highly conserved VZV genome. An important caveat is that the incidence of subclinical VZV reinfection is not known although it has been proved, using molecular methods, that VZV reactivations in the same individual were caused by viruses of different VZV clades [96]. Protection from clinically apparent reinfection may be mediated by neutralizing antibodies present at respiratory sites of inoculation or by a rapid humoral and T cell response if replication is initiated. Adults in close contact with children who have varicella exhibit boosts in both VZV antibody and cell-mediated responses. Administering passive antibodies to VZV within 4 days after exposure of a naïve host can prevent varicella or modify its severity, as demonstrated by varicella zoster immune globulin prophylaxis in immunocompromised children and newborns. Experiments in the SCID mouse model show that infection may be blocked when antibody to the glycoprotein, gH, which has potent neutralizing activity, is given shortly after inoculation of skin xenografts [97].

In one of the most well-established immunologic correlates known, zoster in older adults and immunocompromised patients is associated with reduced T cell proliferation and production of IFN-γ and other cytokines by peripheral blood mononuclear cells stimulated with VZV antigen and with fewer circulating VZV-specific CD4 and CD8 T cells [92, 98]. In contrast, VZV IgG antibody titers are not related to the risk of reactivation; passive antibody adminstration did not alter zoster severity in clinical studies done before antiviral drugs were available. However, antibodies may contribute to modulating cell–cell spread of VZV in

skin and possibly in the affected ganglion. Symptomatic zoster is associated with a dramatic increase in the VZV-specific T response; IgG, IgM and IgA antibody titers are also boosted but the resolution of zoster, like varicella, requires cellular immunity. Of interest, hematopoietic cell transplant recipients may have subclinical reactivation, detected by the presence of VZV DNA in peripheral blood mononuclear cells and recover VZV-specific T cell responses without clinical zoster.

4.5 VZV Vaccines

VZV is the only human herpesvirus for which vaccines are licensed. The live attenuated varicella and zoster vaccines are made from the attenuated Oka virus [99, 100]. Inactivated Oka-derived vaccines have also been evaluated in immunocompromised and healthy patients [101].

Composition. The VZV Oka vaccine seed stock was derived from a clinical isolate, the parent Oka (pOka) virus, which was recovered from a varicella skin lesion; pOka was passaged in guinea pig and human fibroblasts at low temperature. VZV vaccines contain infectious VZ virions made in cells approved for manufacturing live viral vaccines; Oka-derived vaccines also contain viral and host cell proteins and DNA because VZV replication is very highly cell-associated. Oka vaccines are currently manufactured by Biken, Merck and GlaxoSmithKline. Not surprisingly, because of the extreme cell association of VZV, Oka vaccines made by all three manufacturers represent mixtures of VZV genomes. Multiple single nucleotide polymorphisms are identified; some are shared in the various vaccine preparations but others are not and wild type markers are also present [102–104]. The pediatric vaccines, Varivax (Merck) Varilrix (Glaxo) and Okavax (Biken) contain approximately 1,300 pfu of Oka vaccine virus.

Mechanism of attenuation. Attenuation of pOka was achieved empirically by tissue culture passage and verified clinically by the administration of Oka vaccine preparations to susceptible children in Japan [100]. The experience showing attenuation of the Biken Oka vaccine was confirmed in trials of varicella vaccines made from vaccine Oka seed stocks by Merck in the U.S. and by GlaxoSmithKline in Europe.

Investigations in the SCID mouse model demonstrate that vaccine Oka has reduced virulence in skin compared to pOka. In contrast, pOka and vaccine Oka do not differ in their infectivity for T cells and DRG xenografts in vivo [79, 86, 90, 105]. These experiments suggest that attenuation of vaccine Oka in skin is intrinsic, resulting from genetic changes accumulated during tissue culture passage in fibroblasts rather than simply because the vaccine is given by a subcutaneous route of inoculation. The evidence that this attenuation is tissue/cell type specific for skin but not T cells or DRG is consistent with the capacity of Oka vaccine to cause a varicella-like rash in immunocompromised patients and its potential to establish latency in the sensory ganglia of healthy vaccinees [106, 107]. Experiments with pOka/vOka chimeric viruses showed that attenuation in skin was conferred by

different segments of vaccine Oka in the chimera, suggesting that multiple VZV genes have relevant mutations [108]. Identifying mutations that might contribute to attenuation by full genome sequencing is challenging because as noted, varicella vaccines contain mixtures of variants that have various genetic differences [109]. Vaccine Oka mutations do not alter its susceptibility to inihibition by acyclovir and related antiviral drugs.

Measles–mumps–rubella–varicella (MMR-V) multivalent vaccines. Vaccine Oka is also used as a component of a multivalent vaccine containing live attenuated measles, mumps and rubella (MMR-V) [110, 111]. This formulation requires a higher titer of vaccine Oka than the single component vaccine. The Merck vaccine (ProQuad) contains not less than 3.99 log 10 pfu of vaccine Oka; the GlaxoSmith-Kline vaccine (PriorixTetra) contains not less than 3.3 log 10 pfu.

Higher potency vaccines for zoster. Higher potency live attenuated Oka vaccines have been developed and evaluated for their potential to increase VZV cellular immunity in healthy older adults in the U.S. [112]. Dose-finding studies were done using VZV-specific T cell proliferation and responder CD4 T cell frequencies as the endpoint before a large scale efficacy study was undertaken [98]. High potency vaccines boosted VZV T cell responses among 55–87-year-old subjects to ranges observed in younger adults, ages 35–40 years, who had naturally acquired VZV immunity. The infectious virus content of the high potency zoster vaccine manufactured by Merck Inc. is ~20,000 pfu, which is more than 14-fold more than Oka/Merck pediatric vaccines. This higher infectious virus content is presumed to be necessary because zoster vaccine recipients have pre-existing VZV immunity and because immunosenescence diminishes the antiviral T cell responses of older individuals.

Inactivated VZV vaccines. Heat inactivation can reduce the infectious virus content of varicella vaccine to undetectable levels. Heat inactivated vaccine was used to assess effects on T cell responses in healthy elderly individuals [113] and for immune reconstitution and zoster prevention in hematopoietic cell transplant patients [101].

Clinical experience with the efficacy and safety of varicella vaccines. The development of live attenuated VZV vaccines in the U.S. and Europe was first undertaken to protect children with leukemia from varicella [99]. The capacity of vaccine Oka to cause varicella-like illnesses has limited use in immunocompromised children. However, trials of live attenuated varicella vaccines in healthy children led to their introduction as a routine childhood vaccine in North America, Australia and some Europe and Asian countries [99, 114]. Pre-licensure evaluations demonstrated that these vaccines induced both humoral and cell-mediated immunity against VZV, with antibody titers and VZV T cell proliferation responses in the range of those observed after natural VZV infection in childhood. Immunogenicity, measured by serologic and cell-mediated responses, correlated with infectious virus and antigen content. Age was also a factor; a two dose regimen was required to achieve >90% seroconversion rates in those over 12 years old.

The efficacy of varicella vaccine was demonstrated in a small placebo controlled trial in the U.S., leading to licensure in 1995 [99]. Extensive post-licensure

surveillance has supported that the vaccine is effective and safe in healthy children and adults. The recommendation to vaccinate all children at 12–18 months of age and all susceptible older children and adults has had a major impact on varicella incidence, hospitalizations and deaths among the pediatric population and also among persons in older age groups, most of whom benefit indirectly [85, 115]. The annual incidence of varicella decreased by more than 80% when a coverage rate of ~90% was achieved in 2005; hospitalization rates for varicella complications decreased by 88% and age-adjusted mortality was reduced by 66%.

Although the initial recommendation was to give a single dose to children under 12, reports of breakthrough infection in vaccinated children remained relatively common. Efficacy analyses during outbreaks and in surveillance sites showed a single dose vaccine effectiveness rate of ~85%. Although breakthrough varicella cases were typically mild, these cases were a source of VZV transmission to other susceptibles and interfered with the public health objective of varicella control. Therefore, a two dose regimen for all age groups was implemented in the U.S. in 2007 [116]. Whether this pattern reflects waning immunity is debated [117, 118]. However, the single dose regimen is associated with lower seroconversion rates by the most sensitive assay for VZV IgG antibodies, suggesting that primary vaccine failure accounts for many cases of apparent breakthrough varicella in vaccine recipients [119].

Reports about varicella vaccine adverse events to the U.S. vaccine adverse event reporting system (VAERS) showed a rate of 2.6/100,000 doses during the first 10 years after licensure [120, 121]. Varicella vaccine can cause a mild, self-limiting rash in healthy recipients within the first 6 weeks [122, 123]. Some children with severe undiagnosed immunodeficiencies have developed progressive infection caused by Oka vaccine virus; however, treatment with acyclovir has been effective in most cases.

Zoster after vaccination. Zoster can occur in vaccinated individuals. Using sequence differences between Oka and most North American VZV isolates, it has been possible to demonstrate that these cases can be due to either vaccine or wild type VZV [109]. Zoster caused by wild type VZV has been reported in vaccine recipients with no history of breakthrough varicella, indicating that infection can be acquired subclinically, reach neurons and establish latency in sensory ganglia [106]. When evaluated in vaccinated immunocompromised children, zoster was significantly less common than zoster following natural infection. More recently, prospective studies in healthy children demonstrated that the incidence of zoster was 4–12 fold less in vaccinated children under 10 years of age compared to those with natural infection [124] and zoster was rare (27.4 cases/100,000 person years) in 170,000 vaccinated children [125]. Vaccine-related cases of zoster have been mild although cases of meningitis and meningoencephalitis have been reported [107].

Information about how commonly Oka vaccine virus establishes latency in sensory ganglia is limited and consists of VZV DNA sequence analysis of skin lesion specimens from vaccinated people with clinical zoster. However, recent evidence from a postmortem study suggests that vaccine Oka persists in multiple

ganglia for years after vaccination, as observed with wild type VZV and latency was established without vaccine-associated skin lesions [89]. Whether viral load is reduced compared to wild type VZV is not known. These observations are consistent with the capacity of vaccine Oka virus to cause viremia in immuncompromised children and with the evidence that its T cell tropism is intact in the SCID mouse model. The potential for super-infection with wild type VZV in vaccinated individuals along with the persistence of the vaccine virus in ganglia may also permit genetic recombination of wild type and vaccine viruses. Recombination of Oka and wild type VZV has been demonstrated by direct sequencing of VZV DNA from zoster lesions in a previously vaccinated individual.

MMR-V. MMR-V vaccine was licensed in the U.S. based on comparability of the VZV antibody titers against viral glycoproteins, as measured by ELISA. Although the mechanism is not known, MMR-V has been associated with an increased incidence of febrile seizures from 7 to 14 days after the first vaccine dose; rates were 4/10,000 for MMR and 9/10,000 for MMR-V [126]. These observations led to the recommendation to offer an option of giving MMR and varicella vaccines at different sites or to give MMR-V, along with counseling parents about the rare possibility of febrile seizures within 2 weeks after vaccination.

Live attenuated varicella vaccine in high risk patients. Varicella vaccine has been used in clinical practice to immunize children with HIV infection against severe varicella and zoster when their CD4 T cell counts were >15–25%; a recent report found an 82% effectiveness against varicella and 100% effectiveness against zoster in a review of carefully monitored children with HIV [107, 127]. Varicella vaccine has also been given to children with leukemia in remission and solid organ transplant recipients as a safer option than risking natural infection [98]. The rationale is that antiviral therapy can be given if varicella-like illness occurs.

Varicella vaccine issues for the future. Whether the two dose regimen introduced in 2007 will reduce the incidence of breakthrough varicella in childhood requires continued surveillance. Like many viral infections, varicella is more severe in adults. Therefore, as has been true for other childhood viral vaccines, it is important to maintain active surveillance programs to be sure that protection is sustained. Whether those with vaccine-induced immunity will need booster doses is difficult to predict; robust immune responses elicited by a two dose regimen in early childhood may prove to be as long-lasting as natural immunity. Whether intermittent re-exposure to varicella is necessary to maintain natural immunity is not known but interrupting varicella epidemics will obviously reduce such contacts. Based on the assumption that some boosting of memory immunity is required, whether by exogenous re-exposure or endogenous restimulation through subclinical reactivation, some models predict a higher incidence of zoster among those with natural infection, as a consequence of varicella vaccine programs. However, surveillance studies show no increase in zoster [100]. New information indicating that Oka vaccine latency occurs frequently may mean that vaccine-related zoster will be a concern as vaccine recipients become older. If so, as described below, zoster can also be prevented by vaccination. Oka vaccine latency may also result in recombination with wild type

VZV in vaccine recipients who are super-infected. However, it seems unlikely that reversion to wild type patterns of VZV virulence will occur.

Clinical experience with the efficacy and safety of zoster vaccine. The association of zoster-related morbidity in older adults and immunocompromised patients with declining memory T cell immunity along with experience confirming the efficacy and safety of live attenuated varicella vaccines set the stage for developing zoster vaccines [112]. In an early proof of concept study, immunization with a heat-inactivated Oka/Merck vaccine preparation was associated with a reduction in the incidence of zoster from 33 to 13% during the first year after autologous hematopoietic cell transplantation when the vaccine was given as one dose before and three doses after transplantation [101]. Comparing vaccine recipients with matched controls who were unvaccinated showed that VZV specific CD4 T cell responses were reconstituted much earlier among vaccinees, despite their severely immunocompromised state. No vaccine-related adverse effects were observed in recipients of this heat inactivated VZV vaccine. By showing a correlation between restoring VZV T cell immunity and reduced zoster incidence, this study provided direct evidence of the role of cell-mediated immunity in preventing the progression of VZV reactivation to symptomatic zoster.

After dose finding studies, a large placebo-controlled trial was done to evaluate high potency live attenuated Oka vaccine preparations, ranging from 18,700 to 60,000 pfu (median 24,600 pfu) for effects on zoster incidence and severity [128]. Enrolment targeted healthy adults who were >60 years old. Among the 38,546 participants, the median age was 69 in both the vaccine and placebo cohorts; 6.6% of vaccine and 6.9% of placebo recipients were ≥80 years old. Intensive surveillance for zoster was carried out for an average of 3 years; cases were determined by laboratory confirmation and each episode was assessed using pre-established criteria for zoster severity, post-herpetic neuralgia, and health quality of life. The primary endpoint of the study was a zoster burden-of-illness score, representing a composite index reflecting the incidence and severity of zoster. This score was significantly lower in the vaccine cohort compared to the placebo cohort ($P < 0.001$); the effect was independent of sex or age <70 vs. >70 years. Post-herpetic neuralgia (PHN) is the most common debilitating complication of zoster in older individuals. PHN rates were 0.46 cases per 1,000 person-years in the vaccine cohort and 1.38 cases in the placebo cohort ($P < 0.001$); the effect of vaccination on PHN rates was also independent of sex and age stratification. The study was designed with the incidence of zoster per 1,000 person-years as a secondary endpoint. The zoster incidence was 5.42 in the vaccine group and 11.12 per 1,000 person-years in the placebo group ($P < 0.001$). This difference represents a 51.3% efficacy of the high potency vaccine for zoster prevention among individuals >60 years. When the data was stratified by age cohorts, vaccine efficacy for zoster prevention was 63.9% among those <70 years old vs. 37.6% in those who were >70 years old ($P < 0.001$). Thus vaccine recipients in the older cohort were more likely to develop zoster despite immunization. Nevertheless, participants in the vaccine group who were >70 years old experienced less severe zoster than those >70 years in the placebo group. The impact of vaccination on zoster severity was

greater among those >70 years, who were at a higher risk for a more severe episode. Although the vaccine was less effective for zoster prevention in the older age cohort, the benefit of reduced zoster severity maintained vaccine efficacy, assessed from the burden of illness, at 55.4% in healthy adults >70 years old. Overall, the burden of illness score was reduced by 61.1% and PHN incidence was 66.5% lower in men and women >60 years old who were vaccinated.

Serious adverse events were uncommon and rates were equivalent in the vaccine and placebo cohorts. Even though the vaccine contained high concentrations of infectious vaccine Oka, all episodes of zoster were confirmed to be wild type VZV when lesion specimens were tested by PCR and sequencing.

Immunogenicity of zoster vaccine. Zoster vaccine given to healthy older individuals in dose finding studies boosted VZV T cell responses above baseline, with a half life of at least five years [112]. High potency Oka vaccine corrected deficiencies in CD4 T cells that produced IFN-γ or IL-2 and frequencies of CD4 and CD8 effector memory T cells that responded to VZV antigen [94]. Some participants in the efficacy study of zoster vaccine were also evaluated for effects on VZV cellular immunity as measured by responder cell frequencies and ELISPOT assay. Responses were higher within the first 6 weeks when vaccine and placebo recipients were compared and were higher in those who were less than 70 years old when vaccinated compared to those over 70 in the vaccine cohort. Despite a decline by 12 months, VZV T cell responses continued to be above baseline in the vaccine group for the 3 year follow-up period [94].

Zoster vaccine issues for the future. The experience with high potency Oka vaccine demonstrates that symptomatic VZV reactivation can be prevented or its consequences minimized in healthy older people by enhancing their VZV-specific T cell responses. Importantly, safety was maintained despite the high inoculum of vaccine virus. That this intervention diminishes the risk and consequences of VZV reactivation is relevant to the potential for vaccine control of other herpesviruses that also persist for the life of the host and although the effect is on reactivation rather than active replication of a chronic infection, the zoster vaccine can be viewed as a proof of principle that therapeutic vaccination is feasible. Among the unresolved questions are the optimal age for giving zoster vaccine and how long protection against zoster and post herpetic neuralgia will be maintained. More information about protection in the very old is also needed. Whether the vaccine virus reaches sensory ganglia when given to individuals with pre-existing VZV immunity is not known. Given the evidence that super-infection can occur with wild type VZV strains and in varicella vaccine recipients, it is possible that zoster vaccination could lead to recombination events as has been observed in a few instances after varicella vaccination. Whether inactivated Oka vaccine will reduce zoster morbidity in immunocompromised patients in a larger placebo-controlled trial is being evaluated.

Opportunities to improve live attenuated VZV vaccines. The VZV genome can be mutated readily using cosmid and bacterial artificial chromosome methods and the consequences of targeted mutations on VZV virulence in skin, T cells and DRG can be evaluated in the SCID mouse model of VZV pathogenesis. Insights gained

about the molecular mechanisms of VZV pathogenesis have relevance to designing "second generation" live attenuated VZV vaccines that have reduced potential to infect T cells and neurons and are safer for immunocompromised patients. Disrupting T cell tropism would be predicted to prevent vaccine virus delivery to neuronal cells, which would block the establishment of vaccine virus latency in sensory ganglia and eliminate the potential for vaccine virus recombination with wild type VZV as well as vaccine-related zoster.

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Classical Live Viral Vaccines

Thomas P. Monath

Abstract Classical, live viral vaccines have been developed by adapting viruses by serial passages in animals, tissue or cell cultures during which multiple mutations in the viral genome have accumulated. The majority of vaccines in use today were developed in this way and a number of similar investigational vaccines are currently in development. The principal advantage of live vaccines is that they mimic natural infection and induce durable immunity, including cytotoxic T cell responses that are not generated by soluble proteins and inactivated vaccines. Recent studies of gene activation following a live vaccine (yellow fever 17D) have shed light on the role of innate immune responses in provoking strong, polyfunctional adaptive immunity following the administration of live vaccines. The principal disadvantage of live vaccines is that, occasionally, infection caused by the live vaccine causes adverse events resembling the parental (virulent) virus. Such events can be due to reversions in critical attenuating mutations or to host-specific susceptibility factors. The attenuating mutations in live vaccines increase the inapparent: apparent infection ratio compared to the parental virus, but overt infection (adverse events) while far less frequent than in natural infection can still occur. This problem is inconsequential for infections that are typically mild or self-limited, such as measles, mumps, rubella, and varicella, but can be devastating for infections that are frequently lethal such as yellow fever or in individuals who are immunocompromised. Despite these issues, live vaccines have had dramatic benefits in reducing the incidence of the most important infections of humankind, and in one case (smallpox) a live vaccine helped eradicate a viral disease.

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1 Introduction

Classical live viral vaccines are those developed by empirical methods, mostly by adapting the virus to replicate in a host different from that in which the virus grows naturally. Almost all viral vaccines in use today (Table 1) were developed in this way, and they have contributed to the successful control of many major diseases. Each of these vaccines has its own unique history, indications for use, and biological characteristics, the details of which are beyond the scope of this brief review. Therefore, this chapter will focus on the general principles underlying the development, safety, and immunogenicity of classical live vaccines and illustrate the same with specific examples.

2 Timelines for Vaccine Development

Figure 1 displays the evolution of development of approved live viral vaccines, beginning with a description, by Edward Jenner in 1796, of the use of cowpox for protection against smallpox to a modern smallpox vaccine produced in cell culture

Table 1 Viral vaccines approved for use

A. Available as live vaccines
Adenovirus 4, 7 (military use)[a]
Junín (Argentine hemorrhagic fever, available in Argentina)
Measles
Measles–mumps–rubella
Measles–mumps–rubella–varicella
Mumps
Rotavirus
Rubella
Smallpox (vaccinia)
Varicella
Yellow fever
Zoster
B. Available as live and inactivated vaccines
Hepatitis A (live vaccine available in China)
Influenza [live vaccine available in US (Flumist®) and Russia]
Japanese encephalitis (live SA14-14-2 available in Asia)
Poliomyelitis
C. Available only as inactivated or subunit vaccines
Hepatitis B
Rabies
Human papillomavirus
Tick-borne encephalitis
Kyasanur forest disease (India)
Hemorrhagic fever with renal syndrome (Korea, China)

[a]In registration

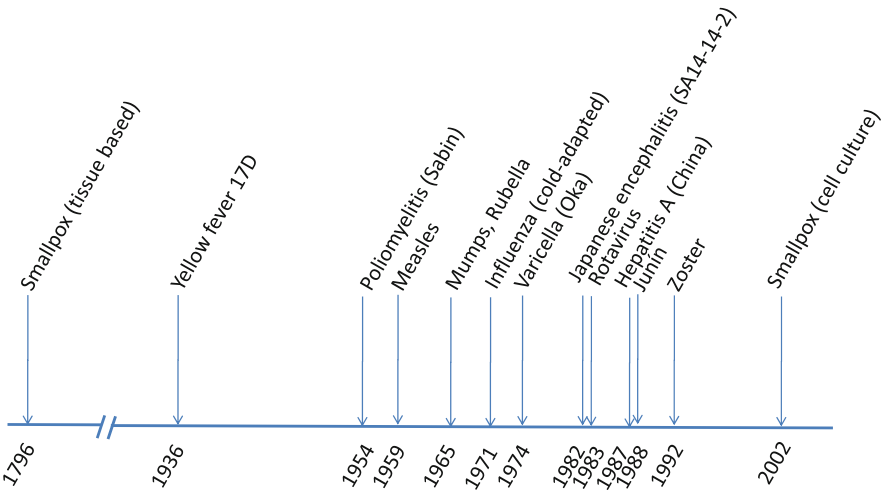

Fig. 1 Year in which currently approved vaccines were first tested in humans, establishing proof of concept and initial data on safety and immunogenicity

(ACAM2000) first tested in humans 206 years later. Jenner referred to the material used in his studies as "vaccine", a term derived from the Latin word for cow (*vacca*). One hundred years later, Louis Pasteur applied the term "vaccination" to the use of other agents for inducing prophylactic immunity. Jenner's invention (use of an animal virus that was poorly adapted for growth in humans but capable of evoking cross-protective immune responses), often referred to as Jennerian vaccination, is still en vogue; for example, the first human rotavirus vaccines were developed using bovine (e.g., Nebraska Calf Diarrhea Virus) or simian rotaviruses and veterinarians have long used measles vaccine to protect animals against the antigenically related canine distemper virus. Vaccinia virus used for smallpox vaccination in modern times is not cowpox, but a different orthopoxvirus (closer to horsepox than cowpox) which arose during uncontrolled passages in animals and humans prior to the establishment of standardized manufacturing in the middle of the 20th Century.

The development of all other live viral vaccines awaited the availability of tools for isolating and efficiently growing obligate intracellular viruses and for identifying, quantitating, and characterizing them in the laboratory. These tools first became available in the late 1920s and early 1930s, when yellow fever, influenza, and polio viruses were shown to infect laboratory rodents (Theiler, Armstrong and others), chorio-allantoic membranes of the developing chick were demonstrated to be susceptible to fowlpox virus (Woodruff and Goodpasture), and various relatively crude tissue culture systems were developed for growing viruses (Maitland, Carrell, and others). Use of the embryonated egg for virus propagation enabled the isolation of causative agents of viral diseases, for example mumps virus (in 1934) and the production of live vaccines, including vaccinia (1933) and yellow fever (1936). Cell culture methods developed by Enders, Weller, and Robbins in 1948 enabled

the propagation of mumps, measles, polio, and other viruses for vaccine production. The advent of molecular biology in the 1960s has allowed the rational design of new live viral vaccines, including reassortant rotavirus vaccines, chimeric, live vaccines, and many other new generation vaccines described elsewhere in this book.

140 years had elapsed since Jenner's smallpox vaccine was described, before a new live, attenuated vaccine for humans was developed (Fig. 1). Following the lead of Pasteur, who had "fixed" rabies virus neurovirulence by serial passage in rabbit brain and had thereby reduced its virulence for dogs, Max Theiler (Rockefeller Institute, New York) passed the French strain of yellow fever virus (a virus evolutionarily adapted to primate hosts) by serial passage in mouse brain until it lost its ability to cause hepatitis in monkeys, while retaining a relatively high level of neurotropism. The virus fixed by intracerebral passage up to 176 times was used to prepare a vaccine from a clarified suspension of mouse brain tissue. In 1931, Sawyer, Kitchen, and Lloyd used Theiler's French neurotropic virus for human immunization [1]. Human immune serum was added to the vaccine, since the virus was thought to be insufficiently attenuated for direct application. By 1936, Max Theiler and Hugh Smith had developed a safer, live attenuated yellow fever vaccine (the 17D strain) by serially passaging another wild-type virus (the Asibi strain) in cultures of minced mouse embryos and embryonated chick embryos; this vaccine was shown in human trials to be safe and highly immunogenic [2], and the 17D vaccine remains in wide use, with over 600 million doses distributed over ensuing decades. Other early attempts were abortive and did not result in approved products. About the same time as yellow fever vaccine was being tested, Smorodintsev et al. in the Soviet Union immunized volunteers with a mouse-adapted strain of influenza virus given by the respiratory route [3], and in 1935, Kolmer prepared a live vaccine candidate against poliomyelitis that had been attenuated by serial passage in monkey spinal cord tissue (the vaccine proved unsafe, causing cases of polio at an incidence of 1 in 1,000) [4].

Live, attenuated viruses represent the most widely used and effective approach to human vaccination. Of the 16 licensed vaccines against human viral diseases available today, 12 (75%) are live, attenuated vaccines and four (25%, polio, influenza, Japanese encephalitis, and hepatitis A) are used as either inactivated or live, attenuated products (Table 1).Only four widely available, approved viral vaccines for humans (hepatitis B, human papillomavirus, rabies, tick-borne encephalitis) and two regional vaccines (Kyasanur Forest disease, hemorrhagic fever with renal syndrome) are available solely as inactivated or recombinant subunit vaccines. Classical, live, attenuated investigational vaccines against respiratory syncytial virus, parainfluenza, dengue, West Nile, Venezuelan equine encephalitis, Rift Valley fever, and chikungunya are in various stages of development. Although considered too risky for human immunization, a live attenuated HIV vaccine (or the SIV equivalent) has demonstrated the feasibility (in the monkey model) of prophylactic immunization against this most challenging disease [5]. The success of live vaccines is based on a suite of recognized advantages, described in the next section and in Table 2. The obstacles to development of live vaccines include (1) the difficulty in finding the correct balance of attenuation (safety, tolerability) and immunogenicity,

Table 2 Advantages of live viral vaccines

Replicate in host, expand antigenic mass
Combine roles of delivery vehicle, adjuvant, and antigen
Often target antigen presenting cells
Wide dissemination of antigen
Contains all antigens of pathogen of interest in native configuration
Strong innate immune response, cytokine environment similar to pathogen of interest
Intracellular replication, MHC-I presentation, cytotoxic CD8+ T cell response
Durable response, large pool of memory B and CD8+ T cells

which is still largely an empirical exercise though facilitated today by knowledge of molecular determinants of virulence and immunity, and (2) the inability to propagate certain viruses in a suitable substrate. The regulatory pathway for live vaccines is increasingly difficult, and it is certain that the time for the development of some older vaccines, such as smallpox, rubella and polio would have been much longer if undertaken in today's regulatory environment due to concerns over safety. The principal problems with live viral vaccines are the potential for serious adverse events related to active infection in individuals with acquired or hereditary susceptibility factors; genetic instability of the virus; lack of thermostability and (historically), contamination with adventitious viruses during manufacture.

3 Development of Classical Vaccines

The principle that attenuation of virulence would occur by passage of a virus in an unnatural host was established first by Pasteur, who found that the virulence of rabies virus became attenuated for dogs after serial passages in rabbit brain. The same principle was applied by Theiler in developing yellow fever vaccine by adaptation in mouse brain or in chick embryo tissue culture [for which he received the only Nobel Prize (1951) for a virus vaccine [6]]; by Koprowski et al. in adapting poliovirus type 2 to mice and cotton rats [7]; by Enders et al. in adapting measles virus by passage in human kidney, human amnion, and chick embryo cells [8]; and by Maassab et al. by adapting influenza virus in primary chick kidney cells [9], to name but a few examples. In some cases, tissue culture passages were done at suboptimal temperature for virus growth as a means of selecting a temperature sensitive virus population that would be less fit for replication in humans at higher body temperature. This approach was employed in the development of measles, influenza, varicella, and the (investigational) respiratory syncytial virus vaccines. The attenuating mutations that occurred in oral polio vaccine strains during passage also rendered them temperature sensitive. The passage history of some vaccines was quite elaborate; in particular, the development of oral polio vaccines by Sabin involved meticulous plaque purification and neurovirulence testing to select candidate strains. Theiler monitored the virulence of yellow fever virus at many points

during its passage series in minced chick embryo tissue culture by inoculating monkeys intracerebrally and found that neurovirulence and viscerotropism were lost between the 89th and 114th passages (Fig. 2) [10]. The passage history of the SA14-14-2 Japanese encephalitis vaccine involved some peculiar empirical passages in rodents by different routes (Fig. 2). Those viruses for which no animal model existed for human virulence, such as measles, mumps, rubella, and varicella posed the problem that attenuation could not be readily assessed as the passage series in tissue cultures progressed. The decision to stop a passage series and perform clinical tests to determine whether the correct balance of attenuation and efficacy had been reached was essentially a "judgment call" and resulted from a series of trials and errors [11].

4 Genetic Basis of Attenuation

For many vaccines, including measles and mumps, the genetic basis of attenuation is unknown. Comparison of the genomic sequences of classical live vaccine strains and their virulent parents, and the use of infectious cDNA clones, in which mutations or reversions that occurred in vaccine strains are investigated for their phenotypic effects, have provided descriptive insights into the molecular basis for attenuation of some vaccines. However, due to the large number of mutations that have arisen during the long series of passages in the derivation of live vaccines and the multigenic nature of virulence, it has often not been possible to pin-point the role of specific determinants. For example, the 17D and parental Asibi strains of yellow fever differ at 20 amino acids and four nucleotide changes in the 3′ noncoding region (NCR). It has not yet been determined precisely which mutations are responsible for the reduced virulence of the 17D vaccine strain [12], although gene switching experiments have shown that the mutations in both the structural genes [principally the envelope (E) glycoprotein] and the nonstructural region are important [13]. Rubella vaccines and their progenitors have been sequenced; the TO-336 vaccine differed from its parent at 21 nucleotides but the phenotypic changes associated with mutations are unknown and no consistent pattern of mutations occurred in other vaccine strains [14]. Similarly, varicella vaccine (Oka) and its wild-type parent have been compared; a number of sequence differences have been found but the determinants responsible for the attenuation remain undefined [13]. In contrast, precise mapping of the molecular determinants of the live poliovirus vaccines has been accomplished. For example, in the case of poliovirus types 2, attenuation is dependent on only two nucleotide changes, one in the 5′ NCR and a threonine→isoleucine mutation in a capsid gene VP1, at position 143 [15]. Poliovirus types 1 and 3 also have a critical attenuating mutation in the 5′NCR (as well as attenuating mutations in the capsid genes.) The polio type 1 vaccine has many more attenuating mutations in the capsid (and one in a nonstructural gene) consistent with the better safety record of that serotype [16].

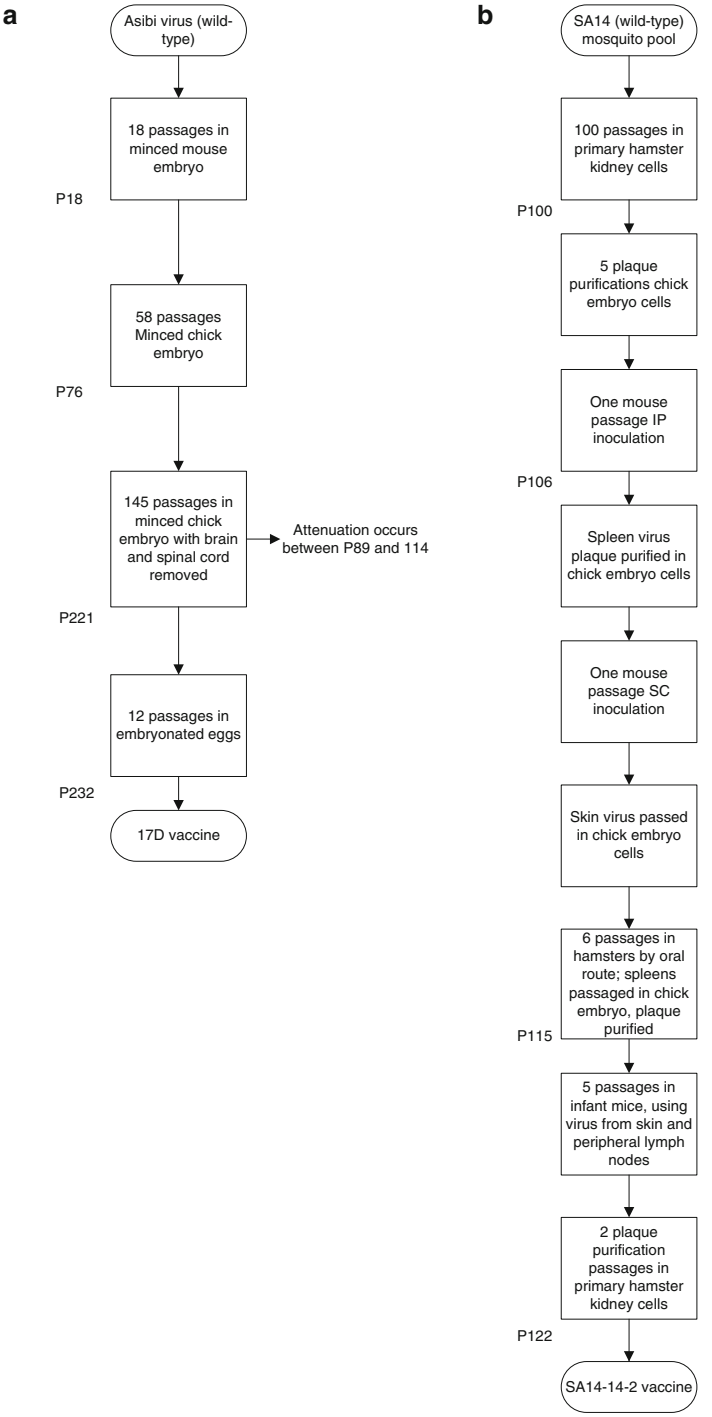

Fig. 2 Passage histories of A. yellow fever 17D and B. the live, attenuated Japanese encephalitis SA14-14-2 vaccine, leading to development of attenuated vaccines

5 Advantages of Live Viral Vaccines

Most naturally acquired viral infections induce a durable state of immunity against reinfection with the homologous virus, which is often life-long. If this immunity is not complete, at least disease expression is modified and less severe. Live viral vaccines mimic this attribute of natural infection, whereas inactivated or subunit vaccines are generally associated with short-lived immunity. The reasons for this difference are not completely understood. Some key factors are the activation of innate immunity by the active infection and the production of type I interferons promoting a strong adaptive immune response, a brisk CD4+ helper T cell response, and a large pool of memory B and CD8+ T cells. Unlike inactivated or subunit vaccines, replicating live vaccines cause an expansion of antigenic mass and present all of the epitopes of the native virus to antigen presenting cells. Antigen processing proceeds in a Class-I-dependent manner, identical to that following natural viral infection. Both humoral and cellular responses are evoked efficiently. Finally, live viral vaccines can be delivered by the natural (e.g., oral, intranasal) route of infection and elicit mucosal immunity protecting the portals of entry of virus infection.

The extraordinary efficacy of yellow fever 17D vaccine has spurred studies aimed at understanding the ability of this vaccine to rapidly elicit strong, life-long immunity in virtually 100% of individuals [17] after a single dose of 100 plaque forming units or less [12]. The adaptive immune response includes early production of IgM antibodies that last for 18 months or more [18], robust neutralizing antibody production that persist life-long [19], a balanced Th1 and Th2 helper cell response, and a CD8+ T cell response [20]. The gene activation signatures following yellow fever 17D vaccination and interaction of the virus with immune cells have been studied in detail [21–23]. Yellow fever 17D virus targets dendritic cells (DCs) as an early site of replication, though productive replication in these cells appears to be limited [24, 25]. The virus activates myeloid and plasmacytoid DCs to produce proinflammatory cytokines (IL-12p40, IL-6 and interferon-α) via toll-like receptors (TLR) 2, 7, 8, and 9 [26]. Other proinflammatory mediators induced in peripheral blood mononuclear cells of vaccinees include IL-1α and CXC-chemokine ligand 10 (CXCL10) [20]. The activation of multiple TLRs is responsible for the mixed Th1 and Th2 helper T cell response seen after 17D immunization. Production of interferon-α by plasmacytoid DCs requires TLR-mediated activation of the mammalian target of rapamycin (mTOR), a regulator of cytokine expression, and activation of downstream mediators of mTOR, p70 ribosomal S6 protein kinases [24].The innate immune receptors RIG-I and melanoma differentiation-associated gene 5 (MDA-5) are stimulated also, as well as the genes that regulate these signaling pathways (e.g., RIG-I-like RNA helicase). Transcription factors that regulate expression of interferon-α are activated (e.g., IRF7, ETS, and STAT1). In addition, genes in the AIM2 (inflammasome) pathway and the complement pathway are switched on. Importantly, gene signatures correlate with the magnitude of the B and T cell responses in individual subjects. Complement protein C1qB, eukaryotic translation factor 2 alpha kinase 4 (EIF2AKA) and solute carrier family 2,

member 6 (SLC2A6) correlated with CD8+ responses [20, 26]. Activation of TNF receptor superfamily, receptor 17 (TNFRSF17), a receptor for B cell activating factor (BAFF), was a correlate of the magnitude of the antibody response. Overall, the gene signatures indicate a marked up-regulation of the innate immune system that persisted for about 2 weeks after vaccination. This response determined, in turn, the strength and persistence of the adaptive polyfunctional immune responses to 17D vaccine. It is likely that analogous patterns of gene activation occur following natural infection, and that they will also be uncovered when other live viral vaccines are subjected to study.

An obvious control for studies of gene activation and the role of innate immune activation in adaptive immune responses would be a comparison of the latter following the administration of an inactivated or subunit vaccine against the same virus. Inactivated (subunit) vaccines tend to elicit Th2 oriented helper T cell responses, weak or absent CD8+ cytotoxic T cell responses, and B cell responses that are relatively short-lived. How these differences are reflected in the gene activation signatures following immunization with inactivated versus live virus vaccines remains to be determined.

Live vaccines have the potential advantage of being administered via the natural route of infection, and of stimulating mucosal as well as systemic immunity. The live attenuated intranasal vaccine against influenza is immunogenic and effective, given as a single dose to adults or two doses to children. Unlike the parenteral (inactivated) influenza vaccine, the intranasal vaccine does not efficiently elicit rises in hemagglutination-inhibiting antibodies, indicating that other mechanisms, which more closely mimic the responses to natural influenza infection, underlie protective immunity [27]. The live vaccine is able to evoke CD8+ T cells [28] and secretory IgA antibodies [29], whereas inactivated vaccine does not. It is likely that mucosal immunity and T cells in the respiratory tract play an important role in vaccine induced protection against infection in the upper and lower airway epithelium.

Similarly, oral polio vaccine is associated with the production of local immunity that not only protects the host but also restricts virus replication in the pharynx and gut and reduces transmission of wild-type viruses, a benefit particularly important in the developing world. Mucosal immunity is evoked in a high proportion of neonates even after a single dose of oral vaccine. Oral polio vaccine is more efficient than inactivated vaccine in inducing mucosal immunity [30].

The application of measles vaccine by aerosol induces higher systemic and mucosal antibody responses than subcutaneous inoculation [31].

6 Benefits of Live Virus Vaccine Utilization

The public health impact of live virus vaccines can hardly be exaggerated. Smallpox vaccine was critical first to the prevention and control of smallpox worldwide, and then, as part of a strategy of surveillance and containment, vaccination contributed

to the eradication of the disease (the first and only example of the eradication of a human infectious disease). The vaccine protects against illness not only if given before exposure but also postexposure when administered during the first week.

The success of poliovirus vaccination is almost as spectacular. Oral polio vaccine was introduced into routine childhood immunization worldwide [32], and this approach combined with mass campaigns has led to significant declines in polio and elimination of the disease in many parts of the world, particularly the Western Hemisphere. Overall vaccine coverage rates approach 80% [33]. Seroconversion rates in industrialized countries are >95% after three doses, but immunogenicity is substantially less in developing countries (~73%) [34] due to interference by immunity to heterologous enteroviruses or ingestion of maternal (milk) antibody. The vaccine not only induces serum neutralizing antibodies that protect the central nervous system but also affords mucosal protection and a reduction in the potential for transmission. Eradication of polio may be feasible, but the use of oral (live) vaccine will need to be replaced with inactivated vaccine because of the problem of persistence and transmission of the live vaccine virus and recombinant viruses.

Measles vaccine is highly effective, resulting in >95% seroconversion rates and efficacy under field conditions of >90% [35]. The vaccine is generally given as a stand-alone vaccine in the Expanded Program of Immunization, or is combined with mumps and rubella in developed and some developing countries. Overall vaccine coverage worldwide approaches 80% [31]. Some countries, e.g., Scandanavia, have been able to eliminate measles altogether through vaccination. Because of the high infectiousness of the virus, where immunization coverage is incomplete or lapses, the disease has reappeared.

Rubella vaccination has led to dramatic declines in the incidence of congenital rubella syndrome, and indeed this disease can be eradicated where immunization practices are vigorously applied.

Two arthropod-borne diseases, yellow fever and Japanese encephalitis, have been successfully controlled through routine vaccination and mass campaigns in many endemic regions. Like the yellow fever vaccine, the live attenuated SA14-14-2 Japanese encephalitis vaccine induces protective immunity after a single dose in >90% of vaccines, although immunity may be less persistent.

7 Problems with Live Vaccines

7.1 Shedding, Viremia, and Recombination

With the exception of oral polio vaccine and vaccinia virus, shedding of live viral vaccines is considered to be brief in duration and low in magnitude, with very low risk of transmission to contacts.

Smallpox vaccine (vaccinia virus) is delivered by epidermal scarification and causes a cutaneous lesion that sheds virus for prolonged periods (~3 weeks) [36],

until scabbing is complete. This can be the source of infection of direct contacts or persons exposed to fomites from contaminated clothing or dressings, who in turn may develop severe adverse events if they have underlying risk factors such as eczema or immune suppression. The application of dressings to limit shedding and precautions to limit fomite spread is recommended [37].

Between 70 and 90% of infants undergoing primary immunization with oral polio vaccine excrete virus in feces [38]. This is a frequent source of infection for family members and nonfamilial contacts. As discussed below, shedding and transmission of virus assumes greatest importance when neurovirulent revertant virus [causing vaccine-associated paralytic poliomyelitis (VAPP)] is spread to immunologically competent or immunodeficient contacts. Individuals with B cell deficiency can become life-long carriers of vaccine derived polioviruses, posing a significant challenge for the eradication of the disease [39].

The cold-adapted live intranasal influenza vaccine is shed in nasal secretions, but at a very low level. Shedding of virus following vaccination occurs transiently (for ~1 day) versus 5 days or more in the case of natural infection, and viral loads are ~1,000-fold lower than in natural infections [40, 41]. The risk of transmission is thus exceedingly small. When monovalent influenza A vaccine was administered to subjects without immunity to the vaccine strain, 40–80% shed virus with relatively low peak titers between 1.5 and 3.0 log $TCID_{50}$/mL [42]. A theoretical concern for use of the live, avian (H5N1) influenza vaccine in the context of active transmission is that the vaccine virus could reassort with wild-type virulent H5N1 virus and produce a virulent virus adapted for interhuman transmission.

The likelihood of horizontal transmission of measles vaccine virus is considered very low, although shedding in respiratory secretions following subcutaneous vaccination has been documented occasionally [43]. Measles virus is shed in urine for 10 days or more after natural infection, and shedding may be prolonged in immune deficient children [44]. Viral RNA or antigen has also been detected in urine of a high proportion of vaccinees [45].

After natural infection, mumps virus is shed in respiratory secretions, principally before the onset of parotitis [46]. Shedding of live mumps vaccine has been documented, and transmission to susceptible contacts recorded, principally in the case of less attenuated vaccine strains such as Urabe and Leningrad-3 [47, 48].

Skin lesions are the source of shedding and potential transmission of the varicella vaccine virus. The vaccine causes skin lesions in ~3% of healthy vaccinees, and these are exceedingly mild. Transmission to unimmunized contacts is extremely rare, and only a few documented cases have been reported. However, in leukemic children vaccination may be associated with increased number of skin lesions and potential for shedding. In one study, 23% of contacts were infected and 17% developed a rash [49]. Tertiary spread is exceedingly rare.

Yellow fever 17D produces a minimal viremia in humans, and the levels (< 2.0 $\log_{10}$ PFU/mL) are ~ 10,000 times lower than observed in wild-type yellow fever infections, and are insufficient to infect mosquitoes [11]. Moreover, yellow fever 17D are incapable of infecting mosquitoes after oral infection [50]. The live, attenuated Japanese encephalitis SA14-14-2 vaccine is infectious for mosquitoes,

but the low viremia levels in vaccinated subjects precludes infection and transmission. Viremia following yellow fever 17D vaccination is the source of neuroinvasion in rare cases of yellow fever vaccine associated neurotropic adverse events (YEL-AND).

7.2 Genetic Instability

The replication of RNA viruses is error-prone resulting in rapid adaptation and evolution, and these viruses characteristically have a quasi-species nature (heterogenous mixtures of sequence variants). High mutation rates are believed to be due to the absence of proofreading enzymes in viral RNA-dependent RNA polymerases or reverse transcriptases. The generally accepted average incidence of mutations is 10^{-4}–10^{-5} per nucleotide per round of RNA replication [51, 52]. However, this rate may differ among RNA viruses; for example one estimate of the mutation rate in the yellow fever 17D polymerase gene was as low as 5.7×10^{-8} per copied nucleotide, suggesting this virus was more genetically stable than most [53]. An additional feature of RNA viruses is the occurrence of "hot spots" for mutation in different functional areas of the genome; these may be related to adaptation for growth in a particular cell type (see the chapter "Recombinant, chimeric, live, attenuated vaccines against flaviviruses and alphaviruses" – for examples).

The genome sequence and biological characteristics of the subpopulations in quasi-species vaccines can differ greatly, and selection of a more virulent subpopulation in the vaccinated host can lead to adverse consequences. For example, the Urabe strain of mumps vaccine (discontinued due to neurovirulence) contains subpopulations that differ in the sequence of the hemagglutinin-neuraminidase gene and the propensity to cause meningitis post-vaccination [54]. The yellow fever 17D vaccine is also a mixed population, and it contains subpopulations with different plaque morphology and reactivity with monoclonal antibodies [11]. This "magic sauce" of virion populations appears to have the right balance of attenuation and efficacy, which could be perturbed with a few passages, since over-attenuation and a higher frequency of serious adverse events occurred during early development when passage level was not controlled by a seed lot system. Specifically, a high incidence of postvaccinal encephalitis was associated with uncontrolled passage of yellow fever 17D sub-strains during the early years of vaccine manufacture [55]. The problem was resolved when stabilization of passage level during vaccine manufacture was instituted in 1941. Similarly, when the Japanese encephalitis SA-14-14-2 vaccine underwent some additional passages and was manufactured in a new substrate (primary dog kidney) [56] instead of the standard primary hamster kidney cell substrate, it was overattenuated when tested clinically.

The quasi-species nature of oral poliovirus vaccine is especially problematic due to revertants in the 5′NCR mutations that are critical to attenuation [57, 58]. Revertants associated with an increase in neurovirulence and failure to pass the monkey safety test occur at a low frequency (0.1% of the total virus population) but

can increase during large scale production. The most important adverse event associated with the use of oral poliovirus vaccine is VAPP, which is most commonly caused by the polio type 3 vaccine component. The incidence of VAPP following primary vaccination is approximately 1 in 900,000 [59]. Immune deficiency, particularly B cell deficiency is a risk factor for VAPP, due in part to uncontrolled replication and an opportunity for the emergence of revertant strains. Analysis of viruses isolated from VAPP patients shows that the majority retain genome sequences that are less than 1% divergent from the original vaccines, but as would be expected sequence divergence depends on the duration of time between vaccination and isolation. Persistent infections occur particularly in immune deficient subjects. Most cases of VAPP result from infection with revertants at determinants associated with increased neurovirulence, particularly for polio 2 and 3 where attenuation is controlled by only 2 or 3 mutations, including the single 5′NCR mutations [60–62]. Recombination events (with oral polio vaccine strains and heterologous enteroviruses) are also implicated in VAPP.

Perhaps because of its lower mutation rate [63], yellow fever 17D vaccine has been less frequently associated with adverse events arising as a result of mutation. Deaths from postvaccinal encephalitis (yellow fever vaccine associated neurotropic disease) are exceedingly rare but from one case (a 3 year-old girl in the US) virus was recovered from the brain which exhibited increased neurovirulence for experimental animals (mice and monkeys). The isolate differed from 17D vaccine at two determinants in the E gene (E155 and E303) and in a nonstructural gene (NS4B76) [63]. The mutation at E303 is very close to a determinant (E305) that distinguishes 17D vaccine from wild-type yellow fever virus and is located in Domain III of the E protein, which contains ligands for cell-receptor interactions. It is possible that mutations in this region could have altered tropism of the vaccine virus for neural tissue.

Genetic instability at determinants controlling replication can result from selective pressure in vitro or in vivo. For example, mutations in loci controlling temperature sensitivity (ts) can be favored under conditions of nonpermissive growth temperatures [64]. Polioviruses isolated from patients with VAPP have lost the ts phenotype and have increased fitness for growth in human intestine [39, 65].

The reader is referred to the chapter on recombinant, chimeric, live, attenuated vaccines against flaviviruses and alphaviruses for further details on problems in vaccine development associated with genetic stability of live RNA virus vaccines.

In contrast to RNA viruses, vaccines using live DNA viruses are inherently stable [63].

7.3 Thermostability and Microbial Contamination

Most live vaccines are relatively unstable unless kept cold or lyophilized, and vaccine failures have been caused by improper storage and handling [66]. The instability of live vaccines has required the establishment of an elaborate cold-chain

infrastructure to support vaccine distribution in the Expanded Program of Immunization and has added significantly to the costs and complexity of childhood immunization in developing countries. In the late 1970s efforts to improve the stability of lyophilized measles and yellow fever viruses, two of the least thermostable vaccines, resulted in significant improvements. However, the improvements are incremental and have not approached the ideal of a liquid, heat-stable formulation that would not require a cold-chain (this has been the subject of significant research efforts stimulated by the Gates Grand Challenge grants). Moreover, once reconstituted to the liquid state, infectivity (potency) of live vaccines is lost rapidly, and the reconstituted vaccines must be administered within 1 h (yellow fever) or 8 h (measles). The live, varicella vaccine is especially thermolabile, must be stored lyophilized in a mechanical freezer, and used within 30 min after reconstitution. Thermostability requirements have been established by the World Health Organization and differ across vaccines. In general, these requirements specify that the vaccine must retain minimum potency and not lose more than a specified amount (e.g., 0.5 or 1 $\log_{10}$) under accelerated conditions (exposure at 37°C temperature) for a specified length of time which may be as short as 2 days (polio) or as long as 30 days (smallpox). Excipients used by some manufacturers to stabilize live vaccines, in particular hydrolyzed porcine gelatin [67], can be responsible for allergic adverse events.

With one exception (smallpox vaccine), it is not possible to include antimicrobial agents in multidose vaccine vials due to the fact that these agents inactivate viruses; this is another reason why the vaccine must be used quickly after reconstitution. There have been a number of reported episodes of bacterial contamination of improperly handled multidose vaccine vials, resulting in serious infections and even deaths [12]. An exception is smallpox (vaccinia), which is not adversely affected by glycerol–phenol preservatives in the diluent used to reconstitute the lyophilized vaccine, which is in a 100-dose container used for repeated percutaneous vaccinations over 30 days after reconstitution.

7.4 Adventitious Viruses

It is obvious that live vaccines would contain any passenger virus that happened to be introduced during manufacture or as a result of contamination of the original isolate used to develop the vaccine. The control of such contamination has steadily improved over the years, and currently stringent steps are taken to reduce the possibility of such contamination and to detect it through the use of both compendial and special testing procedures applied to cell banks, raw materials, virus seeds, and vaccine lots. There has been an effort in the vaccines industry to eliminate sources of such contamination by using continuous cell cultures which can be readily controlled rather than primary cell cultures or tissues (from embryonated eggs and animals) for virus propagation, and to remove bovine serum and other animal-derived proteins from the manufacturing process. This movement was

accelerated after the bovine spongiform encephalopathy epizootic in the 1980s. When a new strategic supply of smallpox vaccine was required (to protect against biological attack), serum-free cell culture methods of manufacture were selected in place of the original calf lymph production [68, 69]. The concern over animal viruses contaminating vaccines has increased with the recognition that many viruses would not be detected by standard testing methods, such as bovine and porcine circoviruses, polyomaviruses, and caliciviruses. The early history of vaccine development is replete with significant problems related to adventitious agents, and a few illustrative examples will be given.

Embryonated hens' eggs were used for the propagation of measles and yellow fever viruses, beginning in the 1930s and primary chick embryo cells have been used to prepare measles and mumps vaccines. Flocks from which eggs were sourced during the early years were not controlled for adventitious virus infections. In 1966, measles and yellow fever 17D vaccines were discovered to be contaminated with avian leukosis virus [70]. Although the presence of leukosis virus is certainly undesirable because of the possibility of insertion of leukosis virus oncogenes, there is no evidence to implicate the virus in human disease. The question was addressed by a retrospective survey for cancer deaths in veterans who had received yellow fever vaccine as early as World War II [71]. The incidence of all cancers, lymphoma, and leukemia was not significantly different (and in fact was lower) in persons vaccinated 5–22 years previously with 17D vaccine than in those not vaccinated. All major manufacturers of 17D currently utilize eggs from ALV and other specific pathogen-free (SPF) flocks. Vaccines manufactured in eggs or chick embryo cells, including yellow fever 17D, test positive by the product-enhanced reverse transcriptase (PERT) assay, reflecting the presence of defective particles containing endogenous avian leucosis or retrovirus sequences [72]. No evidence has been found for infectious or inducible replication competent retroviruses or for infection with avian leucosis or endogenous avian retrovirus in humans [73].

The lack of thermostability of live viral vaccines has required the addition of stabilizers, typically proteins, to improve shelf life and stability under conditions of shipment, storage, and use in the field. In the early days of vaccine development, pooled normal human serum was used as a stabilizing excipient added to vaccines. Yellow fever 17D was originally prepared in this way. In 1937, Findlay and MacCallum reported cases of acute hepatitis in persons who received 17D vaccine [74]. Cases were also reported in Brazil during the vaccination campaigns between 1938 and 1940 and, after careful study, were attributed to an adventitious agent in the vaccine rather than to hepatitis caused by 17D virus [75]. In 1942 a large epidemic of jaundice occurred in US military personnel who had received 17D vaccine, with 28,000 cases and 62 deaths [76]. The use of pooled serum for stabilizing 17D vaccines was discontinued in Brazil in 1940 and in the USA in 1943. Subsequent retrospective serological studies confirmed that the responsible agent was hepatitis B virus [77]. In addition to the acute deaths from fulminant hepatitis B, the vaccinees had a 3.3-fold increased risk of later developing liver cancer [78].

Fetal bovine serum (FBS) is commonly used during expansion of cell cultures for virus propagation. A common contaminant of FBS is bovine viral diarrhea virus (BVDV), a ruminant pestivirus. Before the routine control of raw materials and cell banks for the presence of BVDV, many vaccine lots contained this adventitious agent and some modern vaccines may still contain infectious virus or at least RNA sequences [79, 80]. Some mammalian cells used for manufacturing, such as Vero cells, are permissive to growth of BVDV [81]. BVDV is not a known human pathogen, but antibodies to BVDV have been found in patients with HIV/AIDS [82] and in children with congenital neurological conditions, and antigen has been found in stools of subjects with infantile gastroenteritis [83]. BVDV has also been recovered from human leukocytes [84].

Cache Valley virus, a mosquito-borne bunyavirus, has been recovered from FBS and, on at least one occasion, it contaminated a biological manufacturing process [85]. Cache Valley virus is teratogenic in sheep and may also be in humans. A human case of severe multiorgan failure and encephalitis caused by natural infection with Cache Valley virus has been reported [86].

Poliovirus vaccines (and a parenteral adenovirus vaccine used by the military) were originally manufactured in primary monkey kidney cell culture. Some lots of vaccine were found to be contaminated with simian virus 40 (SV40), a polyomavirus of Asian macaques that causes a progressive multifocal leukoencephalopathy (PML) syndrome in immunosuppressed monkeys and cancer in rodents. Since many children were exposed to SV40 as a result of polio vaccination in the 1950s and 1960s, long term surveys were organized to assess the risk of cancer. It was initially concluded that no increased risk was associated with exposure [87] and this conclusion was supported by a number of additional epidemiological studies. However, many of these studies were flawed based on the fact that exposed and unexposed populations could not be reliably differentiated. The concern about a role of SV40 in human cancers remains, because many different laboratories have reported finding SV40 in a variety of human cancer tissues, and in particular human mesotheliomas [88, 89]. SV40 does not contaminate current poliovirus vaccines.

7.5 Adverse Events due to Unchecked Replication in the Host

Yellow fever 17D vaccine causes a syndrome of hepatitis, multiorgan failure, cardiovascular collapse, and high lethality (>60%), similar to that caused by the wild-type virus. Fortunately the incidence of yellow fever vaccine associated viscerotropic adverse events (YEL-AVD) is low (between 1:50,000 and 250,000 depending on the occurrence of risk factors for this condition) [12, 90, 91]. Unlike VAPP, the occurrence of this syndrome is not dependent on genetic instability, mutation, or selection of a virus variant with enhanced pathogenicity [92, 93]. Instead, YEL-AVD occurs in individual patients who have one of a variety of underlying, familial or acquired risk factors that permit unchecked replication of 17D virus in vital organs. The occurrence of this syndrome and its severity is analogous to progressive vaccinia in individuals with defects in their T cell

responses, who are unable to clear their live vaccine infections. Indeed, some patients who developed YEL-AVD had previously undergone thymectomy and likely had a defect in adaptive immunity [94]. However the underlying defects leading to YEL-AVD appear to be more complex. One case report described possible defects in chemokine receptor 5 (CCR5) and the CCR5 ligand RANTES [95] and another case report described a possible defect in an allele of the 2′5′-oligoadenylate synthetase 1 (OAS1) gene involved in interferon-specified antiviral responses [96]. Mice deficient in type I interferon receptor genes develop a lethal viscerotropic disease [97]. Thus, defects in innate immunity, so important to the control of adaptive immune responses to yellow fever 17D vaccine, appear to be implicated in some cases of severe adverse events.

7.6 Precautions and Contraindications

Live vaccines have a number of precautions and contraindications for use, aimed at protecting individuals who have underlying risk factors for adverse events. Each vaccine has its unique set of such precautions, but there are some common and logical themes. Age is an established risk factor for increased incidence or severity of many natural viral infections, and the same principle applies to vaccine viruses derived from wild-type viruses. Very young infants (<6 months of age) are at increased risk of wild-type yellow fever and of YEL-AND following 17D vaccine, and are thus excluded from vaccination. This adverse event is likely due to immaturity of the blood brain barrier. (Age may also be a factor limiting effectiveness of live vaccines. Where the prevalence of immunity is high, efficacy of vaccination in young infants (< 6 months) may be impaired due to maternally acquired immunity. This is the case for measles vaccine). Advanced age may also be a risk factor for adverse events; the reporting rate of both YEL-AND and YEL-AVD is >twofold higher in persons > 70 years of age than in younger persons [90]. Pregnancy is a contraindication, mainly on theoretical grounds that the live vaccine virus could infect the placenta or fetus, causing stillbirth or congenital malformation. Inherited or acquired immune deficiency or treatment with immunosuppressive drugs or radiation is also a contraindication for use of most live vaccines. Fortunately significant events have been rare; progressive vaccinia in T cell deficient subjects, the occurrence of fatal YEL-AND in a patient with HIV/AIDS [98], and rare cases of pneumonia, severe rashes and hepatitis following varicella vaccination of immunocompromised patients [99] are illustrative examples.

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Part II
Genetically Attenuated Micro-Organisms as Vaccines

Recombinant Live Vaccines to Protect Against the Severe Acute Respiratory Syndrome Coronavirus

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Abstract The severe acute respiratory syndrome (SARS) coronavirus (CoV) was identified as the etiological agent of an acute respiratory disease causing atypical pneumonia and diarrhea with high mortality. Different types of SARS-CoV vaccines, including nonreplicative and vectored vaccines, have been developed. Administration of these vaccines to animal model systems has shown promise for the generation of efficacious and safe vaccines. Nevertheless, the identification of side effects, preferentially in the elderly animal models, indicates the need to develop novel vaccines that should be tested in improved animal model systems. Live attenuated viruses have generally proven to be the most effective vaccines against viral infections. A limited number of SARS-CoV attenuating modifications have been described, including mutations, and partial or complete gene deletions affecting the replicase, like the nonstructural proteins (nsp1 or nsp2), or the structural genes, and drastic changes in the sequences that regulate the expression of viral subgenomic mRNAs. A promising vaccine candidate developed in our laboratory was based on deletion of the envelope E gene alone, or in combination with the removal of six additional genes nonessential for virus replication. Viruses lacking E protein were attenuated, grew in the lung, and provided homologous and heterologous protection. Improvements of this vaccine candidate have been directed toward increasing virus titers using the power of viruses with mutator phenotypes, while maintaining the attenuated phenotype. The safety of the live SARS-CoV vaccines is being increased by the insertion of complementary modifications in genes nsp1, nsp2, and 3a, by gene scrambling to prevent the rescue of a virulent phenotype by recombination or remodeling of vaccine genomes based on codon deoptimization using synthetic biology. The newly generated vaccine

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candidates are very promising, but need to be evaluated in animal model systems that include young and aged animals.

1 The Disease

The SARS-CoV was identified as the etiological agent of an acute respiratory disease causing atypical pneumonia and diarrhea with an average mortality of 10% [1]. SARS-CoV is a zoonotic virus that crossed the species barrier, most likely originating from bats, and has been grown in other species, notably civets [2–4]. The virus emerged in Guangdong Province, China, in late 2002, and rapidly spread to 32 countries [5–10]. After July 2003, only a few community-acquired and laboratory-acquired SARS cases have been reported (http://www.who.int/csr/sars/en/). Of the human CoVs (HCoVs), such as HCoV-229E, HCoV-OC43, SARS-CoV, HCoV-NL63, and Hong Kong University 1 (HKU1)-CoV, SARS-CoV causes the most severe disease [11, 12].

SARS-CoV–like viruses are circulating in bats from different continents [4, 13–15]. In addition, SARS-CoV could be used as a biological weapon, and it has been declared as a category C priority pathogen by the National Institute of Allergy and Infectious Diseases Biodefense (http://www.niaid.nih.gov/Biodefense/bandc_priority.html). A defined efficacious therapy is not yet available for SARS, and the possibility of a reemergence of the virus due to the presence of a high number of bats in different continents infected with SARS-CoV ancestors is a realistic one. Therefore, the design of an effective and safe vaccine to protect against SARS is still high priority.

2 Types of SARS-CoV Vaccines and Prospects of Protection Against SARS by Vaccination

Several types of SARS vaccines have been developed, including inactivated viruses, subunit vaccines, virus-like particles (VLPs), DNA vaccines, heterologous expression systems, and live attenuated vaccines derived from the SARS-CoV genome by reverse genetics (for recent reviews see [16–19]). This chapter will focus on live attenuated vaccines, i.e., on the development of safe, live, recombinant vaccines based on attenuated SARS-CoV, including biosafety safeguards that can be engineered to assure attenuation.

A preliminary issue is whether previous and current attempts for developing a vaccine to protect against SARS have provided promising results. We believe that this is the case based on the results obtained with different types of vaccines [17–19]. Nevertheless, the vaccines developed so far have not been tested in humans for obvious reasons and, in addition, the animal models used to evaluate

the experimental vaccines do not fully reproduce the clinical signs observed in the natural host. In addition, with few exceptions, the evaluation of these vaccines has been made in young animals, and it has been shown that the outcome of challenge experiments, although positive in young animals, frequently showed side effects when performed in old mice [20, 21]. Recently, animal models have been considerably improved, reproducing most of the pathology observed in humans [22, 23]. In particular, a mouse-adapted SARS-CoV model, selected after fifteen passages in mice (SARS-CoV-MA15), reproduces most clinical signs observed in human infections during the SARS epidemic in 2003, including death of infected mice. This animal model is considered the best available. Therefore, vaccine candidates developed so far may have to be reevaluated in this model using young and aged mice.

2.1 Inactivated and Vectored Vaccines Developed to Prevent SARS

Vaccines based on whole purified inactivated virus have the benefit of presenting a complete repertoire of viral antigens, although inactivated vaccines do not in general provide longlasting immunity. These vaccines provide good protection in mice [24], hamster [25], and partial protection in ferrets [25, 26].

In rhesus monkeys, a formaldehyde-inactivated SARS-CoV vaccine showed partial protection [27, 28]. An inactivated SARS-CoV vaccine was also administered to humans. This vaccine was safe and induced neutralizing antibodies, but no efficacy data have been reported [29]. Overall, inactivated vaccines, based on whole purified virus, induced neutralizing antibodies, were apparently safe at least in young animal models and provided good protection.

Subunit vaccines have the advantage of their simplicity, chemical definition, and lack of potential variability [30]. In the case of SARS-CoV, a great advantage is that well-defined S protein domains binding the cell receptor human angiotensin converting enzyme (hACE2) have provided full immune protection [31–34]. This concept is reinforced by the observation that monoclonal antibodies specific for the receptor binding domain elicited protection in several animal models, including African green monkeys [35–41].

In addition to S protein–derived domains or peptides, protein 3a, a large protein of SARS-CoV exposed on its envelope, also elicits virus neutralizing antibodies [42] and could be useful in improving subunit vaccines. Furthermore, immunity to SARS-CoV has also been demonstrated with virus-like particles (VLP) [43]. Overall, the results obtained with subunit vaccines strongly suggest that protection against SARS by vaccination is feasible.

DNA vaccines are safe and nonexpensive but, often, are not very efficient in large mammals. DNA vaccines induce SARS-CoV neutralizing antibodies and protection in mice [44–47].

The use of viral vectors to protect against SARS has been extensively explored. Adenovirus induced good protection in mice [25]. Modified vaccinia Ankara (MVA) provides protection in mice [35] and ferrets [48] although induction of antibody-dependent enhancement of disease (ADED) was reported. Adeno-associated virus induces long-term protection against SARS-CoV [34]. Parainfluenza virus elicits protection in hamsters and monkeys [49, 50]. Recombinant measles viruses expressing the S protein of SARS-CoV induces neutralizing antibodies and immune responses against SARS-CoV [51]. Newcastle disease recombinant virus expressing the S protein of SARS-CoV induces neutralizing antibodies in African green monkeys immunized via the respiratory tract [52]. A recombinant attenuated vesicular stomatitis virus (VSV) protects mice against SARS-CoV challenge 4 months after vaccination [53, 54]. Venezuelan equine encephalitis (VEE) virus expressing the S protein of SARS-CoV induces protection against challenge with virulent virus in the mouse model [20]. Overall, these results indicate that there is a very good prospect for the development of an efficacious and safe vaccine to prevent SARS. Nevertheless, there are relevant aspects that need to be improved in order to achieve a vaccine that can be fully protective and free of side effects both in young and in elderly people.

3 The Virus

SARS-CoV is an enveloped, single-stranded positive-sense RNA virus with a genome of 29.7 kb that belongs to Genus β of the *Coronaviridae* [55–57] (Fig. 1a). The replicase gene is encoded within the 5′ two-thirds of the SARS-CoV genome, including two overlapping open reading frames (ORF) named ORFs 1a and 1b. The latter is translated by a ribosomal frameshift upstream of the ORF 1a stop codon [58, 59] (Fig. 1b). Translation of both ORFs in the cytoplasm of infected cells results in the synthesis of two polyproteins, pp1a and pp1ab, that are processed by two viral proteinases to yield 16 functional nonstructural proteins (nsps) [60, 61]. These nsps are the components of the membrane-anchored replication–transcription complex [62]. All CoVs encode species-specific accessory genes in their downstream ORFs, with a remarkably conserved order: replicase/transcriptase, spike (S), envelope (E), membrane (M), and nucleocapsid (N). The lipid bilayer envelope contains at least three proteins: E and M that coordinate virion assembly and release, and the large peplomer S (Fig 1a). This glycoprotein is located on the virion surface, conferring the virus characteristic corona shape. S is the main mediator of host cell attachment and entry. SARS-CoV ORFs 3a, 6, 7a, and 7b encode additional virus membrane proteins [63–67]. Other accessory proteins are 8a, 8b and 9b. The functions for most of the accessory proteins are still unclear; however, it is known that some of these proteins influence virus–host interaction and viral pathogenesis [68–70].

For SARS-CoV, hACE2 molecule serves as a receptor [71]; CD209L has also been implicated as an alternative receptor in entry [72].

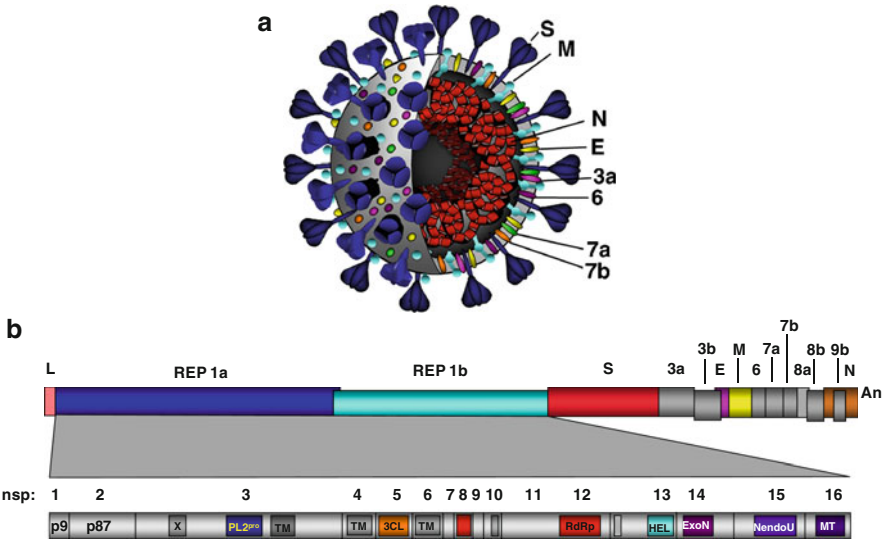

Fig. 1 SARS-CoV structure and genome organization. (**a**) Schematic diagram of SARS-CoV structure. *S*, spike protein; *M*, membrane protein; *E*, envelope protein; *N*, nucleoprotein; *3a*, *6*, *7a*, and *7b*, accessory structural proteins. (**b**) Schematic representation of SARS-CoV genome. *Rep 1a* and *1b*, replicase genes; *3b*, *6*, *8a*, *8b*, and *9b*, nonessential genes. Other genes as in (**a**). In the *bottom* boxes, the putative functional open reading frames of the SARS-CoV replicase are indicated. *Nsp*, replicase nonstructural proteins; *p9* and *p87*, tentative amino-terminus replicase polypeptides; *X*, ADP-O-ribose 1′-phosphatase; *PL2^{pro}*, papain-like cysteine protease 2; *TM*, putative transmembrane domains; *3CL*, 3C-like main protease; *RdRp*, RNA-dependent RNA polymerase; *HEL*, helicase; *ExoN*, 3′-to-5′-exoribonuclease; *NendoU*, Nidoviral uridylate-specific endoribonuclease; *MT*, putative ribose-2′-*O*-methyltransferase

4 Generation of Recombinant SARS-CoV Vaccines Based on the Deletion or Modification of Genes

Live attenuated viruses have generally proven to be the most effective vaccines against viral infections. The production of effective and safe live attenuated vaccines for animal CoVs has not been satisfactory, largely because vaccine strains are insufficiently immunogenic and, in addition, may recombine, resulting in novel viruses with increased virulence [73–75]. Several groups, including ours, have described modifications to the SARS-CoV that are attenuating. These "domesticated" viruses may be useful platforms to develop inactivated or live vaccines.

In general, for RNA viruses, it is essential to develop a reverse genetic system to develop a virus with an attenuated phenotype. This is certainly the case for coronaviruses that have the largest genome known (around 30 kb) for an RNA virus, increasing the technical difficulty of generating an infectious cDNA. We have developed efficient transmissible gastroenteritis CoV (TGEV) and SARS-CoV reverse genetics systems, by inserting infectious cDNA clones of these viruses into bacterial artificial chromosomes (BACs) [76–80] (Fig. 2a). In this system, the

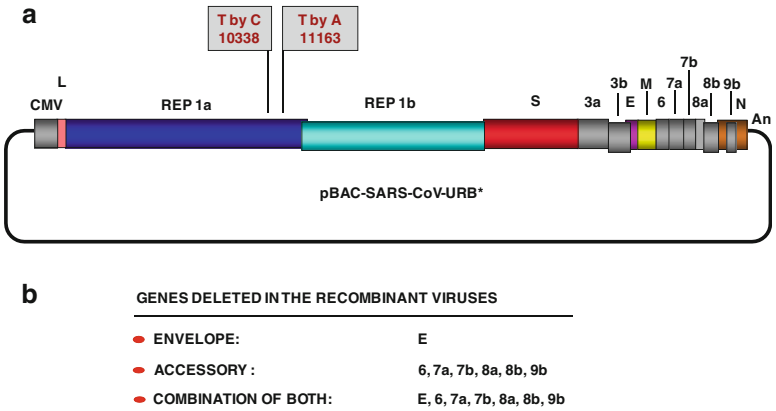

Fig. 2 Structure of the infectious SARS-CoV cDNA cloned in a bacterial artificial chromosome and the derived deletion mutants. (**a**) Schematic structure of the SARS-CoV infectious cDNA of the Urbani strain, cloned in a bacterial artificial chromosome (BAC). The infectious cDNA is expressed under the control of the cytomegalovirus promoter (CMV); *L* leader; the cDNA includes genetic markers (T10338C and T11163A), introduced to differentiate the engineered clone from the wild-type Urbani strain genome; *acronyms* on the top of the bar indicate gene names, as in Fig. 1. (**b**) Table with the genes deleted in three SARS-CoV recombinant viruses. Deletion mutants without E gene led to viruses with attenuated phenotypes

genomic RNA is expressed in the cell nucleus under the control of a cytomegalovirus promoter (first amplification by the cellular polymerase II), with subsequent amplification in the cytoplasm by the viral RNA-dependent RNA polymerase. This reverse genetics system is highly efficient because it implies two amplification steps. In addition, cDNA stability in the BAC is very high. Soon after the BAC technology was applied to assemble an infectious coronavirus cDNA clone, alternative strategies were developed, including (1) a system to assemble a full-length cDNA construct of the TGEV genome by using adjoining cDNA subclones that have unique, flanking, interconnecting junctions [81]. Transcripts derived from the TGEV cDNA assembled using this approach can be used to derive infectious recombinant virus; (2) a system in which the cloning vector is a poxvirus. Using the genome of this poxvirus including the genome cDNA copy as a template, the viral genome is transcribed in vitro, and infectious virus is recovered from transfected cells [82]; (3) a modified procedure was described in which the coronavirus genomic RNA is transcribed inside cells using a poxvirus genome as a template. To this end, the viral genome is cloned under the control of T7 promoter, and the poxvirus DNA including the infectious cDNA is transfected into cells that are infected with a poxvirus expressing T7 polymerase [83]. The generated transcript reconstitutes an infectious CoV.

In the case of SARS-CoV, several genes have been deleted in order to generate viruses with attenuated phenotypes. Nevertheless, deletion of one or more accessory genes did not significantly attenuate SARS-CoV [88]. Fortunately, we showed that deletion of the E gene, encoding the envelope protein, led to a viable SARS-CoV, indicating that E protein is not essential for virus replication. Interestingly, viruses

lacking E protein are attenuated, grow in the lung, and are immunogenic in different animal models [79, 85–87].

Modification or deletion of other SARS-CoV genes has also been considered in the design of vaccines to prevent SARS. Some of these genes (nsp1, nsp14, S, and N) are essential for virus replication, while others (3a, 6, 7a, 7b, 8a, 8b, 9b) are nonessential for virus growth in cell culture or in vivo. The design of SARS vaccines based on deletion of SARS-CoV genes is described below. Nevertheless, most attention is given to the deletion or modification of E, nsp1, nsp2, and 3a genes.

SARS-CoV deletion mutants lacking each of ORFs 3a, 3b, 6, 7a, or 7b did not significantly influence in vitro and in vivo replication efficiency in the mouse model [88, 89]. All recombinant viruses replicated to similar wild-type levels, suggesting that either the group-specific ORFs play a limited role in in vivo replication efficiency or that the mouse model used in the evaluation does not meet the requirements to discriminate the activity of group-specific ORFs in disease [88]. In fact, it was unexpected that the deletion of ORFs such as 3a, 7a, and 7b which encode structural proteins [64, 67, 88, 90, 91] would show little influence on virus replication in the mouse model. Only deletion of ORF 3a showed a minor decrease (below tenfold) in virus growth. Furthermore, deletion of combinations of genes, such as deletion of ORFs 3a and 3b, and ORF6, showed a 10–30-fold titer reduction in Vero cells, but showed a limited effect on virus growth in the murine model at day 2 postinfection. Moreover, the simultaneous deletion of larger combinations of group-specific genes such as 6, 7a, 7b, 8a, 8b, and 9b has led to the production of an infectious SARS-CoV deletion mutant that propagated in cell culture with a titer similar to that of the parental wild-type virus and was not attenuated in transgenic mice that expressed the SARS-CoV receptor (hACE2) [85]. Therefore, the effect of SARS-CoV gene deletions needs to be tested in more relevant animal models.

Interestingly, the deletion of the E gene alone, or in combination with the removal of genes 6–9b, led to mutant viruses that seem to be promising vaccine candidates [79, 85–87], and is described next.

4.1 Vaccines Based on the Deletion of E Protein

The E gene was nonessential for the genus β MHV CoV [92], although elimination of this gene from the MHV genome reduced virus growth in cell culture more than 1,000-fold. In contrast, for the group 1 TGEV coronavirus, expression of the E gene product was essential for virus release and spread. Propagation of E gene–deleted TGEV (TGEV-ΔE) was restored by providing E protein in *trans* [93, 94]. A recombinant SARS-CoV (rSARS-CoV) that lacks the E gene, generated from a BAC (Fig. 2b), was recovered in Vero E6 cells with a relatively high titer (around 10^6 pfu/ml) and also from Huh-7 and CaCo-2 cells with low titers, indicating that SARS-CoV E protein is not essential for virus replication in cell culture [79]. Electron microscopy observation of Vero E6 cells infected with the SARS-CoV

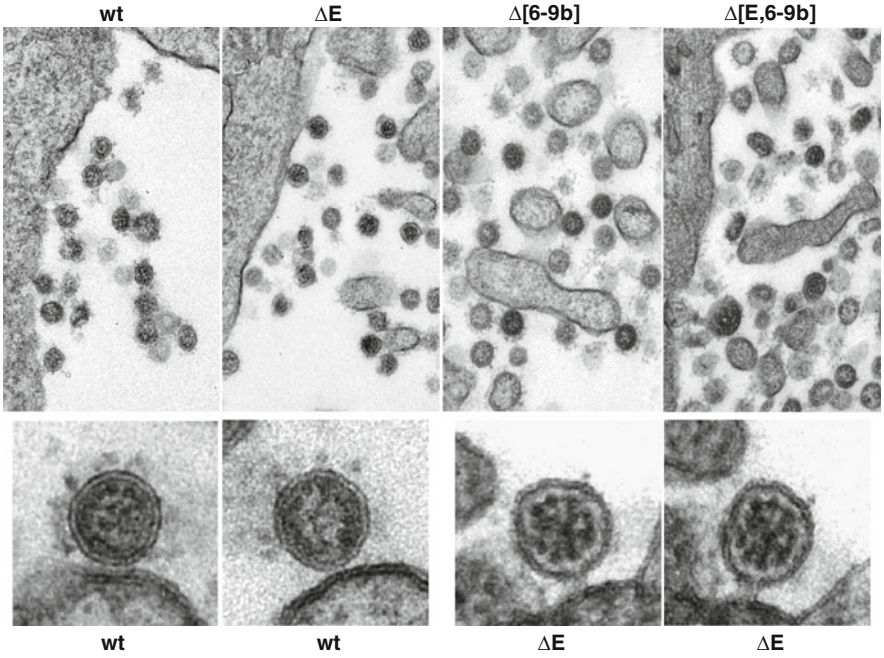

Fig. 3 Electron microscopy of SARS-CoV and envelope protein deletion mutants. (**a**) Extracellular viruses released from cells infected with the SARS-CoVs indicated at the *top*. (**b**) Micrographs of *wt* and SARS-CoV-ΔE mutants in the budding process. In cells infected with the wt virus, 5% of the virions in the final budding step were found bound to the cell, whereas in the E protein-deleted viruses, this number was increased to 16%, suggesting that absence of E protein led to a delay in the "pinch-off step"

wt or the ΔE deletion mutant showed a higher efficiency of assembly and release for the *wt* virus (Fig. 3). In this respect, SARS-CoV-ΔE behaves like MHV, although SARS-CoV-ΔE grows to a considerably higher titer. Vaccine viability and efficacy require the production of viruses with high titers. Interestingly, adaptation of the rSARS-CoV-ΔE virus to grow in Vero cells after 16 passages led to an increase of virus titers reaching values almost identical to those displayed by the full-length virus (around 10^7 pfu/ml) [87]. This titer is close to those required for a competitive live attenuated vaccine.

4.2 Evaluation of SARS-CoV-ΔE Vaccine Candidate in Different Animal Model Systems

While SARS-CoV infects and replicates in several species, including mice, ferrets, hamsters, and nonhuman primates, most of these animals only develop inapparent or mild disease [95]. An ideal animal model that completely reproduces human

clinical disease and pathological findings has not been identified. To evaluate the rSARS-CoV-ΔE vaccine candidate, we have used three animal model systems: hamster, transgenic mice expressing the hACE2 receptor for human SARS-CoV, and conventional mice challenged with the mouse-adapted virus [22, 23, 79, 85–87, 96–98]. These animal model systems are complementary.

The *hamster model* has been used to study SARS-CoV-ΔE virus pathogenicity, because it demonstrates elements present in human cases of SARS-CoV infections including interstitial pneumonitis and consolidation [79, 96, 97]. The hamster model reproducibly supports SARS-CoV replication in the respiratory tract to a higher titer and for a longer duration than in mice or nonhuman primates. Virus replication in this model is accompanied by histological evidence of pneumonitis, and the animals develop viremia and extrapulmonary spread of virus [96]. Although overt clinical disease is absent, the hamster model is a useful model for the evaluation of SARS-CoV infection. Titers of recombinant SARS-CoV (rSARS-CoV) achieved in the respiratory tract of hamsters were similar to those previously reported for the wild-type virus [96] and were at least 100-fold higher than titers of the rSARS-CoV-ΔE virus, suggesting that this mutant virus is attenuated. Histopathological examination of lungs from infected hamsters showed reduced amounts of viral antigen and pulmonary inflammation in rSARS-CoV-ΔE infected than in rSARS-CoV infected animals, indicating that rSARS-CoV-ΔE is attenuated in vivo [79]. In fact, reduction of SARS-CoV titers in patients has been associated with a considerable reduction in pathogenicity and increase in survival rates [99, 100]. rSARS-CoV-ΔE immunized hamsters remained active following wild-type virus challenge while mock immunized displayed decreased activity [86].

The *transgenic mice model* is based on the production of mice expressing the hACE2, the receptor for human SARS-CoV. Transgenic mice models have been obtained in different laboratories by expressing the hACE2 under the control of different promoters [98, 101]. These mice develop moderate respiratory disease, but overwhelming neurological disease with 100% mortality after intranasal infection with SARS-CoV. As such, they are very useful to assess attenuation and vaccine safety and efficacy. We previously showed that infection of these highly susceptible mice with rSARS-CoV-ΔE, or rSARS-CoV with E and several group-specific protein genes 6, 7a, 7b, 8a, 8b, and 9b deleted (rSARS-CoV-[ΔE,6–9b]) resulted in neither weight loss nor death, even after inoculation with very high virus doses [85].

The *mouse-adapted SARS-CoV model* used in the evaluation of the rSARS-CoV-ΔE and rSARS-CoV-Δ[E,6–9b] was based on the recent isolation of a SARS-CoV adapted to growth in mice or rats [22, 102, 103]. This model provided a useful system for vaccine evaluation because some strains of mice and rats infected with these viruses develop severe respiratory disease and even death. A mouse-adapted strain was isolated after 15 passages through the lungs of BALB/c mice (MA15 strain) and, unlike the parental Urbani strain of virus, intranasal inoculation with this virus results in signs of respiratory disease with substantial mortality [22]. We showed that immunization with rSARS-CoV-ΔE or SARS-CoV-Δ[E,6–9b] almost completely protected BALB/c mice from fatal

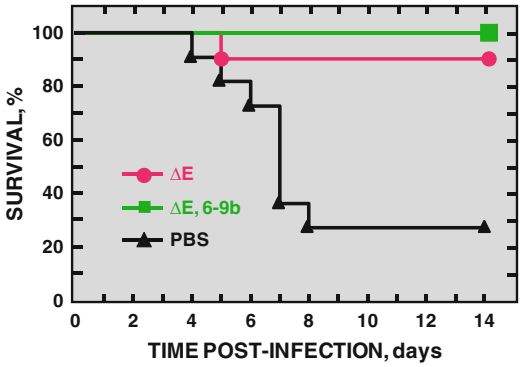

Fig. 4 Protection induced by ΔE mutants against an adapted SARS-CoV in mice. Six-week-old Balb/c mice were immunized with 12,000 pfu of rSARS-CoV-ΔE (*red circles*), rSARS-CoV-Δ[E,6–9b] (*green squares*), or PBS (*black triangles*) and challenged at day 21 post immunization with 1×10^5 pfu of the mouse adapted Urbani strain of SARS-CoV (MA15). Mice were monitored daily for survival

respiratory disease caused by mouse-adapted SARS-CoV (Fig. 4), and partly protected hACE2 transgenic mice from lethal disease [87].

In summary, the immunogenicity and protective efficacy of rSARS-CoV-ΔE has been shown in the three animal model systems described above, hamsters, highly susceptible transgenic mice expressing the hACE2 receptor for human SARS-CoV and conventional mice challenged with the MA15 virus. Interestingly, both homologous and heterologous protection was observed. In fact, hamsters and hACE2 transgenic mice immunized with rSARS-CoV-ΔE developed high serum neutralizing antibody titers and were protected from replication of homologous (SARS-CoV Urbani) and heterologous SARS-CoV (GD03) in the upper and lower respiratory tract [86, 87]. The relevance of this observation is that the GD03 strain of SARS-CoV is one of the serologically most divergent human SARS-CoV identified, in relation to the Urbani strain. In addition, it has been shown that the GD03 strain is closely related to the isolates obtained from animals and if SARS-CoV were to reemerge, it would probably have an animal origin. Despite being attenuated in replication in the respiratory tract, rSARS-CoV-ΔE virus is an immunogenic and efficacious vaccine in hamsters and two mouse models.

4.3 SARS-CoV E Gene Is a Virulence Gene

E gene deletion mutants SARS-CoV-ΔE and SARS-CoV-Δ[E,6–9b] were attenuated in two animal model systems, hamster and transgenic mice, expressing the ACE-2 receptor, as indicated above. In fact, infection with both deletion mutants led to no weight loss, death, or lung immune histopathology, in contrast to infection with virulent SARS-CoV [79, 85–87] (Fig. 5). In addition, a more refined test for virus

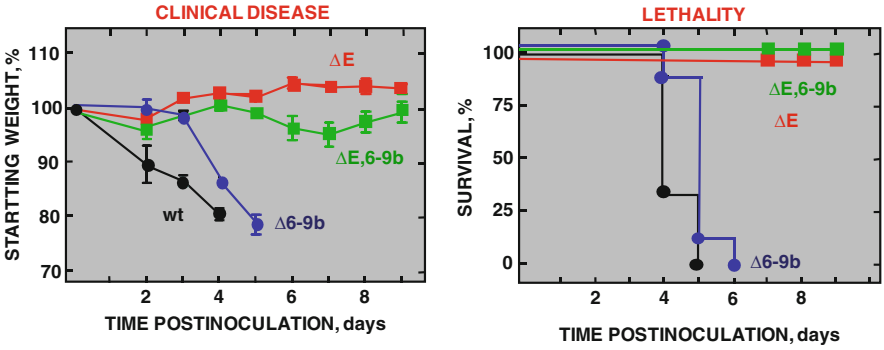

Fig. 5 Effect of SARS-CoV envelope E protein deletion on virus virulence. (**a**). Clinical disease. Six-week-old hACE2 transgenic mice were inoculated with 12,000 pfu of rSARS-CoV-ΔE (*red squares*), rSARS-CoV-Δ[E,6–9b] (*green squares*), wild-type rSARS-CoV (*black circles*), or rSARS-CoV-Δ[6–9b] (*blue circles*) and monitored daily for weight loss (*left*) and survival (*right*)

virulence was performed with hamsters using the activity wheel, and no decrease of hamster activity was detected 7 days after hamster infection with the SARS-CoVs lacking the E gene, in contrast to those infected with a virus with full-length genome. Furthermore, rSARS-CoV-ΔE did not infect the brain of infected transgenic mice, in contrast to the *wt* virus. Overall, these data indicate that E is a virulence gene [79, 85].

The potential mechanism of E gene product in virulence has been investigated in our laboratory. We have shown that the expression of E gene drastically reduced the expression of genes involved in stress and unfolded protein responses [104]. A reduction in stress responses has been associated with a decrease in the innate and specific immune responses [105–108]. As a consequence, we have postulated that deletion of the E gene leads to an increased immune response to the virus, reducing its apparent pathogenicity.

4.4 Future Improvement of rSARS-CoV-ΔE Vaccine

Three complementary strategies are being applied to improve the rSARS-CoV-ΔE vaccine:

4.4.1 To Increase Virus Titers While Maintaining the Attenuated Phenotype

To generate an efficient inactivated or live modified vaccine, virus titers need to be high in order to obtain an economically competitive vaccine. To increase virus titers, we propose a novel approach based on previous findings showing that coronavirus genomes encoding a mutated nsp14 3′-5′-exonuclease (ExoN) display

a mutator phenotype [109]. The engineered SARS-CoV with a mutated or deleted E protein will be modified to include an ExoN that causes the accumulation of mutations throughout the viral genome. The mutated viruses will be passed in cell culture by infecting cells with the highest virus dilution possible. These dilutions should contain only those mutant viruses with the highest titer. Therefore, we expect that serial passages of these dilutions will select virus clones with high titers. Once the desired virus titers have been achieved, it will be confirmed that the high titer viruses are still attenuated in vivo. Virus evolution will be reverted to standard levels by replacing the mutator nsp14 by the native one using the infectious cDNA clone [110]. Selected viruses will be tested for protection as previously described.

4.4.2 Deletion of a Second Gene That Interferes with Host-Immune Response

We have previously shown that rSARS-CoV-ΔE elicited protective immune responses [86, 87]. At the same time, we and others have also shown that it was possible to delete additional nonessential genes to generate viable SARS-CoV [85, 88]. Some of the additionally deleted genes are involved in the inhibition of IFN activation [68, 111]. We propose to delete some of these genes and determine whether removal of any of them increases the immune response to the vaccine candidate.

4.4.3 Construction of rSARS-CoV Mutants with Modified E Protein (E*) Eliciting Higher Immune Responses to the Virus Than rSARS-CoV Without E Protein

SARS-CoV E protein reduced stress, unfolded protein, and immune responses to the virus. We have postulated that efforts to enhance assembly (and levels of viral protein) without diminishing the stress response, which is increased in the absence of E, might increase immunogenicity without compromising safety. As a consequence, we propose the construction of rSARS-CoV mutants with modified E protein (E*) eliciting higher immune responses to the virus than rSARS-CoV-ΔE. In these mutants, an E* coding gene fully functional in virus morphogenesis is inserted within the viral genome. The approach is based on the previous identification of host proteins binding SARS-CoV E protein, influencing virus-induced stress response and the immune response to the virus. E protein ligands were identified by co-immune precipitation and mass spectrometry studies, as we have previously reported [112], and by yeast two-hybrid technologies [113]. The effect of these proteins on the stress and immune response has been identified. We propose to modify specific E protein domains, in order to prevent virus–host cell interactions that counteract the induction of a strong immune response by rSARS-CoV vaccines.

4.5 Live SARS-CoV Vaccines Based on Viruses Attenuated by Modification of Structural or Nonstructural Proteins

We will focus on the modification of three SARS-CoV proteins, as previous findings on these proteins indicate that they are not fully essential for virus viability, and that their modification may lead to attenuated viruses.

4.5.1 Modification of the Replicase nsp1 Gene

Most of the experimental information on the influence of coronavirus replicase protein modification in attenuation has been obtained changing nsp1 and nsp2 [114–119]. In the case of SARS-CoV, it has been shown that nsp1 significantly inhibited IFN-dependent signaling by decreasing the phosphorylation levels of STAT1 while having little effect on those of STAT2, JAK1, and TYK2 [115]. A modification of SARS-CoV nsp1 (mutations R124S and K125E) resulted in a virus that replicated as efficiently as wild-type virus in cells with a defective IFN response, while its replication was strongly attenuated in cells with an intact IFN response [115]. Thus, it is likely that nsp1 mutants will lose virulence and have a reduced pathogenicity.

Alternatively, mutations or deletions in the nsp1 gene could be introduced, similar to those described in the MHV replicase [114, 116] that led to an attenuated CoV phenotype. These types of mutants could be investigated for their relevance in the generation of attenuated SARS-CoV phenotypes that could be tested for vaccine candidates.

4.5.2 Modification of Replicase nsp2 Gene

Deletion of nsp2 in MHV and SARS-CoV viruses caused 0.5–1 log10 reductions in peak titers in single-cycle growth assays, as well as a reduction in viral RNA synthesis and growth [117, 119]. These findings indicate that nsp2 is not essential for virus replication and that its deletion may lead to viruses with an attenuated phenotype. In addition, recent studies with MHV and HCoV-229E suggest that this protein may have functions in pathogenesis [117, 120]. Therefore, nsp2 seems a promising candidate to complement the safety of a rSARS-CoV-ΔE vaccine.

4.5.3 Modification of Protein 3a

This O-glycosylated accessory protein of 274 amino acids forms a K^+-permeable channel-like structure [91]. It is not essential for growth in tissue culture cells, but deletion of the 3a gene leads to a small (5–10-fold reduction) virus titer reduction both in vitro and in vivo [88]. Protein 3a may also be involved in triggering high levels of proinflammatory cytokine and chemokine production [121–123], and its deletion may reduce SARS-CoV virulence. Gene 3a maps at a distal position from

genes nsp1 or nsp2. Therefore, a recombination event that restores the wild phenotype for gene 3a and genes nsp1 or nsp2 in one event seems very unlikely.

5 Development of a SARS-CoV Vaccine by Modification of the Transcription-Regulating Sequences

Coronavirus transcription is regulated by highly conserved sequences preceding each gene. These transcriptional regulatory sequences (TRSs) are almost identical to sequences located at the 5′ end of the genome, just downstream of the leader sequence. The TRS preceding each gene encodes a complementary sequence in the newly synthesized RNA of negative polarity. These RNAs have to hybridize with the TRS located next to the leader in the process of discontinuous RNA synthesis, typical of CoVs. An alternative approach for developing safer, recombination-resistant live coronavirus vaccines has been developed by Baric's group [84]. The novel procedure involves the modification of the TRSs in a SARS-CoV vaccine strain, to a sequence incompatible with the TRS of any known circulating CoVs. It was postulated that recombinant events between *wt* coronaviruses and TRS remodeled SARS-CoV would result in genomes containing incompatible mixed regulatory sequences that block expression of subgenomic mRNAs. Using a molecular clone, the SARS-CoV TRS network was remodeled from ACGAAC to CCGGAT [84]. This rewiring of the genomic transcription network allows efficient replication of the mutant virus, icSARS-CRG. The icSARS-CRG recombinant virus replicated to titers equivalent to *wt* virus and expressed the typical ratios of subgenomic mRNAs and proteins. It has been shown that this vaccine candidate provides protection against challenge with virulent SARS-CoV.

6 Potential Side Effects of SARS-CoV Vaccines

Previous studies using animal CoVs have provided experimental evidence for humoral [124–133] and T cell–mediated responses to animal coronaviruses that exacerbate disease [134], as previously summarized [17]. This safety concern was increased in the case of SARS-CoV by two studies. In one report [135], antibodies that neutralized most human SARS-CoVs also enhanced virus entry mediated by two civet cat SARS-CoVs. These viruses had S glycoproteins related to the SARS-CoV GD03 isolate. In a second report, it has been shown that the administration of MVA-based SARS-CoV S vaccine into ferrets, but not MVA alone, followed by live SARS-CoV challenge, resulted in enhanced hepatitis [136]. Nevertheless, these side effects have not been described in other studies with SARS-CoV in mice, hamster, ferrets, and African green monkeys [24, 35, 36, 44, 96, 137–140].

In general, immunization with vaccine candidates has resulted in the absence of side effects. Nevertheless, there are still three concerns that remain unaddressed.

One is that specific viral proteins, such as SARS-CoV N expressed by a Venezuelan Equine Encephalitis (VEE) virus vector has resulted in enhanced immunopathology following viral challenge [20], similar to the immune pathology observed following vaccination with formalin-inactivated respiratory syncytial virus (RSV) [141–143]. A second main concern is the observation that SARS-CoV vaccines that provide protection in the absence of side effects in young mice show immunopathological complications in aged mice [20]. A third consideration is that most vaccine candidates have been tested in animal models that do not fully reproduce the clinical symptoms observed in humans, and, with one exception, no phase I clinical trials in humans have been performed. Therefore, SARS vaccine candidates would require additional rigorous clinical and immunological evaluation, using the SARS-CoV mouse-adapted virus model, and potential side effect assessment both in young and in aged animals.

7 Future Trends to Increase Biosafety of Live Modified SARS-CoV Vaccines

Live virus vaccine formulations should include rational approaches to minimize the potential reversion to the *wt* phenotype and simultaneously resist recombination repair. In principle, a combination of SARS-CoV genome modifications could lead to viruses with an attenuated phenotype that could be considered safe and effective vaccine candidates.

While rSARS-CoV-ΔE or the selected rSARS-CoV-E* will be attenuated, in principle, reversion to the virulent phenotype could take place by the reintroduction of the E gene into the virus, by recombination with a closely related coronavirus present in the environment. Furthermore, it cannot be excluded that compensatory mutations increasing virus fitness could cause reversion to the virulent phenotype. To minimize these possibilities, additional modifications have to be introduced into the final vaccine candidate, including the modifications of ORFs encoding proteins nsp1, nsp2, or 3a, described above. The advantage of combining deletions or mutations in the E protein with those in nsp1 or nsp2 ORFs reside in that these genes map into distal positions of the genome (more than 20 kb 5′ separation), making it very unlikely that a single recombination event could restore the wt virus phenotype. In addition, other creative reorganizations of the virus genome have been described that could increase SARS-CoV safety (described below).

7.1 Gene Scrambling to Prevent the Rescue of a Virulent Phenotype by Recombination

CoVs have a characteristic, strictly conserved genome organization with genes occurring in the order 5′-Pol-S-E-M-N-3′. MHV virus mutants with the genes encoding the structural proteins located in a different order were constructed, and

it was shown that the canonical coronavirus genome organization is not essential for in vivo replication [144]. Some of the mutants showed an attenuated phenotype. Interestingly, rearrangement of the viral genes may be useful in the generation of CoV with reduced risk of generating viable viruses by recombination with circulating field viruses. In fact, potential recombination between viruses with different gene orders most likely will lead to nonviable viruses lacking essential genes.

7.2 Vaccines Based on Codon Deoptimization of Viral Genome

As a result of the degeneracy of the genetic code, all but two amino acids in the protein coding sequence can be encoded by more than one synonymous codon. The frequencies of synonymous codon used for each amino acid are unequal and have coevolved with the cell's translation machinery to avoid excessive use of suboptimal codons, which often correspond to rare or otherwise disadvantaged tRNAs [145, 146]. This results in a phenomenon termed "synonymous codon bias" which varies greatly between evolutionarily distant species [147]. While codon optimization by recombinant methods has been widely used to improve cross-species expression, the opposite direction of reducing expression by intentional introduction of suboptimal synonymous codons has seldom been chosen [146].

De novo gene synthesis with the aim of designing stably attenuated polioviruses and SARS-CoV is a novel strategy to construct virus variants containing synthetic replacements of virus coding sequences by deoptimizing synonymous codon usage. Infection with equal amounts of poliovirus particles revealed a neuroattenuated phenotype and a striking reduction of the specific infectivity of poliovirus particles [145]. Similar attempts have been made by Baric's group to design SARS-CoV vaccines. These vaccine candidates provide protection in the mouse model system after challenge with virulent virus (Ralph Baric, personal communication). Due to the distribution effect of many silent mutations over large genome segments, codon-deoptimized viruses should have genetically stable phenotypes, and they may prove suitable as attenuated substrates for the production of vaccines.

8 Concluding Remarks

The production of effective and safe vaccines for animal coronaviruses, previously reported, has not been satisfactory [17, 18, 73, 74]. In contrast, the production of inactivated, subunit, vaccines based on DNA and recombinant vectors or vaccines generated by reverse genetics using SARS-CoV genomes seem more promising. Vaccine candidates need to be tested in the SARS-CoV mouse-adapted model, and in macaques, in all cases using both young and aged animals. Later, the absence of side effects and safety has to be assessed in human phase I clinical trials.

Vaccine manufacturers have the tendency to use well-defined inactivated vaccines. Unfortunately, this approach has limited efficacy and elicits immune responses with relatively short immunological memory. A possible balance between efficacy and safety is the development of RNA replication-competent propagation-defective vaccine candidates, based on viral replicons that can generate one-cycle viruses using packaging cell lines [148].

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Live-Attenuated *Shigella* Vaccines. Is Encouraging Good Enough?

Yves Germani and Philippe J. Sansonetti

Abstract Several strategies have been used to develop vaccines against *Shigella* infection. Among these, the most tested has been the construction of live attenuated, orally administered vaccine candidates in which defined mutations were introduced in specific genes. Two major options exist: (1) altering key metabolic pathways affecting bacterial growth in tissues or (2) knocking out virulence genes selected upon their expected capacity to affect one or several key steps of the infectious process. In certain cases, the two options have been combined.

Live-attenuated *Shigella* vaccine candidates have shown great promise. They elicited, in general, significant immune responses when administered orally to volunteers. They have the capacity to confer protection by eliciting both mucosal and systemic immune responses, particularly the intestinal production of secretory IgAs directed against the O-antigens, a series of complex surface sugars accounting for the bacterial serotypes, which are known to mediate protection following natural infection. These responses have been measured by evaluating antibody-secreting cells, serum antibody levels, and fecal IgA to O-antigens and individual virulence-related protein antigens for a dozen of vaccine candidates. Live-attenuated vaccines also offer the potential to elicit strong IFN-γ responses, a cytokine that is known to provide protection against *Shigella* infection. With regard to possible T-cell–mediated responses, much basic research is still warranted to optimize vaccine approaches. Owing to the wide range of *Shigella* serotypes and subtypes, there is a priori a need for a multivalent vaccine representing the prevalent species and serotypes. The barrier to the use of live vaccine candidates against shigellosis is

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the issue of multivalency and indications for an average to poor immune responses observed in infants and children in endemic areas. In addition, identification of the correlates of protection is needed to accelerate the development of these vaccines.

1 Introduction

More than 100 years after Shiga's discovery, shigellosis is still a global human health problem and there is neither a licensed vaccine nor a consensus as to the mechanism[s] of host immunity to *Shigella* and optimal vaccine strategy. Still, advances in our understanding of the molecular mechanisms of virulence of *Shigella* have enabled the development of a new generation of live-attenuated candidate vaccines. But progress in attaining a balance achieving safe and effective *Shigella* vaccines remains a challenge.

Bacillary dysentery is endemic throughout the planet, although essentially a major health concern in its most impoverished areas with substandard hygiene and unsafe water supplies. Various surveys carried out in treatment centers show that *Shigella* is associated with 5–15% of cases of diarrhea and 30–50% of cases of dysentery [1]. The incidence of disease declines with the duration of stay in high-risk settings [2].

Shigellosis can be caused by any serotype belonging to four groups: group A (*Shigella dysenteriae* with 17 serotypes), group B (*Shigella flexneri* with 14 serotypes and subserotypes), group C (*Shigella boydii* with 20 serotypes), and group D (*Shigella sonnei* with a single serotype).

The ability of *Shigella* to cause diarrheal illness is restricted to human and higher nonhuman primates (NHP). The disease is characterized in its classical forms, by a short period of watery diarrhea with intestinal cramps and general malaise, followed by the appearance of a dysenteric syndrome that comprises intestinal cramps and tenesmus, leading to permanent emission of bloody, often mucopurulent stools. *Shigella* species cause bacillary dysentery by invading the large intestinal epithelium in which they promote strong inflammation in human and NHP [3]. Acute complications may occur in absence of quick antibiotic treatment, such as toxic megacolon, peritonitis, and septicaemia that is mostly observed in severely malnourished children. Conversely, repeated shigellosis episodes may lead to severe malnutrition, thus a vicious circle.

The serotype 1 of *S. dysenteriae* (i.e., the Shiga bacillus) emerges as one of particular concern, due to expression of the Shiga toxin, a potent cytotoxin that not only aggravates intestinal lesions but also causes major systemic complications such as the Hemolytic Uremic Syndrome (HUS). When poor conditions are concentrated in a single epidemiological crisis, like in refugee camps, the attack and mortality rates may be quite high, as observed in Goma, Zaire, in 1994 in the course of a *S. dysenteriae* 1 epidemic [4].

Projections based on methodologically convincing epidemiological studies from the three previous decades allowed, back in 1999, to evaluate the number of cases of shigellosis to 165 million per year, with a death rate ranging between 500,000 and 1.1 million, 69% being children below 5 years in the developing world [5]. These

impressive figures have undoubtedly led the community to realize that shigellosis is a high-impact disease, particularly in the poorest populations.

Current figures may not be that high, however, although the epidemiological situation is evolving and figures are lacking in key areas, particularly in Africa. Recent surveys indicate that, in general, the incidence of diarrheal diseases remains stable worldwide, although mortality shows a sustained decrease, being currently evaluated at an incidence of 4.9/1,000 per year [6]. A recent epidemiological survey conducted in six Asian countries [1] has established that shigellosis was likely to be following a similar trend with a stable incidence of cases [4.6% of cases of diarrhea], and decreased severity and mortality. The rationale for this change in disease profile is still unknown. Several pieces of explanation may be proposed, such as better nutrition and hygiene paralleling economic development of the Asian continent, absence of current epidemics of *S. dysenteriae* 1, better education of mothers, improvement of primary health care, and extended use of oral rehydration solution (ORS) and antibiotics.

The issue of antibiotic (multi)resistance is likely to be an important one, however. Beyond the possibly positive impact of free, uncontrolled use of antibiotics on the disease profile at this stage, one may soon face a new crisis associated with massive multidrug resistance. In some areas, the prevalence of strains resistant to all first-line antibiotics, including fluoroquinolones, reaches 5% and is clearly on the rise [7].

Shigella infection appears to be more ubiquitous in impoverished Asian populations than previously thought, and new antibiotic-resistant strains of different species and serotypes are emerging in this part of the world [1]. It is also unlikely that the epidemiological situation in Asia can be generalized, thus a need for an exhaustive evaluation of the incidence of shigellosis, particularly in the sub-Saharan part of the African continent. Current economic stagnation and frequent social instability are creating conditions for shigellosis to remain a leading cause of morbidity and mortality. In order to facilitate such studies, there is a need for efficient and durable surveillance networks benefiting from good microbiological expertise and novel quick, reliable, and robust diagnostic tools such as immunochromatographic dipsticks that could be used directly on fecal samples [8].

All elements considered, including the permanent risk of massive re-emerging epidemics, the need for a *Shigella* vaccine clearly remains. Its major target would be the pediatric population of the developing world, essentially infants around the age of 1 year, and possibly also the elderly population that represents the other peak of disease susceptibility. Such a vaccine could also benefit travelers to high-risk areas, particularly those working or intervening in these areas, e.g., members of nongovernmental organizations (NGOs), army personnel.

2 The Need for an Epidemiologically Valid *Shigella* Vaccine

Shigella flexneri is endemic in developing countries and accounts for most *Shigella* infections worldwide [9]. Pandemics of *S. dysenteriae* 1 dysentery, as they occurred in Central America from 1968 to 1972 [10], South Asia in the 1970s [11], Central

Africa in the 1980s [12], and East Africa in the 1990s [13, 14], profoundly influence the global mortality burden that can be attributed to *Shigella* [5, 14]. During *S. dysenteriae* type 1 epidemics, all age groups are affected, but in endemic areas, the incidence of shigellosis peaks during the first 5 years of life and declines thereafter, suggesting that immunity develops after repeated exposures during childhood [15]. The lack of *S. dysenteriae* 1 endemicity results in lowbackground immunity in populations, so epidemics of *S. dysenteriae* 1 dysentery affect adults and children alike, and the target ages for the use of a Shiga vaccine would be similarly broad [10–12].

S. sonnei incidence tends to increase in countries where living standards improve, thus dominating as an endemic strain in Western countries. This serotype persists in these transitional countries causing sporadic diarrhea and occasional outbreaks in epidemiological niches [such as day-care centers] [16, 17]. Shigellosis due to *S. boydii* or *S. dysenteriae* serotypes other than type 1 is uncommon [1, 5, 18]. *Shigella* is also a primary cause of traveler's diarrhea in individuals from industrialized countries visiting developing areas [19]. They mainly acquire *S. sonnei* and *S. flexneri* infections [20].

Owing to the wide range of *Shigella* serotypes and subtypes, there is a need for a multivalent vaccine representing prevalent species and serotypes. Furthermore, the protective performance of a *Shigella* vaccine in any particular setting will depend on the representation of serotypes in the vaccine and the relative epidemiological occurrence of different serotypes in this setting. Thus, knowledge of the distribution of serotypes among clinical isolates is a key in designing new vaccines for public health programs.

Ideally, an epidemiologically valid *Shigella* vaccine would provide protection at least against *S. dysenteriae* 1, the dominant *S. flexneri* serotypes and *S. sonnei* [5, 21, 22]. The WHO has set it at the top of its priority list, along with ETEC, for the development of a vaccine, and this has recently emerged as a "*Shigella*-ETEC vaccine initiative" by the Bill & Melinda Gates Foundation.

3 Pathogenesis

Shigella is a pathovar whose pathogenic characteristics have been acquired following acquisition of a large virulence plasmid, a series of adaptive mutations in the chromosome, as well as acquisition of bacteriophages and pathogenicity islands. The virulence plasmid supports a large pathogenicity island that encodes a type III secretion system and some protein effectors (i.e., Ipa [invasion plasmid antigen] proteins) that account for invasion of epithelial cells [23, 24]. Once bacteria have penetrated into cells, they lyse the phagocytic vacuole and escape into the cytoplasm where they move thanks to their capacity to induce polar nucleation and assembly of actin filaments via the plasmid-encoded outer membrane autotransporter protein IcsA/VirG [25]. Motility allows cell-to-cell spread, thus plays a major role in epithelial invasion [26]. Chromosomal sequences account for development of the

infectious process in invaded mucosal tissues, a striking example being the aerobactin operon encoding an iron-chelating complex in *S. flexneri* and *S. sonnei* that is essential for bacterial growth in tissues [27]. Shigella enterotoxins have also been identified. ShET1 is encoded by the chromosome of *S. flexneri 2a* [28, 29] and Sen is encoded by the virulence plasmid of the various subgroups [30, 31].

4 Immune Response to Natural Infection with Wild *Shigella* spp.

Wild-type *Shigella* infection confers protective immunity and prevents disease during subsequent exposures. An individual convalescent from *S. flexneri* 2a infection is protected against reinfection only with the homologous serotype. Compelling evidence of serotype-specific natural immunity comes from the longitudinal study of a cohort of children in whom primary *Shigella* infection conferred 76% protective efficacy against reinfection with the same serotype [18]. This has been the basis for resumed interest in LPS determinants for immunization, even by the parenteral route [32].

Lipopolysaccharide (LPS) O-side chains, the major bacterial surface antigens, are the main target of hostadaptive immunity. Of great relevance to vaccine development is the observation that this immunity is serotype specific. Adult volunteers experimentally infected with either *S. sonnei* or *S. flexneri* were significantly protected against illness only following rechallenge with the homologous strain (64–74% protective efficacy) [33, 34].

Antibody response to the somatic antigens of *Shigella* appear early in infection and follow the typical course for anti-LPS antibodies, that is, an IgM response that peaks within weeks and decreases slowly. Anti-LPS antibodies are elicited upon infection, both locally as secretory IgA and systemically as serum IgG. Antibody-mediated protection has been shown to be mostly serotype specific [35], pointing to the O-specific polysaccharide moiety of LPS, also termed O-antigen (O-Ag), as the target of the protective immune response. Indeed, *Shigella* serotypes are defined by the structure of their O-Antigen repeating unit [36].

This has been a strong incentive to consider that protection was an achievable goal with an oral vaccine reproducing key steps of the natural infectious process. Still, natural protection is not absolute and rather short lasting, and again, essentially serotype specific [37] not necessarily "good news" for *Shigella* vaccine development.

5 Rational Selection of Genes to Develop Live-Attenuated Deleted Mutants and Clinical Trials

Live *Shigella* vaccine candidates can be administered by the oral route, thus avoiding the need for needle injection. They are easier to manufacture than other potential formulations. Clinical trials in adult human volunteers have been invaluable for

evaluating candidate *Shigella* vaccines. A challenge dose of 10–1,000 virulent organisms, preceded by a bicarbonate buffer to reduce gastric acidity, is sufficient to consistently induce the symptoms of shigellosis [34].

5.1 Pioneer Vaccine Candidates

In the attempts to develop a live-attenuated vaccine as reported in [38], David Mel from the Military Medical Institute (Belgrade) conducted controlled phase III field trials on 36,000 adults and children in Yugoslavia in the 1960s [39]. Using streptomycin-dependent (SmD) mutants of *S. flexneri* and *S. sonnei*, he showed that oral vaccination was an achievable goal. He also showed that multiple strains could be mixed in a combination vaccine and that protection was serotype specific [39–41]. To produce this pioneer vaccine candidate, *Shigella* strains were serially cultivated on streptomycin-containing media until they acquired streptomycin resistance and dependence. SmD, i.e., inability to grow in the absence of exogenous streptomycin [42], lost their ability to cause purulent keratoconjunctivitis in Guinea pigs (i.e., Sereny test). This vaccine candidate was administered in four doses (2×10^{10}, 3×10^{10}, 4×10^{10}, and 5×10^{10} CFU) over 11 days. It was clinically rather well tolerated in adults, healthy children, and debilitated institutionalized children [43–47]. Only a small percentage of recipients showed vomiting following administration of the first dose [39, 41, 45–48]. Protection endured for 1 year. A single booster dose extended protection for an additional year [41].

The pioneer SmD vaccine strains, however, had drawbacks because the bases of attenuation were unknown and occasional lots reverted to streptomycin dependency even though the revertants remained negative in the Sereny test. Furthermore, difficulties occurred in the large-scale manufacture and process control [47, 49]. Nevertheless, this first vaccine candidate provided proof of concept for future multivalent vaccines aiming to confer broad-spectrum protection, and this has remained the gold standard over the years, in spite of the possible reversion of the mutation, stressing the need for an association of attenuated mutations consisting in gene deletions, whose selection needs to be rationally based on the increasing knowledge in the pathogenic mechanisms of *Shigella* [50].

Another pioneering work was the construction of a mutant-hybrid *S. flexneri* 2a vaccine strain by Formal et al. [51]. The ability of this hybrid mutant to propagate in the lamina propria was diminished even if epithelial invasion still occurred [52]. In response to this, a *Shigella flexneri* 2a mutant that had lost the ability to invade intestinal epithelial cells has been selected. The xylose–rhamnose region of *Escherichia coli,* which diminishes the ability to propagate in the lamina propria (even if the epithelial invasion occurs) was transferred into it. This mutant-hybrid vaccine candidate was well tolerated and immunogenic but conferred only partial protection [45, 48].

Another vaccine strain was developed by Istrati following successive passages [53, 54]. The genetic basis of the attenuation of the *S. flexneri* 2a strain T_{32}-Istrati

was subsequently shown to correspond to a large deletion of 32-MDa in the virulence plasmid encompassing part of the pathogenicity island, *icsA*/*virG* and *sen*. When evaluated in Romania, *S. flexneri* 2a strain T_{32}-Istrati was shown to be well tolerated and protective [55, 56]. That was confirmed in a randomized placebo-controlled field trial performed in China [54]. Interestingly, this vaccine candidate was also reported to provide protection against shigellosis due to *S. flexneri* 1b and *S. boydii* [rev. in 37].

5.2 Rational Selection of Genes

Following these encouraging initial results, attempts were indeed made at rationally attenuating virulence of candidate strains representing the most frequently isolated serotypes, such as *S. flexneri* 2a and *S. sonnei*, as well as *S. dysenteriae* serotype 1, due to severity of cases.

Because there is only a small margin between the risk caused by moderate attenuation and poor immunogenicity by a strong attenuation, two major strategies have been considered:

1. Altering key metabolic pathways affecting bacterial growth in tissues or
2. Knocking out virulence genes selected upon their expected capacity to affect one or several key steps of the infectious process.

The consequences of introducing different mutations into wild-type *Shigella* strains have been evaluated in clinical trials.

To develop a live bivalent *S. flexneri* 2a and *S. sonnei* vaccine, a hybrid strain expressing both *S. flexneri* 2a and *S. sonnei* O-antigens was developed in China (Lanzhou Institute) [57, 58] by introducing a *S. sonnei* form I plasmid with deletions of *ipa* and *virF* into *S. flexneri* 2a T32 [59]. In *S. sonnei*, the O-antigen is encoded by the *rfb* locus located on the form I invasion plasmid. This bivalent vaccine was evaluated in large numbers of volunteers [54]. Its protective efficacy was 61–65% against *S. sonnei* 57–72%, and 48–52% against heterologous *Shigella* serotypes. Although the use of three high doses of vaccine strain ($> 2 \times 10^{10}$ CFU) remains a practical problem, this vaccine was licensed in China in 1997, but no other clinical trial was performed outside China [58].

More recent vaccine candidates have combined both approaches. *aro D* and *A* mutations were initially considered because they abrogate synthesis of aromatic amino acid, thus impairing growth of bacteria in vivo. An *S. flexneri aro* mutant (SFL124) expressing the *S. flexneri* group antigen Y was constructed [60–62] in an attempt to obtain cross protection across the *S. flexneri* serotypes. The advantage of SFL124 is the possibility to convert it to other *S. flexneri* serotypes, using glucosylating and/or acetylating phages [63–68]. This mutant appeared too attenuated when tested in medical students in Vietnam, thus very well tolerated by volunteers in clinical trials, but weakly immunogenic [60, 62]. This vaccine candidate raised an important issue regarding the bases of its attenuation. It is likely that the

wild-type strain that had been selected was already weakly pathogenic; therefore, its further attenuation by *aro* mutation likely caused insufficient colonization potential and poor immunogenicity.

A recent review has stressed the need to confirm full pathogenicity of the strains that serve as a basis for vaccine construction [37]. This is ethically complicated, but the mere isolation from a patient may not guarantee that the isolate shows "optimal" pathogenicity.

Other metabolic mutations have been considered, particularly *guaAB* that introduces a severe auxotrophy impairing synthesis of nucleic acids [69], as well as mutations impairing the strain's capacity to scavenge ferric iron (Fe^{3+}), a property required to compete for vital Fe^{3+}, via the production of siderophores (i.e., aerobactin or enterochelin), against iron-chelating molecules of mucosal surfaces (i.e., lactoferrin) or tissues (i.e., transferrin) [27]. The most recent *Shigella* vaccine candidates have undergone a combination of metabolic and virulence mutations. This combination can lead to various degrees of attenuation. Current vaccine candidates, on these bases, can fall into the category of weakly attenuated or strongly attenuated strains.

In the category of weakly attenuated candidates belong *icsA*/*virG*-based mutants. IcsA/VirG is an outer membrane protein of *Shigella* that nucleates cellular actin, thereby allowing intracellular motility and cell-to-cell spread of the microorganism. Mutation in this gene impairs the capacity of *Shigella* to spread extensively in the epithelium, away from its initial site of entry [25]. It has been shown that such mutants were directly targeted to colonic solitary nodules, the actual inductive sites of the mucosal immune response [70].

Combined with a deletion of the aerobactin operon (*iuc iut*), in *S. flexneri* 2a, *icsA*/*virG* has provided a vaccine candidate, SC602 (a derivative of wild-type strain Pasteur Institute *S. flexneri* 2a 494), that has undergone phase I and II clinical trials (Walter Reed Army Institute of Research and US Army Institute for Research in Infectious Diseases) whose results were considered encouraging in Western volunteers [71, 72]. In brief, when fed to North American volunteers, dosages $> 10^6$ CFU were unacceptably reactogenic, with fever or significant constitutional symptoms and diarrhea in about 15% of the recipient volunteers. But the strain was strongly immunogenic, eliciting a high percentage of circulating plasmocytes producing anti-LPS IgA by the ELISPOT assay. By contrast, at a dosage level of 10^4 CFU, adverse clinical reactions were uncommon and mild, yet the induced immune response remained moderately robust [71]. When vaccinees who had received a dose of 10^4 CFU as vaccine inoculum were challenged with a wild-type pathogenic *S. flexneri* strain of similar serotype, they appeared fully protected against dysentery, and subsequent studies carried out in the USA and Israel demonstrated the absence of accidental transmission [71]. These data showed that in the experimental challenge model, even a single dose of an engineered vaccine strain could confer significant protection against severe shigellosis. They also demonstrated the difficulty of finding a proper balance between clinical acceptability and immunogenicity in adult volunteers in developed countries.

In a trial in Bangladesh, SC602 was well tolerated (in all age categories, including 1-year-old infants with inocula up to 10^7 CFU), colonization appeared limited, and immunogenicity very weak [73]. One possible explanation for the poor performance in Bangladesh could be that the volunteers had higher background immunity due to previous exposure. On the other hand, several possibly combined hypotheses may account for this issue: the protective role of breast feeding against the vaccine strain in infants, the high level of innate stimulation of the intestinal mucosa by recurrent enteric infections in a highly endemic zone, thereby severely affecting the capacity of the vaccine strain to colonize the mucosa, the high exposure of children, at an early age, to multiple enteric pathogens, including the most prevalent serotypes of *Shigella*, thus a quickly acquired status of adaptive immunity. In any event, these observations are important to consider because they are very unlikely to apply only to this particular category of vaccine candidate. Considering at least two oral doses as a possible solution, it would require a second phase II study in similar epidemiological conditions and thus SC602 awaits further evaluation.

The WRAIR developed a *S. flexneri* 2a vaccine strain with deletions in the *icsA*/*virG*, *sen*, and *set* genes. This intranasal WRSF2G11 vaccine candidate showed higher immunogenicity and protective efficacy than strain SC602 [74], probably because a Δ*icsA*/*virG*-based vaccine, which lacks enterotoxin genes, has lower levels of reactogenicity without hampering immune responses.

A *ΔicsA*/*virG S. sonnei* vaccine candidate was constructed by scientists at WRAIR (WRSS1), although the virulence of this strain had not been demonstrated in volunteers. The form I plasmid of most wild-type *S. sonnei* strains is highly unstable [33, 75]. This invasiveness plasmid is required for expression of O-antigen by this serotype. In contrast with most of wild *S. sonnei* strain, investigators selected this strain because its form I invasiveness plasmid was stable [33, 75]. In a Phase I trial, a strong O-antigen–specific IgA antibody-secreting cells and moderate interferon (IFN)-γ responses in peripheral blood mononuclear cells were observed [73], but low-grade fever or mild diarrhea was recorded in 22% of North American vaccinees given a single dose of 10^6 CFU of WRSS1. Another phase I trial was performed in adults with a single dose of 10^3, 10^4, or 10^5 CFU [76]. At the two lower dosage levels, the vaccine was well tolerated (1 of 30 subjects developed moderate diarrhea and five mild diarrhea). At 10^5 CFU, 2 of 15 subjects developed fever and four experienced moderate diarrhea. At the 10^4 CFU dose, all subjects manifested IgA anti–O-antigen antibody secreting cell responses and 73% of the vaccinees showed more than 50 IgA anti–O-antigen antibody secreting cells per 10^6 PBMC. This dosage level provided the better balance of immunogenicity and clinical acceptability. Recently, Collins et al. [77] administered two *Shigella sonnei* vaccines, WRSs2 and WRSs3, along with WRSS1 to compare their rates of colonization and clinical safety in groups of five rhesus macaques. The primate model provides the most physiologically relevant animal system to test the validity and efficacy of vaccine candidates. In this pilot study using a gastrointestinal model of infection, the vaccine candidates WRSs2 and WRSs3, which have undergone additional deletions in the enterotoxin and LPS modification genes, provided better safety and comparable immunogenicity to those of WRSS1.

Recently, a *S. dysenteriae* 1 vaccine candidate (SC599, Institut Pasteur) has been tested in phase I and II trials (Saint George Vaccine Institute, London, UK, and Centre de Vaccinologie Cochin-Pasteur, Paris, France). The vaccine was well tolerated with only mild constitutional symptoms and was minimally shed in stool; however, serum antibody titers were modest or absent [78, 79]. In this strain, three deletions have been introduced: Δ*icsA*/*virG*, Δ*ent fep fes* (genes encoding the enterochelin system), and Δ*stxA*, the gene encoding the catalytic subunit of Shiga toxin. Unlike its *S. flexneri* and *S. sonnei* Δ*icsA*/*virG* counterparts, this strain has shown good tolerance, limited systemic immunogenicity (as judged by seric IgM, IgG, and IgA titers), and average to good mucosal immunogenicity as judged by percentage of anti-LPS IgA measured by ELISPOT, in comparison to SC602 and WRSS1 [80].

WRSd1 is another *S. dysenteriae* 1 vaccine strain (deletion of *icsA*/*virG*, *fnr*, and *stxAB* genes) developed by WRAIR [81] from the wild-type strain 1617 that was isolated during a dysentery outbreak in Guatemala in 1969. This oral vaccine was evaluated (40 volunteers). Immunogenicity is poor; the reactogenic dose was minimal [9], suggesting that the *fnr* gene affects gastrointestinal tract colonization.

WRSS1, SC602 and WRSd1, are attenuated principally by the loss of the IcsA/VirG protein. One drawback has been the reactogenic symptoms of fever and diarrhea experienced by the volunteers, that increased in a dose-dependent manner. WRSs2 and WRSs3, second-generation IcsA/virG-based *S. sonnei* vaccine candidates, are expected to be less reactogenic while retaining the ability to generate protective levels of immunogenicity. Besides the loss of IcsA/VirG, WRSs2 and WRSs3 also lack plasmid-encoded enterotoxin ShET2-1 and ShET2-2. WRSs3 further lacks MsbB2 that reduces the endotoxicity of the lipid A portion of the bacterial LPS. Studies in cell cultures and in gnotobiotic piglets demonstrate that WRSs2 and WRSs3 have the potential to cause less diarrhea due to loss of ShET2-1 and ShET2-2 as well as alleviate febrile symptoms by loss of MsbB2 [102].

In the absence of clear correlates of protection, it is currently difficult to anticipate the potential of this family of vaccines for the future. This is a particularly important issue, as the serotype-dependent nature of protection would necessitate further construction of strains, particularly *S. flexneri* 3a, 1b, and 6, in order to cover a broader spectrum of serotypes [82], and testing of a combination of these strains to address issues such as interference. Only a phase III efficacy trial conducted in an endemic area may provide the final piece of information required to validate the approach.

Several attenuated derivatives from the *S. flexneri* 2a wild strain 2457T were constructed at the Center for Vaccine Development (University of Maryland). Lessons have also been learnt from clinical trials with the series of attenuated derivatives of this *S. flexneri* 2a strain 2457T. CVD candidate 1203 harbors deletions of *aroA* and *icsA*/*virG* [83]. In a Phase I clinical trial in adults, CVD 1203 was well tolerated at a dose of 10^6 CFU, but highly reactogenic and giving a strong immune response at 10^8 CFU or higher doses [61]. The reactogenicity was probably correlated to the high tumor-necrosis factor-α concentrations assayed in stools and

serum. To achieve a satisfactory degree of attenuation, alternative mutations were evaluated.

CVD 1204 carries deletions in *guaAB*. Tested in a phase I trial, CVD 1204 showed strong immune responses in North American adults but induced a reactogenicity [62].

In our effort to obtain more attenuated candidate, CVD 1207 [31] was developed. This candidate belongs to the category of strongly attenuated strains undergoing a *guaBA* [69] mutation combined with *icsA*/*virG* [83], *set* [28], and *sen* [84] mutations, thereby knocking out the genes encoding two putative enterotoxins of this serotype. CVD 1207was very well tolerated in phase I trials with escalating single doses of up to 10^8 CFU [85]. At 10^9 and 10^{10} CFU, only a few volunteers experienced mild diarrhea [85]. But its immunogenicity was insufficient.

CVD 1208, another isogenic derivative of strain 2457T that carry deletions in *guaAB*, *sen*, and *set* [85], has been tested in a phase I trial [85, 86]. These strains differ from isogenic CVD 1207 by having an intact *icsA*/*virG* gene. This design permitted researchers to assess the impact of inactivating ShET1 and ShET2. The tolerance appeared excellent, including the lack of residual diarrhea, validating the elimination of *Sen* and *Set* expression, thereby allowing administration of vaccine doses up to 10^9 CFU without side effects. At such doses, systemic and mucosal responses reached good levels, similar to those observed with SC602 with a 10^4 CFU inoculum, if one tries a comparison [71]. The enterotoxin-negative strain CVD 1208 was considered a highly attractive vaccine candidate that reflects the desired balance of clinical acceptability and robust immunogenicity. This is clearly an alternative option that also needs to be validated in further trials, including in the field.

Recently, Simon et al. [87] evaluated B memory responses in healthy adult volunteers who received one oral dose of live-attenuated *Shigella flexneri* 2a vaccine. Oral vaccination with live-attenuated *S. flexneri* 2a elicits B[M] cells to LPS and IpaB, suggesting that B[M] responses to *Shigella* antigens should be further studied as a suitable surrogate of protection in shigellosis.

6 Alternative Strategy: Hybrid Live Vector *Shigella* Vaccines

Attempts in the late 1970s to construct hybrid live-attenuated vaccine strains were based on the new specific knowledge of pathogenesis describing the importance of host cell invasion, immune responses to the Ipa proteins and the role of mucosal, cell-mediated immune as well as systemic immune responses in protection. In this strategy, the objective was to express *Shigella* O and antigens (Ipa for instance) to maintain the invasive phenotype of *Shigella* in well-tolerated *E. coli* or attenuated *Salmonella enterica typhi* backgrounds.

The vaccine strain PGAI 42-1-15 was constructed by introducing into *E. coli* O8 the genes encoding synthesis of group- and type-specific O-antigens of *S. flexneri* 2a. Phase I showed PGAI 42-1-15 was well tolerated in US adult volunteers; the vaccine strain was excreted for several days [88], but the vaccine failed to protect volunteers challenged with 10^4 CFU or 10^2 CFU virulent *S. flexneri* 2a strain 2457T [88].

Reason of this failure was unclear, but one hypothesis is that to be protective, an *E. coli* live vector strain must also have the capacity to invade epithelial cells.

An invasive *E. coli* live vector EcSf2a-1 strain was constructed at WRAIR. The invasion plasmid of *S. flexneri* 5 and the genes encoding synthesis of group- and type-specific O-antigens of *S. flexneri* 2a were introduced into *E. coli* K12 [89]. At a dose of 10^9 CFU, 31% of vaccinees developed fever, diarrhea, or dysentery. It was acceptably reactogenic at lower dosage levels but failed to protect vaccinees against experimental challenge with *S. flexneri* 2a [89]. The *aroD*-deleted derivative strain EcSf2a-2 was constructed to diminish reactogenicity [90]. EcSf2a-2 which retained the ability to invade epithelial cells caused adverse reactions. Instead of a strong immunogenicity, only 36% of vaccines were protected against illness during experimental challenge [90].

Investigators at WRAIR developed an oral *Salmonella* Typhi live vaccine strain Ty21a expressing the *S. sonnei* O polysaccharide [91]. This 5076-1C live vector strain was abandoned because it showed lot-to-lot variability in its immunogenicity and ability to protect volunteers in challenge studies against wild-type *S. sonnei* strain 53G [33, 92, 93].

7 Vaccine Candidates in Less Developed Countries

Live bacteria have generally been used in attempts to induce mucosal immunization against enteric pathogens, as they are thought to be more immunogenic than killed cells and, in some cases, could offer the possibility of immunization with a single dose. But the intestinal microbiology and physiology of the healthy population living in developing countries differ significantly from that of populations living in industrialized countries. This has significant influence on the design of a live-attenuated vaccine. Commonly, persons (mainly the pediatric population) living in impoverished areas usually have heavy colonization in their proximal small intestines. This state is accompanied by a local inflammatory state [94, 95] and involves heightened activity by the innate immune system. These changes in the intestine may contribute to the observed blunting of immune responses to orally administered attenuated vaccines [94–96]. For example, some enteric vaccines (cholera, polio, rotavirus) that are reactogenic at low dose in adult volunteers living in developed countries have exhibited lower immunogenicity in volunteer subjects living in developing countries [97–99]. Their immunogenicity is generally successfully enhanced by increasing the number of vaccine organisms per dose or by administering additional doses of vaccine [97].

However, regarding bacillary dysentery, although vaccine candidate SC602 administered at a low dose (10^4 CFU) had been reactogenic, had been heavily excreted and conferred protection in North American adult volunteers, during phase I and II trials in Bangladesh, none of adults and children who ingested up to 10^6 CFU excreted the vaccine strain or mount a significant immune response [100]. One hypothesis is that Bangladeshi volunteers have decreased levels of accessible iron in tissues compared with subjects living in developed countries, so that SC602 is more crippled in

individuals living in developing countries. Furthermore, the immune response in these volunteers may also be depressed by micronutrient deficiencies, especially zinc or vitamin A [101]. Vaccination strategies in the under developed countries setting need also to include consideration of the possible impact of maternal immunity on conferring passive immunity to infants [58].

8 Conclusion

Vaccination offers the greatest hope as an effective and sustainable strategy against shigellosis. An oral *Shigella* live-attenuated vaccine would have distinct advantages over subunit vaccines for use in developing countries. It provides an ideal solution if it is easy and cheap to manufacture, safely stored, and distributed without losing viability. Results from the phase I and II clinical trials of live-attenuated *Shigella* vaccine candidate (CVD 1208, SC602, SC599, WRSS1, WRSd1) are promising. They are safe and immunogenic, and SC602 protects against dysentery. Adverse effects (mild fever, diarrhea) sometimes observed have been reduced by elimination of the *sen* and *set* genes from the vaccine strain CVD1208. Among the issues, one is to obtain the strongest possible immunogenicity combined with the highest level of cross protection, in the simplest possible vaccine preparation. Furthermore, optimal storage conditions to maintain the stability of the vaccine at different temperatures and packaging of the vaccines in single-dose format remain to be developed. Another major question is to define the optimal serotype numbers to be introduced in an oral vaccine, considering the increasing serotype diversity observed depending on the region considered.

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New Generation BCG Vaccines

Michael V. Tullius and Marcus A. Horwitz

Abstract Bacille de Calmette et Guérin (BCG) was attenuated from a virulent strain of *Mycobacterium bovis* a century ago and has since been administered as an anti-tuberculosis (TB) vaccine to more than four billion people, making it the most widely used vaccine of all time. Although BCG provides significant protection against disease and death due to childhood and disseminated forms of TB, the efficacy of BCG against adult, pulmonary disease is inconsistent. Thus, despite near universal vaccination with BCG in TB endemic areas, TB remains a heavy burden worldwide, especially in developing nations. In recent years, BCG has been utilized in two major vaccine development strategies. First, BCG has been used as a vector to express foreign antigens in studies aimed at developing new vaccines against a variety of viral, parasitic, and bacterial pathogens, and against cancer and allergic diseases. More recently, in a new vaccine paradigm, BCG has been used as a homologous vector to overexpress native mycobacterial antigens in studies aimed at developing improved vaccines against TB. As a vaccine vector, BCG has several major advantages including a very well-established safety profile, high immunogenicity (excellent CD4+ and CD8+ T-cell responses and strong TH1-Type immune responses), and low manufacturing cost. As a vector for recombinant TB vaccines, BCG has the additional advantages of providing a broad array of relevant mycobacterial antigens in addition to the recombinant antigens, moderate efficacy to begin with, high acceptability as a replacement vaccine for BCG in TB endemic countries, and the capacity to express *M. tuberculosis* proteins in native form and release them in a way that results in their being processed similarly to *M. tuberculosis* proteins. In addition to the overexpression of native proteins to improve their immunogenicity and protective efficacy against TB, recombinant BCG vaccines have been developed that express immunomodulatory cytokines or have been

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engineered for enhanced antigen presentation. Several new recombinant BCG vaccines against TB have demonstrated improved protective efficacy against *M. tuberculosis*, *M. bovis*, and *M. leprae* in small animal models. Against non-TB targets, results have been variable, but several recombinant BCG vaccines have demonstrated excellent immunogenicity and protective efficacy; stable and high-level expression of foreign antigens in recombinant BCG, in a way that will make them available for proper processing and presentation, have been recurrent challenges.

1 Introduction

Bacille de Calmette et Guérin (BCG), an attenuated strain of *Mycobacterium bovis*, was developed as a vaccine against human tuberculosis (TB), which is primarily caused by the closely related species *Mycobacterium tuberculosis*. Although its efficacy against TB is suboptimal, BCG is the most widely used vaccine of all time, having been administered to more than four billion people since 1921. In addition to its use as a prophylactic agent against TB, BCG has also been used as a therapeutic agent in the treatment of bladder cancer.

BCG is an intracellular bacterial parasite that survives and multiplies in mononuclear phagocytes. Akin to *M. tuberculosis*, inside the human macrophage, BCG resides in an endosomal compartment that does not fuse with lysosomes [1, 2] and is not highly acidified [3].

In the past two decades, BCG has been used as a vaccine vector for a number of purposes. First, it was used as a heterologous vector to express foreign antigens of a variety of pathogens, such as HIV and *Borrelia burgdorferi*, the agent of lyme disease [4–9]. Subsequently, in studies initiated in this laboratory, it was used in a new vaccine paradigm as a homologous vector overexpressing native antigens, so as to improve its protective efficacy against TB [10]. Most recently, a variety of additional modifications of BCG have been explored in an effort to improve its protective efficacy against TB. These include endowing it with immunomodulatory cytokines capable of directing the immune response; altering its life cycle in antigen-presenting cells by engineering it to escape its phagosome and enter the cytoplasm, thereby enhancing class I MHC antigen presentation, and arming it with a protease to enhance its ability to present antigens on MHC molecules. This chapter will focus on these new approaches to improving the immunogenicity and protective efficacy of BCG against TB and on the use of BCG as a vector for new recombinant vaccines against non-tuberculous pathogens.

2 A Brief History of BCG

Albert Calmette and Camille Guérin developed BCG as a TB vaccine by passaging *M. bovis* 230 times between 1906 and 1919 on medium consisting of pieces of potato cooked and soaked in ox bile containing 5% glycerine, after which the

organism lost its pathogenicity for animals. Their strategy was essentially an amalgam of Jenner's smallpox vaccine strategy centered on using a less-virulent species (at least for humans) closely related to the target pathogen and Pasteur's anthrax and rabies vaccine strategy centered on the attenuation of pathogens by culture under nonnatural conditions. The vaccine was first administered to children in 1921. Although controlled studies were not conducted at that time, the vaccine was believed effective in reducing the mortality of TB in children below estimated levels prior to its use, and the vaccine gained wide acceptance in Europe and subsequently elsewhere [11].

Modern molecular analyses have elucidated genetic differences between BCG and *M. tuberculosis*. Many of these differences, involving about 3% of the ~4,000 genes of these organisms and clustered in gene segments or Regions of Difference, reflect genomic differences between *M. bovis* and *M. tuberculosis* [12, 13]. In addition, during its original attenuation from *M. bovis*, the original BCG strain lost a gene segment containing nine open reading frames known as Region of Difference 1 or RD1 [13]. After BCG was distributed to different countries and strains propagated and maintained separately, genetic differences among BCG strains evolved including gene deletions and duplications [12, 13]. Genealogically, strains with relatively few subsequent gene deletions are characterized as "early" strains, and include BCG Russia, Moreau, and Japan, and strains that have acquired additional deletions are characterized as late strains, and include BCG Tice, Connaught, Pasteur, Glaxo, and Danish.

Controlled studies conducted in 1937 and afterwards demonstrated that BCG was efficacious in reducing the incidence and mortality of TB in children and in reducing the incidence of disseminated forms of TB such as meningitis and miliary TB [14, 15]. However, the efficacy of BCG against adult pulmonary TB was inconsistent, ranging from −35% to +80% [16]. A large meta-analysis calculated the overall efficacy of BCG against adult TB at 50% [14], but this figure disguises the fact that trials tended to divide into those demonstrating either high or low efficacy, rather than conform to a normal distribution. Trials in nontropical regions of the world have tended to have high efficacy and trials in tropical regions of the world have tended to have low efficacy [14].

In 2007, the most recent year for which data are available, there were an estimated 9.27 million cases of tuberculosis worldwide [17]. Most of these cases occurred in populations where BCG vaccination is near universal and hence these cases can be viewed as vaccine failures. Thus, whatever the efficacy of BCG, there is considerable room for improvement of this century-old vaccine.

3 Why Is the Efficacy of BCG so Inconsistent?

A number of hypotheses have been advanced as to why BCG protects well against TB in some trials and not in others. They are as follows.

3.1 Prior Exposure to Atypical Environmental Mycobacteria That Mask or Interfere with the Immune Response to BCG

In support of a direct antagonistic effect of environmental mycobacteria, Brandt et al. [18] reported that mice pre-sensitized with a mixture of three environmental bacteria (*Mycobacterium avium*, *Mycobacterium scrofulaceum*, and *Mycobacterium vaccae*) clear BCG more rapidly and are less well protected against *M. tuberculosis* challenge. On the other hand, in a different experimental murine model, pre-sensitization with *M. vaccae* and *M. scrofulaceum* before BCG vaccination enhanced protection against *M. tuberculosis* challenge, and pre-sensitization with *M. avium* had no influence on the protective efficacy of BCG [19].

3.2 Malnutrition That Interferes with the Development of a Protective Immune Response

In support of this idea, McMurray et al. have demonstrated that, in contrast to well-nourished guinea pigs, protein-deficient guinea pigs fail to develop mature well-organized granulomas; moreover, when vaccinated with BCG, the protein-deficient guinea pigs are less well protected against *M. tuberculosis* aerosol challenge than well-nourished guinea pigs [20].

3.3 Use of Different BCG Strains

Some have postulated that some strains of BCG are more attenuated than others and that they consequently induce an inferior protective immune response [12, 21]; Brosch et al. postulated that evolutionarily early strains, with fewer gene deletions, may be more potent than evolutionarily late strains. Against this idea, a study in this laboratory showed that an evolutionarily early strain (BCG Japan) and several evolutionarily late strains of disparate genealogy (BCG Tice, Connaught, Pasteur, and Danish) were comparably efficacious in protecting against *M. tuberculosis* aerosol challenge in the demanding guinea pig model of pulmonary tuberculosis [22]. Moreover, a large meta-analysis failed to find differences in efficacy among strains of BCG or for that matter between BCG and *M. microti* (vole bacillus) [14, 23].

3.4 Exposure to Helminths

Helminth infection is highest in tropical regions of the world. Helminths induce TH2-type immune responses, which may interfere with the capacity of BCG to induce a protective TH1 type of immune response against *M. tuberculosis*. In support of

this, Elias et al. found that worm-infected volunteers have significantly diminished TH1-type responses compared with dewormed controls [24].

3.5 High Levels of IL-4

Rook et al. have proposed that high levels of IL-4 in tropical regions, in part as a result of helminth infection, interfere with the protective immune response to BCG [25].

4 Recombinant BCG Overexpressing Native Proteins as Vaccines Against Tuberculosis

4.1 Rationale for the Choice of BCG as Vector

In addition to recombinant BCG vaccines, many different types of vaccines have been evaluated for efficacy against tuberculosis in animal models including subunit vaccines, DNA vaccines, and attenuated *M. tuberculosis*. With the exception of attenuated *M. tuberculosis*, none of these vaccines matches, let alone surpasses, the efficacy of BCG in the most challenging animal models. Because of the risk of reversion to virulence, attenuated *M. tuberculosis* vaccines are burdened with a significant safety concern; multiple gene deletions will almost certainly be required to obtain regulatory approval of these vaccines, and with these additional deletions, the vaccines are likely to exhibit reduced efficacy. Thus, at present, improving BCG offers the greatest potential for a vaccine that is more efficacious and at least as safe as BCG.

Aside from its moderate efficacy to begin with, BCG has several major advantages as a vector for new recombinant BCG vaccines against tuberculosis (Table 1). First, it has a very well-established safety profile, having been administered to over

Table 1 Advantages of BCG as a vector for recombinant vaccines

General advantages
Well-established safety profile – administered to >4 billion people
Live vaccine – high immunogenicity
Unaffected by maternal antibodies – can be given in a single dose at birth
Elicits excellent CD4+ and CD8+ T-cell responses
Elicits strong TH1-Type immune responses
Relatively inexpensive to manufacture
Additional advantages for recombinant TB vaccines
Provides a broad array of shared mycobacterial antigens in addition to recombinant antigens
Moderate efficacy against *M. tuberculosis* to start with
High acceptability as a replacement vaccine for BCG in high-incidence countries
Similar intracellular compartment to *M. tuberculosis* and hence similar antigen processing
Expresses *M. tuberculosis* proteins in native form

four billion persons. Serious adverse effects are exceedingly rare except in immunocompromised individuals, for whom the vaccine is not recommended. Second, as a live vaccine, it has high immunogenicity. Third, and of great importance, modified versions of BCG that are superior to BCG have very high acceptability as a replacement vaccine for BCG in regions of the world where the burden of tuberculosis is greatest; for all of its shortcomings, BCG is life preserving in such parts of the world, especially in infants, and health care workers are reluctant to accept an alternative vaccine in a vaccine trial that is not clearly at least as efficacious as BCG. In such parts of the world, recombinant BCG is considered "BCG+" and is therefore readily acceptable as an alternative to BCG in vaccine trials, provided of course that it has demonstrated sufficient safety and efficacy in preclinical studies. Fourth, BCG occupies the same intraphagosomal compartment as *M. tuberculosis* in host cells, and consequently processes and presents antigens similarly. In human mononuclear phagocytes, both BCG and *M. tuberculosis* multiply extensively if not exclusively within a phagosome; a small minority of *M. tuberculosis* may exit the phagosome at very late stages of infection (Clemens and Horwitz, unpublished data) as has been reported in myeloid cells [26], possibly as a prelude to lysis of the host cell, but the majority of mycobacterial multiplication takes place in an endosome-like compartment that favors antigen presentation via class II MHC molecules. Fifth, BCG expresses *M. tuberculosis* proteins in native form. Nonmycobacterial vectors may express highly conserved *M. tuberculosis* proteins in native form, but a mycobacterial host is frequently required to express proteins unique to mycobacteria, such as the mycolyl transferases, in native form [27]. Finally, BCG can be manufactured cheaply; cost is of course a significant consideration in the developing regions of the world where tuberculosis has the highest prevalence.

4.2 rBCG30

rBCG30, the first vaccine demonstrated more potent than BCG against tuberculosis, is also the first vaccine of any kind incorporating the strategy of utilizing a homologous vector to overexpress native antigens [10, 28]. This is a powerful vaccine strategy that combines the approach of using a live attenuated homologous vector with the approach of immunizing with key immunoprotective antigens of the target pathogen. In the case of rBCG30, the live attenuated homologous vector is BCG and the key native antigen is the *M. tuberculosis* 30 kDa major secretory protein, a mycolyl transferase also known as the alpha antigen or Antigen 85B.

4.2.1 Rationale for Selecting the *M. tuberculosis* 30 kDa Protein for a Recombinant BCG Vaccine

The rationale for using BCG as a homologous vector is discussed above. The rationale for overexpressing the major secretory protein of *M. tuberculosis* is

derived from the Extracellular Protein Hypothesis for vaccines against intracellular pathogens [29–34]. This hypothesis holds that proteins secreted or otherwise released by intracellular pathogens are key immunoprotective antigens because they are available for proteolytic processing by the host cell and presentation on the host cell surface as MHC–peptide complexes, thus allowing the immune system to generate a population of T cells capable of recognizing the MHC–peptide complexes and exerting an antimicrobial effect against the host cell. The hypothesis further holds that appropriate immunization of a naïve host with such proteins incorporated into a vaccine allows the immune system to generate a functionally equivalent population of T cells later capable of recognizing and exerting an antimicrobial effect against host cells infected with the target intracellular pathogen. Finally, the hypothesis holds that among the extracellular proteins released by intracellular parasites, the most abundant ones will figure most prominently because they would provide the richest display of MHC–peptide complexes on the host cell surface.

The *M. tuberculosis* 30 kDa mycolyl transferase is the most abundant protein released by *M. tuberculosis*, making up almost one-quarter of the total extracellular protein released [33]. The 30 kDa protein (Antigen 85B) is highly homologous with two other mycolyl transferases of ~32 kDa mass – Antigen 85A and Antigen 85C [35]. The 30 kDa protein is not only the major protein secreted into broth culture but also among the major proteins of all types expressed by *M. tuberculosis* in infected human macrophages [36]. The 30 kDa protein is highly immunogenic and, when administered as a purified protein with adjuvant, it induces strong cell-mediated and protective immunity in the guinea pig model of pulmonary tuberculosis [33].

4.2.2 Construction of rBCG30

rBCG30 is a recombinant BCG Tice strain overexpressing the 30 kDa protein from plasmid pMTB30 [10], derived from the *Mycobacterium–Escherichia coli* shuttle vector pSMT3 [37]. The plasmid pMTB30 contains the full-length *M. tuberculosis* 30 kDa protein gene and flanking 5′and 3′ regions including the promoter region. rBCG30 expresses approximately 5.5-fold the amount of 30 kDa protein that the parental BCG strain expresses. The *M. tuberculosis* and BCG 30 kDa proteins are nearly identical, differing from each other by one amino acid. Other commonly used BCG strains including Connaught, Glaxo, Japanese, Copenhagen, and Pasteur produce amounts of 30 kDa protein comparable to that produced by BCG Tice [28].

4.2.3 Preclinical Studies

rBCG30 was tested in the guinea pig model of pulmonary tuberculosis, a model noteworthy for its resemblance to human disease clinically, immunologically, and pathologically, and the gold standard among small animal models of tuberculosis.

BCG protects well in this model, in which animals are immunized and then challenged with *M. tuberculosis* by aerosol. Compared with sham-immunized animals, BCG-immunized animals are protected against weight loss, a hallmark of TB, and death, and they have significantly less lung pathology and a lower burden of *M. tuberculosis* in the lung and spleen (~1.5 – 2 logs fewer 10 weeks after challenge).

Despite the fact that the 30 kDa protein is a relatively abundant secreted protein of BCG, parental BCG induces negligible immune responses to the protein in guinea pigs. In contrast, rBCG30 induces strong cell-mediated immunity, manifest by cutaneous delayed-type hypersensitivity to the 30 kDa protein, and humoral immunity, manifest by high serum antibody titer to the 30 kDa protein.

Paralleling these immune responses, in guinea pigs immunized with BCG or rBCG30 and challenged 10 weeks later by aerosol with virulent *M. tuberculosis* Erdman strain, rBCG30 induces greater protective immunity than BCG. Ten weeks after challenge, rBCG30-immunized guinea pigs had fewer CFU in the lung (0.8 ± 0.1 log fewer) and spleen (1.1 ± 0.1 log fewer) than BCG-immunized animals [34]. In a survival study, rBCG30-immunized guinea pigs survived significantly longer than BCG-immunized animals (Fig. 1) [28]. Remarkably few rBCG30 organisms are required to induce strong cell-mediated and protective immunity [38].

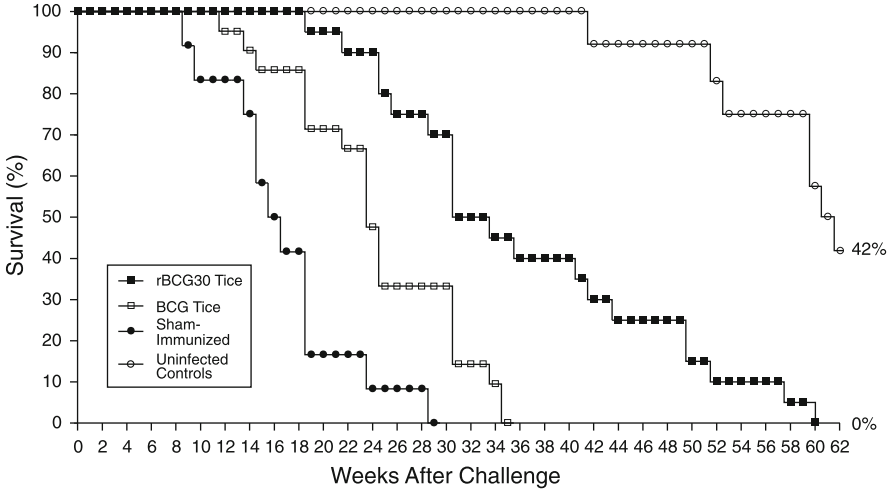

Fig. 1 rBCG30-immunized animals survive longer than BCG-immunized animals after *M. tuberculosis* aerosol challenge. Animals in groups of 20 or 21 were sham immunized or immunized with BCG or rBCG30 Tice, and 10 weeks later challenged by aerosol with virulent *M. tuberculosis*. A group of uninfected animals served as controls. Sham-immunized animals died most rapidly; BCG-immunized animals survived significantly longer than sham-immunized animals; and rBCG30-immunized animals survived significantly longer than BCG-immunized animals. Thirty-five percent of rBCG30-immunized animals survived to the point where uninfected control animals began to die off. Reproduced with permission of the American Society for Microbiology from Horwitz et al. [28].

In addition to enhanced protective efficacy against *M. tuberculosis*, rBCG30 induces greater protective immunity than BCG against *M. bovis*, the primary agent of tuberculosis in domesticated animals, in the guinea pig model and against *M. leprae*, the agent of leprosy, in a murine model (see below) [39, 40].

In preclinical safety studies, rBCG30 was well tolerated. rBCG30 is cleared at the same rate as BCG in guinea pigs, i.e., rBCG30 and BCG are comparably avirulent [28]. No adverse effects were observed in guinea pigs or mice in safety studies conducted by the Aeras Global TB Vaccine Foundation.

4.2.4 Clinical Studies

rBCG30 was tested in a Phase 1 human study, the first live recombinant BCG vaccine against tuberculosis to enter clinical trials [41]. The trial was double blinded with volunteers randomized to rBCG30 or parental BCG Tice. There was no significant difference between the two vaccines in clinical reactogenicity. rBCG30, but not BCG, induced significantly increased Antigen 85B-specific immune responses including significantly increased lymphocyte proliferation, interferon-γ (IFNγ) secretion, IFNγ enzyme-linked immunospot responses, direct ex vivo CD4+ and CD8+ T-cell IFNγ responses, and CD4+ and CD8+ memory T cells capable of expansion. Moreover, in a novel assay of effector cell function, rBCG30 but not BCG significantly increased the number of antigen-specific T cells capable of inhibiting the growth of intracellular mycobacteria (Fig. 2). Thus, rBCG30 was well tolerated and more immunogenic than BCG.

4.3 rBCG Expressing Other Native M. tuberculosis *Proteins*

Recombinant BCG expressing other native *M. tuberculosis* proteins and *M. tuberculosis* fusion proteins have been constructed and evaluated. Noteworthy studies in which protective efficacy has been investigated are discussed below.

4.3.1 rBCG/Antigen 85A

Sugawara and colleagues studied the protective efficacy of a recombinant BCG overexpressing Antigen 85A in guinea pigs, cynomolgus monkeys, and rhesus monkeys [42–44]. Cynomolgus and rhesus monkeys exhibit different susceptibility to *M. tuberculosis* [45]. The rhesus monkey is highly susceptible to progressive infection culminating in death, whereas the cynomolgus monkey is relatively resistant and able to contain low challenge doses [45].

Compared with guinea pigs vaccinated with parental BCG before aerosol challenge with *M. tuberculosis*, guinea pigs vaccinated with rBCG/Antigen 85A showed a trend toward fewer CFU in the lung and spleen [42].

In cynomolgus monkeys vaccinated before intrathecal challenge with *M. tuberculosis* H37Rv, both the parental and recombinant vaccines protected

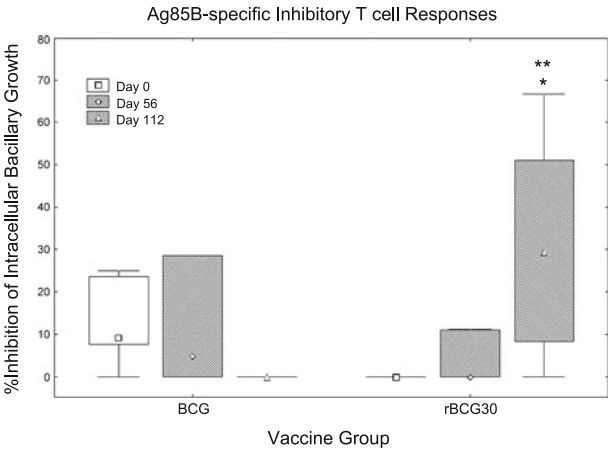

Fig. 2 Phase I human trial of rBCG30: rBCG30 but not BCG-immunized recipients show increased Ag85B-specific T-cell inhibitory activity against intracellular mycobacteria. In a double-blind Phase 1 human trial in which recipients were vaccinated with BCG Tice or rBCG30 Tice, peripheral blood mononuclear cells were harvested from ten recipients of each vaccine prevaccination and on days 56 and 112 postvaccination and stimulated with recombinant 30 kDa Antigen 85B (Ag85B) protein for 7 days. These Ag85B-specific expanded T cells were then cocultured with BCG-infected autologous macrophages for 3 days. The macrophages were lysed; viable CFU of BCG were enumerated on Middlebrook agar plates; and the percent inhibition mediated by Ag85B-specific T cells vs. medium rested T cells was calculated. Shown are the median values (*points*), mid-50% values (*boxes*), and nonoutlier ranges (*whiskers*). *, $p < 0.05$ comparing pre- and postvaccination responses by Wilcoxon matched pairs test. **, $p < 0.05$ comparing rBCG30 and BCG vaccination groups by Mann–Whitney U test. In other assays, rBCG30- but not BCG-immunized recipients showed significantly increased Antigen 85B-specific lymphocyte proliferation, interferon-γ (IFNγ) secretion, IFNγ enzyme-linked immunospot responses, direct ex vivo CD4+ and CD8+ T-cell IFNγ responses, and CD4+ and CD8+ memory T cells capable of expansion. Reproduced with permission of the University of Chicago Press from Hoft et al. [41].

relative to sham-immunized controls, reducing CFU in the lung and spleen by ~2 logs. Compared with cynomolgus monkeys vaccinated with the parental BCG vaccine, monkeys vaccinated with rBCG/Antigen 85A had fewer CFU in lung sections, but not in spleen sections [43].

In rhesus monkeys vaccinated before intrathecal challenge with *M. tuberculosis* H37Rv, monkeys vaccinated with rBCG/Antigen 85A had significantly fewer CFU of *M. tuberculosis* in the lung and spleen than monkeys vaccinated with parental BCG [44].

4.3.2 rBCG/Antigen 85C

Jain et al. [46] investigated a recombinant BCG vaccine overexpressing Antigen 85C, a member of the 30–32 kDa Antigen 85A, B, C family of mycolyl transferases. Compared with BCG, the vaccine gave enhanced protection in the guinea pig model

against high-dose aerosol challenge with *M. tuberculosis* H37Rv, including significantly reduced CFU in the lung and spleen, reduced pathology in the lung, liver, and spleen, and reduced pulmonary fibrosis.

4.3.3 rBCG/ESAT-6 (± CFP10)

Pym et al. evaluated a recombinant BCG vaccine complemented with the RD1 region that is missing in BCG, having been deleted from all BCG strains during its attenuation from *M. bovis* [47]. The vaccine secretes both ESAT-6 and CFP10, two proteins encoded by the RD1 region. The vaccine was tested in both the mouse and guinea pig models. Compared with mice immunized with BCG before intravenous or aerosol challenge with *M. tuberculosis*, mice immunized with the recombinant vaccine had comparable numbers of *M. tuberculosis* in the lung but fewer CFU in the spleen; differences in the spleen were significant in two of four experiments. In a single guinea pig study, animals immunized with the recombinant vaccine before aerosol challenge with *M. tuberculosis* had comparable numbers of *M. tuberculosis* in the lung but fewer CFU in the spleen than animals immunized with control BCG. The recombinant vaccine, however, was more virulent than BCG in severely immunocompromised SCID mice [48], and clinical development of the vaccine has not proceeded.

Brodin et al. studied a potentially safer version of the vaccine constructed in *Mycobacterium microti* [49]. In SCID mice, the recombinant *M. microti* vaccine complemented with the RD1 region was less virulent than the recombinant BCG vaccine complemented with the RD1 region but still much more virulent than a BCG control. In a mouse model, in which immunized mice were aerosol challenged with *M. tuberculosis*, mice vaccinated with the recombinant *M. microti* strain had significantly fewer CFU in the spleen than mice immunized with the control BCG vaccine at two of three time points. In the guinea pig model, the recombinant *M. microti* vaccine and control BCG vaccine were comparably protective.

Bao et al. studied two recombinant BCG vaccines expressing ESAT-6 in a murine model [50]. One recombinant BCG secreted ESAT-6 and one expressed ESAT-6 as part of a nonsecreted fusion protein. There was no significant difference in protective efficacy between either of the two recombinant BCG vaccines and BCG.

4.3.4 rBCG/38 kDa Protein

Castanon-Arreola et al. investigated a recombinant BCG vaccine overexpressing a secreted *M. tuberculosis* 38 kDa glycoprotein and reported that mice immunized with the recombinant vaccine before challenge with either *M. tuberculosis* H37Rv or *M. tuberculosis* Beijing strain survived longer than mice immunized with the parental BCG Tice vaccine [51].

4.3.5 rBCG/19 kDa Protein

Rao et al. investigated a recombinant BCG vaccine overexpressing a 19 kDa lipoprotein of *M. tuberculosis* and found that it abrogated the protective effect of BCG [52]. Compared with splenocytes of mice immunized with BCG, splenocytes of mice immunized with the recombinant vaccine exhibited an enhanced TH2-type immune response to BCG sonicate (increased IL-10 and decreased IFNγ and IgG2a:IgG1 ratio). In guinea pigs, BCG but not the recombinant vaccine induced immunoprotection against subcutaneous *M. tuberculosis* challenge.

4.4 rBCG Expressing M. tuberculosis *Fusion Proteins*

4.4.1 rBCG/72f

Kita et al. investigated a recombinant BCG secreting a hybrid of two proteins (Mtb39 + Mtb32) named 72f in the cynomolgus monkey model [53]. The recombinant vaccine induced immune and protective responses but they were not significantly different from BCG controls.

4.4.2 rBCG/Antigen 85B-ESAT-6

Shi et al. tested recombinant BCG vaccines secreting fusion proteins of Antigen 85B and ESAT-6 in a mouse model [54]. The amount of the fusion protein secreted was not quantitated but appeared to be small on the Western blots on which it was detected. Splenocytes from mice immunized with the recombinant vaccines produced significantly more IFNγ in response to *M. tuberculosis* culture filtrate proteins than splenocytes from mice immunized with control BCG. However, there was no significant difference between the recombinant vaccine and BCG in protective efficacy.

Xu et al. evaluated recombinant vaccines expressing Antigen 85B, an Antigen 85B-ESAT-6 fusion protein, and an Antigen 85B-ESAT-6-mouse IFNγ fusion protein [55]. The biological activity of the mouse IFNγ was not evaluated and was not likely active. In the mouse model, the recombinant vaccines appeared to give slightly better protection than BCG in the lung but not the spleen at late time points.

4.4.3 rBCG/Antigen 85B-Mpt64$_{190–198}$-Mtb8.4

Qie et al. investigated a recombinant BCG vaccine expressing a fusion protein of Antigen 85B, an immunodominant peptide of Mpt64 and Mtb8.4 [56]. In a murine model, the recombinant vaccine had comparable or slightly better efficacy than BCG.

5 Recombinant BCG Overexpressing Native Proteins as Vaccines Against Leprosy

BCG has shown efficacy against leprosy in addition to TB, but as with TB, protection is inconsistent. Two recombinant vaccines have been compared with BCG for efficacy against leprosy in murine models of leprosy.

5.1 rBCG30

Gillis et al. immunized BALB/c mice with BCG, rBCG30, or a recombinant BCG vaccine carrying plasmid pNBV1 encoding the *M. leprae* 30 kDa Antigen 85B (rBCG30ML), and then challenged the animals 2.5 months later by administering viable *M. leprae* into each hind foot pad [40]. Seven months later, the number of *M. leprae* per foot pad was enumerated. In addition, splenocytes and lymph node cells from immunized animals were evaluated for lymphocyte transformation to *M. tuberculosis* Purified Protein Derivative (PPD). All vaccinated groups showed sensitization to PPD; splenocytes from mice immunized with rBCG30 and rBCG30ML showed the highest responses. In the one experiment in which an efficacy comparison was feasible, rBCG30 and rBCG30ML gave protection superior to BCG and the difference between rBCG30 and BCG was statistically significant, as was the difference between the two rBCG30 groups combined and BCG.

5.2 rBCG/Antigen 85A, Antigen 85B, and MPB51

Ohara et al. examined the protective efficacy of a recombinant BCG vaccine overexpressing Antigen 85A, Antigen 85B, and MPB51 [57]. C57Bl/6 mice vaccinated with recombinant vaccine but not with the control BCG vaccine had significantly reduced *M. leprae* in footpads 30 weeks after challenge with *M. leprae*. Compared with unimmunized controls, BALB/c mice vaccinated with either the control BCG or recombinant BCG had reduced numbers of *M. leprae* in footpads. While there was no significant difference in the number of footpad *M. leprae* between mice immunized with the recombinant BCG or control BCG, there was a trend toward fewer *M. leprae* in the footpads of recombinant BCG-immunized mice.

6 Recombinant BCG Overexpressing Native Proteins and Attenuated *M. bovis* as Vaccines Against Bovine Tuberculosis

M. bovis is the principal etiologic agent of tuberculosis in domesticated animals. *M. bovis* infection of domesticated animals exacts a significant economic toll; for example, in cattle it results in reduced fertility, milk production, and meat value

[58]. Control measures, where they can be afforded, center on testing animals for a cell-mediated immune response to *M. bovis* antigens (indicative of exposure) and culling herds of animals testing positive. One approach to reducing the incidence of *M. bovis* infection in domesticated animals is vaccinating the domesticated animals and/or the wild animals that serve as reservoirs of infection. BCG has been tested as a vaccine in cattle, but as in humans, its efficacy is suboptimal [59] (<50%), prompting a search for vaccines that are better. Standard tests for TB in domesticated animals frequently rely on assessment of a cell-mediated immune response to PPD. Since BCG vaccination can interfere with tests involving PPD, the use of a BCG vaccine in domesticated animals may need to be coupled with the use of a diagnostic test employing antigens absent in BCG but present in *M. bovis*, such as members of the ESAT-6 family.

Two new generation vaccines have been compared with BCG for efficacy against *M. bovis* infection.

6.1 rBCG30

rBCG30, described above, was tested in a guinea pig model of *M. bovis* infection in which vaccinated animals were challenged with *M. bovis* by aerosol. Compared with BCG, rBCG30-immunized animals had a lower burden of *M. bovis* in the lung and spleen [39].

6.2 WAg533

WAg533 is a newly attenuated strain of *M. bovis*. It was tested for efficacy in brushtail possums, an important reservoir of *M. bovis* infection in New Zealand; vaccinated animals were challenged with *M. bovis* by aerosol. Compared with animals immunized with BCG, animals immunized with WAg533 by three different routes (conjunctival/intranasal, oral, and subcutaneous) had reduced severity of illness and lower CFU burdens in the lung and spleen [60].

7 Recombinant BCG Overexpressing Native Proteins and Additionally Attenuated for Safety in HIV-Positive Persons

The tuberculosis and AIDS pandemics are closely intertwined. Approximately 12 million people throughout the world are infected with both *M. tuberculosis* and HIV, or about a third of all persons infected with HIV. These coinfected people have the greatest susceptibility to developing active tuberculosis, the major opportunistic infection in AIDS patients.

BCG is an extremely safe vaccine in immunocompetent people, but it can cause serious and even fatal disseminated disease in immunocompromised individuals, including AIDS patients. The World Health Organization has advised against administering BCG to HIV-positive infants because of their increased risk of disseminated BCG infection [61]. This has created a need for a vaccine that is safe and effective in HIV-positive persons, especially infants. Since the HIV status of infants in high-risk regions of the world is often unknown, such a vaccine may be a prudent choice for all infants of unknown HIV status in regions of the world where HIV prevalence is high.

Tullius et al. approached this problem by developing versions of rBCG30 that are readily grown in the laboratory but are replication limited in the host [62]. Although their replication is limited, it is nevertheless sufficient to induce a strong immunoprotective response.

*7.1 rBCG(*mbtB*)30*

rBCG*(mbtB)*30 is the first vaccine that is safer than BCG in the SCID mouse and yet more potent than BCG in the guinea pig model of pulmonary tuberculosis [62]. rBCG*(mbtB)*30 was rendered siderophore dependent by deletion of the gene *mbtB*, which encodes an enzyme necessary to produce the iron siderophores mycobactin and exochelin. The vaccine grows normally in vitro in broth culture and in human macrophages provided the iron-loaded siderophore mycobactin is provided, and during in vitro growth, it is able to store iron mycobactin. This stored iron mycobactin allows the vaccine to multiply for several divisions in vivo, sufficient to induce cell-mediated and protective immunity. In the SCID mouse, rBCG *(mbtB)*30 is much safer than BCG. In the guinea pig, rBCG*(mbtB)*30 is cleared much faster than BCG; nevertheless, in contrast to BCG, it induces a strong cell-mediated immune response to the 30 kDa protein. Most importantly, rBCG*(mbtB)* 30 induces protective immunity that is significantly greater than that induced by BCG (Fig. 3).

*7.2 rBCG(*panCD*)30*

rBCG(*panCD*)30 was rendered pantothenate dependent by deletion of the *panCD* genes [62]. This vaccine can multiply in vitro in broth culture or human macrophages in the presence of high concentrations of pantothenate, but multiplication is highly limited in vivo. In the SCID mouse, rBCG(*panCD*)30 is much safer than BCG. In the guinea pig, rBCG(*panCD*)30 is cleared very rapidly, allowing high doses to be administered safely. In guinea pigs administered high doses of rBCG (*panCD*)30, protection is comparable to BCG.

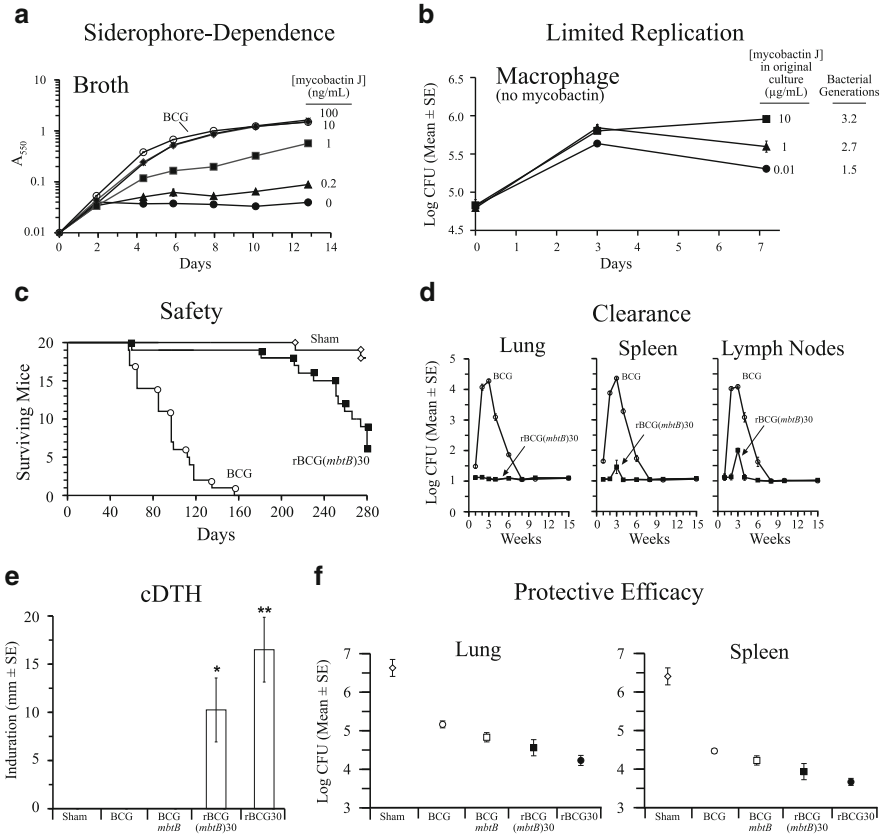

Fig. 3 rBCG(*mbtB*)30, a replication-limited but highly immunoprotective TB vaccine designed specifically for HIV-positive persons. (**a**) Siderophore dependence of rBCG(*mbtB*)30 grown in broth culture. rBCG(*mbtB*)30 was cultured in medium containing 0.01 μg/ml mycobactin J and washed before inoculation into broth containing 0–100 ng/ml mycobactin J (as indicated to the *right* of the *graph*). Growth of the parental BCG strain (grown without mycobactin) is shown for comparison. (**b**) Preloading of rBCG(*mbtB*)30 with mycobactin–iron results in greater residual growth in human THP-1 macrophages. rBCG(*mbtB*)30 was cultured in medium containing 0.01, 1, or 10 μg/ml mycobactin J (as indicated to the *right* of the *graph*) and washed before addition to THP-1 monolayers. CFU were enumerated 0, 3, and 7 days after infection. Data are the mean log CFU ± SE for duplicate wells. The number of bacterial generations over the 7-day course of infection is indicated to the far *right* of the growth *curves* (data are means for two independent experiments). (**c**) Attenuation of rBCG(*mbtB*)30 in SCID mice. SCID mice in groups of 20 were injected with 10^6 CFU of BCG or rBCG(*mbtB*)30, or sham treated with PBS via the tail vein, and survival was monitored over a 40-week period. SCID mice vaccinated with rBCG(*mbtB*)30 survived significantly longer than mice vaccinated with BCG ($p < 0.0001$). (**d**) Limited replication of rBCG(*mbtB*)30 in guinea pigs. Guinea pigs in groups of 24 were immunized by intradermal administration of 10^6 CFU of BCG or iron mycobactin-loaded rBCG(*mbtB*)30. At 1–15 weeks after immunization, as indicated, three animals per group were euthanized, and CFU of BCG and rBCG(*mbtB*)30 in the lungs, spleen, and inguinal lymph nodes were assayed. Data are mean log CFU ± SE. The limit of detection was 1 log CFU. (**e**) Immunogenicity of rBCG(*mbtB*)30 in guinea pigs. Guinea pigs in groups of six were immunized by intradermal administration of 10^3 CFU BCG

8 Recombinant BCG Expressing Immunomodulatory Cytokines

Cytokines play a central role in the workings of the immune system, potentiating some responses and dampening others. Recognizing this, investigators have attempted to beneficially modulate immune responses to vaccines by incorporating cytokines into them. Early studies by O'Donnell et al. and Murray et al. explored the effect of recombinant BCG secreting cytokines on immune responses in mice [63, 64]. O'Donnell et al. constructed a recombinant BCG secreting IL-2 and showed that, compared with wild-type BCG, it induced enhanced splenocyte secretion of interferon-γ (IFNγ) [64]. Murray et al. constructed BCG secreting a number of different cytokines and studied a variety of immune responses in mice immunized with the constructs. Compared with splenocytes from mice immunized with parental BCG, splenocytes from mice immunized with BCG secreting interleulin-2 (IL-2), granulocyte macrophage colony stimulating factor (GM-CSF), or IFNγ, but not BCG secreting interleukin-4 (IL-4) or interleukin-6 (IL-6), had increased lymphocyte proliferation and increased production of cytokines, especially IFNγ, IL-2, and IL-10, upon stimulation with PPD [63].

Later studies have focused on the efficacy of cytokine-secreting BCG vaccines in protection against tuberculosis, and in the therapy of bladder cancer and allergy.

8.1 Cytokine-Secreting Vaccines for Tuberculosis

8.1.1 rBCG/GM-CSF

Ryan et al. studied mice immunized with BCG secreting murine GM-CSF [65]. Compared with mice immunized with control BCG, mice immunized with BCG secreting GM-CSF had greater numbers of antigen-presenting cells ($CD11c^+$

Fig. 3 (continued) or rBCG30 or with 10^6 CFU of iron mycobactin-loaded BCG *mbtB* or rBCG (*mbtB*)30, or were sham immunized with PBS. Ten weeks after immunization, the animals were skin tested by intradermal administration of highly purified *M. tuberculosis* 30-kDa major secretory protein (Antigen 85B), and the degree of induration was assessed 24 h later. Data are mean diameters of induration ± SE. *, $p \leq 0.01$; **, $p \leq 0.001$ (ANOVA; compared with BCG-immunized guinea pigs). (**f**) Protective efficacy of rBCG(*mbtB*)30 in guinea pigs. Guinea pigs in groups of 15 (except for the sham-immunized group of nine animals) were immunized by intradermal administration of BCG, rBCG30, BCG *mbtB*, or rBCG(*mbtB*)30 as in (**e**) above. Ten weeks after immunization, the animals were challenged with a low-dose aerosol of *M. tuberculosis* Erdman strain. Ten weeks after challenge, the animals were euthanized and CFU of *M. tuberculosis* in the lungs and spleen were assayed. Data are mean log CFU ± SE. *Open* symbols indicate sham-immunized animals and groups immunized with BCG strains not overexpressing the 30-kDa protein. *Closed* symbols indicate groups immunized with BCG strains overexpressing the 30-kDa protein. For (**e**) and (**f**), one representative experiment of three is shown. Reproduced with permission of the American Society for Microbiology from Tullius et al. [62].

$MHCII^+$ cells) in draining lymph nodes; increased numbers of IFNγ-secreting spleen cells stimulated ex vivo with BCG lysate 5 and 17 weeks after immunization; and ~1 log fewer CFU in the spleen after aerosol challenge with *M. tuberculosis*.

8.1.2 rBCG/IFNγ and rBCG30/IFNγ

Tullius et al. have constructed rBCG and rBCG30 expressing various forms of human IFNγ, including strains encoding monomeric and covalently linked dimeric forms, and studied their effect on antigen presentation in human monocytes. Infection of human monocytes with these constructs results in upregulation of class I and II MHC molecules and enhanced presentation on an MHC class II molecule of a peptide of the 30 kDa Antigen 85B major secretory protein (Tullius and Horwitz, unpublished studies).

8.1.3 rBCG/IL-2

Young et al. studied the capacity of recombinant BCG secreting murine IL-2 to counter a Type 2 immune response in mice and to alter the immune profile of immunosuppressed mice [66]. As noted above, one theory as to why BCG is sometimes poorly effective is that it is administered in a setting in which a TH2 type of immune response predominates, e.g., as a result of helminth infection. The investigators showed that rBCG/mIL-2 but not control BCG can induce a TH1 profile in mice immunosuppressed with dexamethasone and in IL-4 transgenic mice. Compared with BCG-immunized mice, the rBCG/mIL-2–immunized mice exhibit greater splenocyte proliferation and IFNγ production in response to PPD and a higher IgG2a:IgG1 antibody ratio (consistent with a TH1-type immune response) in both types of mice.

In a separate study, Young et al. investigated the protective efficacy of rBCG/mIL-2 in mice [67]. Although the IL-2–secreting BCG vaccine induced a longer lasting splenic lymphocyte proliferative response to PPD, a higher IFNγ level in response to PPD, and a higher IgG2a:IgG1 antibody ratio than control BCG, the recombinant vaccine was not more protective against *M. bovis* aerosol challenge than control BCG.

Slobbe et al. studied a BCG vaccine secreting cervine IL-2 in outbred red deer [68]. rBCG/cIL-2 induced a smaller delayed-type hypersensitivity (DTH) response to PPD than parental BCG. RT-PCR studies of lymphocytes from the deer revealed that IL-2 and IFNγ levels were similar in deer vaccinated with rBCG/cIL-2 or parental BCG but that IL-4 levels were reduced in the deer vaccinated with rBCG/cIL-2.

8.1.4 rBCG/IL-18

Young et al. evaluated a recombinant BCG secreting murine IL-18 [67]. Disappointingly, this vaccine induced significantly less IFNγ in splenocytes of immunized mice

that control BCG, and paralleling this finding, it was significantly less protective than control BCG.

8.1.5 rBCG/IL-15

Tang et al. studied a recombinant BCG vaccine secreting a fusion protein of Antigen 85B and murine IL-15 [69]. Whether the IL-15 portion of the fusion protein was biologically active was not investigated. In any case, the investigators report that the vaccine was cleared more rapidly than a control recombinant vaccine secreting only Antigen 85B and that mice immunized with the recombinant vaccine secreting the fusion protein, compared with mice immunized with the control vaccine, had greater absolute numbers of CD4+ and CD8+ T cells in lung, spleen, and peritoneal exudate cells, greater numbers of IFNγ-secreting CD4+ cells to two *M. tuberculosis* antigens, and a lower bacterial burden in the lung, but not the spleen, after intratracheal challenge with *M. tuberculosis*.

8.2 Cytokine-Secreting Vaccines for Therapy of Bladder Cancer

In addition to its use as a vaccine against TB, BCG plays an important immunotherapeutic role in the treatment of superficial bladder cancer. Such treatment is associated with induction of TH1 cytokines [70]. Approximately 30% of patients do not respond to current BCG therapy and 50% of patients suffer a recurrence [71]. This has prompted investigators to explore new generation BCG vaccines secreting TH1-inducing cytokines for the treatment of bladder cancer.

8.2.1 rBCG/IFNγ

Arnold et al. tested the immunotherapeutic efficacy of rBCG expressing murine IFNγ in a mouse model [70]. They found that rBCG/mIFNγ upregulated class I MHC expression in murine bladder cancer cells to a greater extent than a BCG control strain transfected with an empty vector. Intravesicular instillation of rBCG/mIFNγ resulted in greater recruitment of CD4+ T cells into the bladder and increased expression of IL-2 and IL-4 compared with intravesicular instillation of control BCG. Finally, rBCG/mIFNγ but not BCG treatment of orthotopic bladder cancer significantly prolonged survival compared with untreated animals; however, while survival of rBCG/mIFNγ-treated animals was greater than that of BCG-treated animals, the difference did not reach statistical significance.

8.2.2 rBCG/IFNα

IFNα has been shown to improve the response of patients to BCG therapy [71], prompting investigators to evaluate the immunogenicity of rBCG secreting IFNα

2B in vitro. Luo et al. found that rBCG/IFNα induced more IFNγ and IL-2 from human PBMC in vitro than BCG [72] and Liu et al. found that rBCG/IFNα induces more potent PBMC cytotoxicity than BCG against human bladder cancer cell lines and the effect was dose dependent [71]. The addition of neutralizing antibodies against IFNα, IFNγ, or IL-2 to PBMC cultures stimulated with rBCG/IFNα reduced PBMC cytotoxicity against the cancer cells.

8.2.3 rBCG/IL-2

Yamada et al. studied the cytotoxic effect of recombinant BCG secreting murine IL-2 on murine bladder cancer cells in vitro [73]. They constructed a recombinant BCG secreting murine IL-2 fused to the signal sequence of the 30 kDa Antigen 85B of BCG and reported that the fusion protein was functional. Peritoneal exudate cells (PEC) incubated with rBCG/mIL-2 produced greater amounts of IFNγ, TNFα, and IL-12 and were more cytotoxic than PEC incubated with BCG. The enhanced cytotoxicity was neutralized by the addition of anti–IL-2 antibody.

8.2.4 rBCG/IL-18

Luo et al. found that, compared with splenocytes from BCG-immunized mice, splenocytes from mice immunized with rBCG/mIL-18 had increased IFNγ, TNFα, and GM-CSF levels and increased lymphocyte proliferation in response to BCG antigens [74]. Mouse PECs (>90% macrophages) stimulated in vitro with rBCG/mIL-18 also had greater cytolytic activity against a mouse bladder cancer cell line than PEC stimulated with BCG.

8.3 Cytokine-Secreting Vaccines for Allergy

Antigen-specific TH2-type cells figure prominently in allergic reactions, and TH1 cells, via the secretion of IFNγ, may counter allergic responses by dampening TH2 cell activity [75].

8.3.1 rBCG/IL-18

Biet et al. studied recombinant BCG secreting biologically active (increased NF-κB) mouse IL-18 [75]. IL-18 acts synergistically with IL-12 to induce IFNγ, and since BCG itself induces IL-12, Biet et al. hypothesized that rBCG secreting Il-18 might exert a more potent dampening effect on TH2-type immune responses than BCG [76]. rBCG/mIL-18 enhanced TH1 and diminished TH2-type immune responses in mice. Compared with splenocytes from BCG-immunized mice, splenocytes from

rBCG/mIL-18–immunized mice had increased IFNγ and GM-CSF production in response to PPD. In contrast, rBCG/mIL-18–immunized mice had decreased serum IgG to BCG antigens [75].

Biet et al. explored the therapeutic potential of rBCG/mIL-18 in a murine model of pulmonary allergic inflammation, in which mice were sensitized to ovalbumin [76]. The investigators found that lymph node cells from sensitized mice immunized with rBCG/mIL-18 and challenged with ovalbumin produced more IFNγ when stimulated with ovalbumin than lymph node cells from sensitized mice immunized with BCG and challenged with ovalbumin; in contrast, IL-5 production was suppressed. Moreover, ovalbumin-sensitized mice immunized with rBCG/mIL-18 had less bronchoalveolar eosonophilia after ovalbumin challenge than BCG-immunized mice.

9 Recombinant BCG with Enhanced Antigen Presentation

In addition to engineering BCG that secretes various cytokines, two other approaches have been used to improve antigen presentation. One approach is aimed primarily at enhancing MHC class I antigen presentation and another approach is aimed at enhancing MHC class II antigen presentation.

9.1 BCG with Altered Intracellular Pathway

Grode et al. engineered a recombinant BCG vaccine secreting listeriolysin, to promote perforation of the phagosomal membrane, and with a deleted urease gene, to reduce the pH in the phagosome to nearer the pH optimum of listeriolysin [77]. The rationale for this vaccine was to increase class I MHC antigen presentation by allowing egress of BCG antigens to the cytosol. As this vaccine also was proapoptotic in macrophages, it additionally promoted presentation of mycobacterial antigens via cross-priming. In a murine model, this vaccine was more potent than BCG against challenge with *M. tuberculosis* Beijing/W, reducing CFU in the lung and spleen by 1–2 logs. The vaccine was tested for safety in SCID mice; mice administered the recombinant vaccine intravenously survived significantly longer than mice administered the parental BCG vaccine. In a study in the guinea pig model, the vaccine was not more potent than BCG [78].

9.2 rBCG/Cathepsin S

Sendide et al. [79] reported that IFNγ-induced surface expression of mature MHC class II molecules is suppressed in THP-1 macrophages infected with wild-type

BCG compared with macrophages incubated with killed BCG, that the suppression is correlated with reduced cathepsin S activity, and that the reduced cathepsin S activity is mediated via BCG-induced IL-10 secretion. This prompted Soualhine et al. to engineer and evaluate a recombinant BCG secreting the mature form of human cathepsin S [80]. The rBCG/hCathepsin S strain had increased IFNγ-induced surface MHC class II expression and increased expression of an MHC class II–Antigen 85B peptide complex. Whether a recombinant vaccine secreting cathepsin S was capable of inducing improved protective immunity in an animal model was not investigated.

10 Recombinant BCG Overexpressing Native Proteins and Escaping the Phagosome

Sun et al. [81] engineered a novel recombinant BCG vaccine that combined the approach of overexpressing native antigens, first employed by Horwitz et al. [10], with the approach of phagosome escape, first employed by Grode et al. [77]. To expand the antigenic repertoire of the vaccine, the investigators engineered it to overexpress, in addition to Antigen 85B (the antigen overexpressed in rBCG30, described above), Antigen 85A and TB10.4, a low molecular mass protein in the ESAT-6 family. Instead of using listeriolysin to promote phagosome membrane lysis, these investigators used a mutant form of perfringolysin O. The vaccine, designated AFRO-1, was safer than BCG in SCID mice. Vaccination of mice and guinea pigs with AFRO-1 induced stronger immune responses to the overexpressed antigens than vaccination with the parental BCG vaccine. In mice vaccinated before aerosol challenge with the hypervirulent *M. tuberculosis* strain HN878 (Beijing-type clinical outbreak strain), the group vaccinated with AFRO-1 survived significantly longer than mice vaccinated with the parental BCG vaccine.

11 Recombinant BCG Expressing Foreign Antigens

With the development of techniques to genetically manipulate mycobacteria [82–85], researchers rapidly looked to exploit recombinant BCG as a multi-component vaccine vector by expressing foreign antigens from various pathogens [4–9, 86–89]. As noted above, BCG possesses a number of potential advantages as a vaccine (Table 1): it has been extensively used for many decades, it has a very good safety profile, and it is inexpensive to produce [4, 90]. BCG is unaffected by maternal antibodies, so it can be given in a single dose at birth, and it is capable of inducing a long-lasting cellular immune response. Due to its intracellular location in the phagosome of macrophages and dendritic cells, BCG primarily elicits a CD4+ cellular immune response through MHC class II. However, BCG also elicits a humoral immune response, and potent

antibody responses to foreign antigens expressed by recombinant BCG have been obtained in some cases (see Tables 2–6). Recombinant BCG expressing foreign antigens can also elicit a CD8+ cytotoxic T-lymphocyte response through MHC class I. This feature of recombinant BCG is particularly important for protection against viral pathogens and those bacterial pathogens that invade the cytoplasm of the host cell. Presentation of antigen through MHC class I may occur via cross-priming.

Recombinant BCG targeting viral (Table 2), parasitic (Table 3), and bacterial (Table 4) diseases have been developed. Several studies have examined recombinant BCG vaccines expressing toxins to enhance the adjuvant effect of BCG (Table 5) and recombinant BCG vaccines against cancer and allergy as well (Table 6). Despite the promise of recombinant BCG vaccines, results have been decidedly mixed. Good to excellent immune responses and/or protection have been achieved in a number of studies, but many studies have also demonstrated weak to moderate immune responses and no protection or modest protective efficacy at best. Many studies have used high doses (greater than the human dose of ~10^6 CFU administered intradermally) and/or multiple doses of recombinant BCG in an attempt to obtain better immune responses. Heterologous boosting of recombinant BCG has been effective in generating a more potent immune response in several studies [91, 96, 114]. While a boosting regimen that yields an effective vaccine has benefit, it does negate one purported advantage of being able to give recombinant BCG in a single dose at birth. The type of immune response that is obtained with any particular rBCG vaccine cannot be predicted, and it is difficult to draw many generalizations from the numerous immunological studies of rBCG vaccines due to the large number of variables among studies (animal model, route of vaccination, dose and timing of vaccine, method of growth and preparation of vaccine, vaccine viability, expression level and cellular location of the foreign antigen, stability of foreign antigen expression, etc.) [173–176]. However, the expression level of the foreign antigen and its cellular location (intracellular, secreted, or membrane anchored) have had profound effects on immune responses generated by recombinant BCG vaccines in some studies. In general, higher expression levels and targeting of the foreign antigen to the membrane or extracellular space have yielded more potent vaccines [173, 175]. To achieve high expression of a foreign antigen, episomal plasmids with strong promoters have typically been required. Unfortunately, this has been associated with instability of expression of the foreign antigen in more than a few studies. Integrated vectors are more stable but result in less expression and often lower immune responses. High-level and stable expression of foreign antigens in recombinant BCG have been rather difficult to achieve in practice.

11.1 HIV

rBCG expressing HIV and SIV antigens were among the first rBCG multi-component vaccines constructed. rBCG expressing Gag, Nef, Env, Pol, and RT, as complete genes or as smaller fragments, have been developed and tested for their

Table 2 Viral diseases targeted by recombinant BCG vaccines

Disease (organism) Antigen	Animal model	Immunogenicity	Protective efficacy	References
HIV and SIV				
Gag, Pol, Env (HIV)	BALB/c mice	Weak Ab to Gag and Env IFNγ secretion (SP) and CD8+ CTL (SP) to Gag [only Gag tested]		[8]
Gag, Pol, Env, RT (HIV)	BALB/c mice	Weak Ab; CD8+ CTL (SP) [only Env tested]		[4, 6, 7]
HIVA (HIV) [synthetic construct containing ~73% of Gag fused to a multi-CTL epitope from the Gag, Pol, Nef, and Env proteins]	BALB/c mice	No CD8+ T-cell response alone, but a good response obtained after heterologous boost	Protection against surrogate recombinant Vaccinia virus challenge	[91]
Gag, Pol, Env, Nef (SIV) (cocktail)	Rhesus macaques	IgA and IgG secreted by PBMC; CTL (PBMC)		[92]
Gag, Env, Nef (SIV) (cocktail)	BALB/c mice	Mucosal IgA, serum IgG; CTL (SP)		[93]
Gag, Env, Nef (SIV) (cocktail)	Cynomolgus macaques	IFNγ secretion and CTL (PBMC)	No protection against rectal challenge	[94]
Gag, Nef (SIV) (bivalent strain)	BALB/c mice	Serum IgG; IFNγ secretion (CD4+ T-lymphocytes, spleen)		[95]
Gag, Pol, Env (SIV) (cocktail)	Rhesus macaques	Weak IFNγ ELISPOT and tetramer binding (PBMC) which could be significantly enhanced with heterologous boosting		[96]
Gag (HIV) [p17gag B-cell epitope (aa 92–110) fused to *M. kansasii* α-antigen]	BALB/c mice	High titer Ab induced in 3 of 7 mice		[97]
Gag (HIV)	Chacma baboons	No Ab; no or weak IFNγ ELISPOT (PBMC) which could be significantly enhanced with heterologous boosting		[98]

Gag (HIV)	BALB/c mice	40-fold higher expression using codon optimization resulted in stronger immune response; T-cell proliferation and IFNγ ELISPOT (SP); serum IgG		[99]
Gag (HIV)	BALB/c mice	Heterologous prime-boost resulted in prolonged CTL (SP)		[100]
Gag (HIV)	BALB/c mice	CTL and T-cell proliferation (SP); no serum Ab		[101]
Gag (SIV)	Rhesus macaques	CD8+ CTL (PBL)		[87]
Gag (SIV)	Rhesus macaques	Heterologous prime-boost; good CTL (PBL); no Ab	No protection against cell-free intravenous SIV challenge	[102]
Gag (SIV)	Cynomolgus macaques	Heterologous prime-boost; IFNγ ELISPOT (PBMC)	Protection against mucosal challenge with SHIV	[103]
Gag (SIV)	Guinea pigs, Hartley strain	Long lasting, very high titer serum IgG; IFNγ mRNA elevated (PBMC)		[104]
Gag (SIV)	Guinea pigs, Hartley strain	DTH; T-cell proliferation (PBMC); IFNγ mRNA elevated (PBMC, SP); very high titer serum IgG		[105]
Env (HIV) [15 aa V3 CTL epitope fused to *M. kansasii* α-antigen]	BALB/c mice	CD8+ CTL (SP)		[106]
Env (HIV) [19 aa V3 CTL epitope fused to *M. kansasii* α-antigen]	BALB/c mice and Guinea pigs, Hartley strain	DTH; CTL (SP); moderate serum Ab; Neutralizing Ab		[107]
Env (HIV) [19 aa V3 CTL epitope fused to *M. kansasii* α-antigen]	C57BL/6J mice	serum IgG; Neutralizing Ab; IFNγ and IL-2 secretion by CD4+ T-cells		[108]
Env (HIV) [19 aa V3 CTL epitope fused to *M. kansasii* α-antigen]	Guinea pigs, Hartley strain	DTH; T-cell proliferation (PBMC, SP, I-IEL)		[109]

(*continued*)

Table 2 (continued)

Disease (organism) Antigen	Animal model	Immunogenicity	Protective efficacy	References
Env (HIV) [19 aa V3 CTL epitope fused to *M. kansasii* α-antigen]	Guinea pigs, Hartley strain	DTH; serum IgG and IgA; Neutralizing Ab		[110]
Env (HIV) [19 aa V3 CTL epitope fused to *M. kansasii* α-antigen]	Rhesus macaques	Serum Ab; Neutralizing Ab; weak IFNγ ELISPOT (PBMC)	No protection against pathogenic SHIV	[111]
Env (HIV) [15 aa V3 CTL epitope (PND) fused to *M. tuberculosis* chaperonin-10]	BALB/c mice	T-cell proliferation (SP); serum IgG		[112]
Env (HIV) [11 or 12 aa V3 CTL epitope fused to *M. kansasii* α-antigen]	Guinea pigs	Moderate serum IgG; Neutralizing Ab		[113]
Env (HIV)	BALB/c mice	IFNγ ELISPOT (SP, lung, FRT); No serum Ab although could be elicited with heterologous boost		[114]
Env (SIV)	BALB/c mice and Guinea pigs	CD8+ CTL (LN); serum IgG; fecal IgA; Neutralizing Ab		[115]
Nef (HIV)	BALB/c mice	T-cell proliferation (LN)		[9]
Nef (HIV)	BALB/c mice	T-cell proliferation (LN); No serum Ab		[116]
Nef (SIV)	BALB/c mice	T-cell proliferation and CD8+ CTL (LN)		[117]
Nef (SIV)	BALB/c mice	T-cell proliferation (PGLN, MLN, CLN, SP); IFNγ and TNFα ELISPOT (PP, I-IEL, PGLN, MLN, SP); CD8+ CTL (I-IEL, MLN, SP)		[118]
Measles virus				
Nucleocapsid (N) protein (measles virus)	C3H/He mice	Moderate serum Ab but only when rBCG given twice; T-cell proliferation (SP); low levels of Neutralizing Ab after infection	Partial protection	[119]
Nucleocapsid (N) protein (measles virus)	Rhesus macaques	CTL and T-cell proliferation (PBMC); no serum IgG	Partial protection	[120]

Human papillomavirus (HPV)				
HPV type 6b late protein L1, HPV type 16 early protein E7	C57BL/6J or BALB/c mice	DTH; serum Ab (weak but could be boosted); T-cell proliferation and low CTL (SP)	No protection against tumor challenge	[121]
Cottontail rabbit papillomavirus (CRPV)				
L1 (major capsid protein)	Outbred New Zealand white rabbits	Serum Ab; neutralizing Ab	Good protection	[122]
L2, E2, E7 or L2E7E2 fusion	Outbred New Zealand white rabbits		Therapeutic vaccine: 71% of papilloma sites had complete regression with rBCG expressing L2E7E2 fusion protein	[123]
Porcine reproductive and respiratory syndrome virus (PRRSV)				
GP5, M (cocktail)	BALB/c mice	Serum Ab; neutralizing Ab; IFNγ secretion (SP)		[124]
GP5, M (cocktail)	Crossbreed F1 (Landrace × Large White) pigs	Serum Ab, neutralizing Ab in 3 of 5 pigs; negative IFNγ ELISPOT (PBMC)	Partial protection	[125]
Hepatitis B virus (HBV)				
HBsAg-Middle S, HBsAg-Large S	BALB/c mice	Serum Ab		[126]
Hepatitis C virus (HCV)				
NS5a (nonstructure protein 5a) [12 aa CTL epitope of HCV-nonstructure protein 5a (NS5a) as a chimeric protein with α-antigen of *M. kansasii*]	BALB/c × C3H/HeN (CC3HF1) mice	CD8+ CTL (SP); increased IFNγ, IL-2, and IL-12 mRNA (LN)	Good protection against surrogate recombinant vaccinia virus challenge	[127]
CtEm [multi-epitope antigen, including the major epitope domains of the truncated core, mimotopes from E2, and six HLA-A2–restricted CTL epitopes from NS3, NS4 and NS5]	HHD-2 mice (transgenic for HLA-A2.1)	Serum Ab; T-cell proliferation, IFNγ and IL-2 secretion, and CTL (SP)	Good protection against surrogate recombinant vaccinia virus challenge	[128]

(*continued*)

Table 2 (continued)

Disease (organism) Antigen	Animal model	Immunogenicity	Protective efficacy	References
Rotavirus				
VP6	BALB/c mice, Guinea pigs, Rabbits	No serum Ab	Partial protection	[129]
Respiratory syncytial virus (RSV)				
N, M2	BALB/c mice	No serum IgG; IFNγ and IL-2 secretion and increased CD69+ T-cells (SP)	Very good protection	[130]
Encephalomyocarditis virus (EMCV) D-variant (Human type I diabetes model)				
VP1 (major outer capsid protein)	SJL/J mice, inbred Guinea pigs	Serum IgG; Neutralizing Ab; DTH; T-cell proliferation (SP)	Long-lasting (>10 months) protective immunity, very good protection (required at least 6 weeks for full immunity)	[131]
Rabies virus				
N (nucleoprotein) [B-cell and T-cell epitopes fused to *M. leprae* 18-kDa protein]	BALB/c mice	Serum IgG		[132]

Abbreviations: *aa* amino acid, *Ab* antibody, *CLN* cervical lymph nodes, *CTL* cytotoxic T-lymphocytes, *DTH* delayed-type hypersensitivity, *ELISPOT* enzyme-linked immunosorbent spot, *FRT* female reproductive tract, *I-IEL* intestinal intraepithelial lymphocytes, *LN* lymph nodes, *MLN* mesenteric lymph nodes, *PBL* peripheral blood lymphocytes, *PBMC* peripheral blood mononuclear cells, *PGLN* periglandular lymph nodes, *PP* Peyer's patches, *SP* splenocytes

Table 3 Parasitic diseases targeted by recombinant BCG vaccines

Disease (organism) Antigen	Animal model	Immunogenicity	Protective efficacy	References
Malaria (*Plasmodium* spp.)				
CSP (circumsporozoite protein of *Plasmodium falciparum*)	BALB/c and BALB/k mice	No serum Ab; no IFNγ secretion or T-cell proliferation (SP) in response to CSP Th2R peptide (CD4 + T-lymphocyte epitope)		[89]
CSP (circumsporozoite protein of *Plasmodium falciparum*)	BALB/c mice	Serum IgG; T-cell proliferation and IFNγ and IL-2 secretion (SP)		[133]
CSP (circumsporozoite protein of *Plasmodium yoelii*) [B-cell epitope of CSP fused to *M. kansasii* α-antigen]	BALB/c mice	Serum Ab in only one of seven mice		[134]
MSP-1 (merozoite surface protein 1 from *Plasmodium yoelii*) [15 kDa C-terminal region of MSP-1 fused to *M. kansasii* α-antigen]	C3H/He mice	IFNγ secretion (SP); no serum Ab	Very good protection against challenge with 10^4 *P. yoelii* 17XL-parasitized erythrocytes	[135]
MSP-1 (merozoite surface protein 1 from *Plasmodium yoelii*) [15 kDa C-terminal region of MSP-1 fused to *M. kansasii* α-antigen]	C3H/He mice		Long-lasting (9 month) protection against intraperitoneal challenge with 10^4 *P. yoelii* 17XL-parasitized erythrocytes but protection was substantially less than at 1 month postvaccination	[136]
MSA2 (merozoite surface antigen 2 from *Plasmodium falciparum*)	BALB/c mice	Serum IgG; T-cell proliferation and IFNγ and IL-2 secretion (SP)		[137]
F2R(II)EBA (fragment 2 region II of EBA-175), $(NANP)_3$, as well as two T-cell epitopes of the *M. tuberculosis* ESAT-6 antigen	BALB/c mice	No expression data presented; serum IgG; T-cell proliferation (SP); increased splenocyte CD4+ IFNγ+, CD4+ IL-2+, CD4+ IL-4+ cells in response to $(NANP)_3$; increased splenocyte CD4+ IL-4+ cells in response to F2R (II)EBA; immune responses also obtained against *M. tuberculosis* ESAT-6 epitopes		[138]

(*continued*)

Table 3 (continued)

Disease (organism) Antigen	Animal model	Immunogenicity	Protective efficacy	References
Leishmaniasis (*Leishmania* spp.)				
gp63 (*Leishmania major* surface proteinase)	BALB/c and CBA/J mice		Good protection against *L. mexicana* promastigotes and amastigotes, poor protection against *L. major* promastigotes in BALB/c mice	[88]
gp63 (*Leishmania major* surface proteinase)	BALB/c and C57BL/6 mice		Partial protection against a challenge with *L. major* amastigotes in BALB/c-mice	[139]
LCR1 (antigen cloned from amastigote *L. chagasi* library)	BALB/c mice		Weak protective immunity against *L. chagasi* infection	[140]
Schistosomiasis (*Schistosoma* spp.)				
Glutathione S-transferase (*Schistosoma mansoni*, Sm28GST)	BALB/c, C57BL/6, and C3H/HeJ mice	T-cell proliferation (LN but not SP) [BALB/c and C57BL/6 mice]		[141]
Glutathione S-transferase (*Schistosoma mansoni*, Sm28GST)	BALB/c mice	High titer and long-lasting (1 year) serum IgG; Neutralizing Ab		[142]
Glutathione S-transferase (*Schistosoma haematobium*, Sh28GST)	BALB/c mice	High titer serum IgG; serum and BALF IgA; Neutralizing Ab		[143]
Glutathione S-transferase (*Schistosoma japonicum*, Sj26GST)	BALB/c mice	T-cell proliferation and IFNγ secretion (SP)		[144]
Sm14 (from *Schistosoma mansoni*)	BALB/c and outbred Swiss mice	No serum Ab; IFNγ secretion (SP)	Partial protection against subcutaneous challenge with 100 *S. mansoni* cercaria; not able to boost protection with rSm14 protein or rBCG	[145]

Sm14 (from *Schistosoma mansoni*, codon optimized)	BALB/c and outbred Swiss mice	No serum Ab; IFNγ secretion (SP); codon optimization increased expression level but did not improve immune response	Partial protection against subcutaneous challenge with 100 *S. mansoni* cercaria; not able to boost protection with rSm14 protein	[146]
Toxoplasmosis (*Toxoplasma gondii*)				
GRA1 (a major secreted antigen)	OF1 outbred mice, Suffolk crossbred sheep	Mice: no serum Ab; no DTH Sheep: no serum Ab; T-cell proliferation and IFNγ secretion (PBMC)	Mice: very weak protection against oral challenge Sheep: shorter duration of pyrexia following oral challenge	[147]
ROP2 (rhoptry protein 2)	BALB/c mice	Serum Ab; IFNγ and IL-2 secretion (SP)	Slightly increased survival after intraperitoneal challenge	[148]
Coccidiosis (*Eimeria tenella*)				
Rho (rhomboid gene)	Chickens	Serum IgG	Partial protection	[149]

Abbreviations: *Ab* antibody, *BALF* bronchial alveolar lavage fluid, *DTH* delayed-type hypersensitivity, *LN* lymph nodes, *PBMC* peripheral blood mononuclear cells, *SP* splenocytes

Table 4 Bacterial diseases targeted by recombinant BCG vaccines

Disease (organism) Antigen	Animal model	Immunogenicity	Protective efficacy	References
Lyme disease (*Borrelia burgdorferi*)				
OspA (outer surface protein A)	BALB/c, C3H/HeJ, and outbred Swiss Webster mice	High titer serum IgG	Complete protection	[5]
OspA (outer surface protein A)	BALB/c mice	Prolonged systemic IgG and mucosal IgA	Complete protection	[150]
OspA (outer surface protein A)	Human phase I clinical trial	None of the 24 volunteers developed anti-OspA Ab		[151]
OspA (outer surface protein A)	White-tailed deer	Serum Ab		[152]
Bacterial pneumonia, otitis media, meningitis (*Streptococcus pneumoniae*)				
PspA (Pneumococcal surface protein A)	BALB/c, C3H/HeJ, and CBA/N (Xid) mice	High titer serum Ab	50–100% protection against intraperitoneal challenge	[153]
Leptospirosis (*Leptospira* spp.)				
LipL32 (Leptospira interrogans external membrane protein)	Golden Syrian hamsters	Serum IgG	Partial protection against intraperitoneal challenge with *L. interrogans*	[154]
LipL32	BALB/c mice	Serum Ab		[155]
Listeriosis (*Listeria monocytogenes*)				
p60 (major secreted antigen)	BALB/c mice	CD8+ T-cells (SP)	Complete protection with rBCG expressing membrane-anchored p60	[156]
DPT (Diphtheria–Pertussis–Tetanus)				
S1-TTC (hybrid protein of S1 subunit of pertussis toxin fused to fragment C of tetanus toxin)	BALB/c mice	High titer serum anti-TTC IgG but no anti-S1 IgG; toxin neutralizing Ab; IL-2 secretion (SP)		[157]

S1 subunit of pertussis toxin	BALB/c mice	Long-term serum IgG (up to 8 months) and memory response (15 months)		[158]
S1 subunit of pertussis toxin (genetically detoxified)	BALB/c and outbred Swiss mice	Weak serum Ab; IFNγ secretion and T-cell proliferation (SP)	Good protection against intracerebral *Bordetella pertussis* challenge	[159]
S1 subunit of pertussis toxin (genetically detoxified)	Outbred Swiss mice (neonates)	No serum Ab; IFNγ secretion (SP)	Good protection against intracerebral *Bordetella pertussis* challenge	[160]
CRM197 (mutated nontoxic derivative of diphtheria toxin)	BALB/c mice	Weak serum Ab (nonneutralizing); rBCG capable of priming a humoral response to DT vaccine		[161]
FC (tetanus toxin fragment C), CRM197 (mutated nontoxic derivative of diphtheria toxin) (cocktail)	BALB/c and outbred NIH mice, Guinea pigs	Mice: weak serum anti-FC IgG; rBCG capable of priming a humoral response to DT vaccine guinea pigs: Neutralizing Ab against tetanus toxin and diphtheria toxin	75% protection against a challenge with 100 minimum lethal doses of tetanus toxin	[162]
FC (tetanus toxin fragment C, ToxC)	Outbred NIH Swiss mice	Serum Ab		[4]
FC (tetanus toxin fragment C, ToxC)	Outbred NIH Swiss mice	Serum Ab	Partial to complete protection against a challenge with 100 minimum lethal doses of tetanus toxin	[7]
Bovine anaplasmosis (*Anaplasma marginale*)				
MSP1a	BALB/c mice	Weak serum Ab; IFNγ secretion (SP)		[163]

Abbreviations: *Ab* antibody, *SP* splenocytes

Table 5 Bacterial proteins used to enhance the adjuvant effect of recombinant BCG vaccines

Antigen	Animal model	Immunogenicity	References
CTB (cholera toxin B subunit)	BALB/c mice	Increased IgA and TGF-β1in bronchial alveolar lavage fluid	[164]
LTB (B subunit of *E. coli* heat labile enterotoxin)	BALB/c mice	Serum IgG and IgA; oral rBCG also induced mucosal IgA	[165]
LTB (B subunit of *E. coli* heat labile enterotoxin) fused to R1 repeat region of P97 adhesion from *Mycoplasma hyopneumoniae*	BALB/c mice	LTB used for adjuvant effect; serum anti-R1 IgG and IgA (greater response to LTB-R1 fusion than to R1 alone)	[166]

immunological properties primarily in mice and guinea pigs, although several of these vaccines have been tested in nonhuman primate models as well (Table 2). Developing a vaccine against HIV has proven extremely challenging [177, 178] and rBCG–HIV vaccines are no exception. It is generally agreed that both broadly neutralizing antibodies and antiviral cytotoxic T-lymphocytes are needed for a highly effective vaccine, although CD4+ T cells also have a role in mediating these effects. Due to the great difficulty in generating broadly neutralizing antibodies, recent HIV vaccine research has focused more on T-cell vaccines. As rBCG–HIV vaccines have been reviewed several years ago [173], this section will focus on some of the most recent studies.

11.1.1 Recent Studies

Cayabyab et al. constructed rBCG strains expressing SIV Gag, Pol, and Env localized to the mycobacterial cell wall with the 19 kDa lipoprotein signal under the control of the α-antigen promoter [96]. Rhesus macaques were vaccinated intradermally or intravenously with 10^6–10^9 CFU rBCG (given as a cocktail of all three strains) and boosted with an identical dose 23 weeks later. Twenty weeks after the second immunization, all the monkeys were boosted with 10^{10} virus particles of recombinant adenovirus 5 expressing the same SIV antigens as the rBCG strains. The monkeys developed very weak CD8+ T-cell responses to the SIV antigens even with two doses of rBCG. However, after the heterologous prime boost with adenovirus, the monkeys developed strong responses to all three SIV antigens, as measured by PBMC IFNγ ELISPOT responses to Gag, Pol, and Env peptide pools. The responses to Gag and Pol were greater for the rBCG immunized animals compared with naïve animals, but a similar response was obtained for Env in rBCG immunized and naïve animals, which was attributed to instability of expression of Env by the rBCG vaccine.

Promkhatkaew et al. constructed an rBCG expressing HIV Gag intracellularly from the strong *hsp60* promoter (0.26–0.45 mg/L of culture) [101]. Expression was reported to be stable. BALB/c mice were vaccinated subcutaneously with 0.1 mg (2×10^6 CFU) and cell-mediated immune responses were measured 2 weeks to

Table 6 Cancer and allergic disease targeted by recombinant BCG vaccines

Antigen	Animal model	Immunogenicity	Protective efficacy	References
OVA	C57BL/6 mice	IFNγ-secreting CD8+ T cells and CTL (SP)	Significant protection against challenge with OVA-expressing tumor cells (B16-OVA)	[167]
OVA (SIINFEKL epitope)	C57BL/6 and TAP1-/- mice	Weak CTL	Partial protection against challenge with OVA-expressing tumor cells (B16-OVA)	[168]
MUC1 (22 variable-number tandem repeats) + mIL-2	SCID mice reconstituted with 10^7 human PBL	IFNγ secretion; low-level serum IgG and IgM; rBCG secretes functional IL-2 but effect of cytokine is unclear as rBCG expressing MUC1 alone was not tested		[169]
MUC1 (1, 4, or 8 variable-number tandem repeats) + hGM-CSF	SCID mice reconstituted with 5×10^7 human PBL	Increased IFNγ ELISPOTand CTL; no data on whether coexpressed hGM-CSF was functional	Partial protection against tumor challenge	[170]
S1 subunit of pertussis toxin (genetically detoxified)	C57BL/6 mice	Increased TNFα mRNA (qPCR)	Reduction of bladder tumor volume	[171]
Der p I (a major allergen from house dust mites, immunodominant peptide containing T- and B-cell epitopes)	C57BL/6J and BALB/b mice	IFNγ secretion (SP)		[172]

Abbreviations: *CTL* cytotoxic T-lymphocytes, *ELISPOT* enzyme-linked immunosorbent spot, *PBL* peripheral blood lymphocytes, *SP* splenocytes

2 months later. Gag-specific CTL to multiple epitopes as well as lymphocyte proliferation was induced in response to vaccination, but no anti-Gag antibodies were detected in sera. In a follow-up study, mice were boosted 1 month or 6 months after subcutaneous or intradermal rBCG vaccination with a replication-deficient vaccinia virus strain also expressing full-length Gag and cell-mediated immune responses were measured 1 month later [100]. The prime-boost regimen resulted in a more persistent CTL response than the single vaccination with rBCG.

Chege et al. tested two rBCG vaccines expressing HIV Gag localized to the mycobacterial cell wall with the 19 kDa lipoprotein signal for immunogenicity in a baboon model using a prime-boost regimen [98]. Both humoral and cellular Gag-specific immune responses to rBCG alone were very weak, but rBCG succeeded in priming the immune system for a Gag VLP boost (assessed by assaying the IFNγ ELISPOT response to Gag peptides and Gag-specific antibody). rBCG was administered at very high doses (10^8 CFU at 0, 14, 24, and 40 weeks) and boosted twice with Gag VLPs at 92 and 104 weeks. The exact expression level of Gag by the rBCG strains was not reported, but the strain with higher expression, which produced better results, was reported to be less stable. Low expression and/or instability of the rBCG vaccines may have resulted in a poor immune response in this study.

Kawahara and colleagues examined the long-term immune response of rBCG expressing full-length SIV Gag in guinea pigs [104, 105]. Gag was expressed intracellularly under the control of a strong promoter (P_{hsp60}) at 0.5 ng Gag/mg of rBCG. Guinea pigs were immunized intradermally (0.1 mg) or orally (80 mg × 2) using typical human doses. A strong immune response was achieved from both routes of immunization for up to 3 years as evidenced by: Gag-specific serum IgG 10^6-fold greater than control (IgG2 > IgG1), DTH, proliferation of PBMC and splenocytes in response to Gag, and increased IFNγ mRNA in PBMC and splenocytes in response to Gag, mediated largely through CD4+ T-cells. The high levels of antibody produced contrasted with the authors' previous work with an rBCG secreting a 19 amino acid CTL epitope from Env fused to *M. kansasii* α-antigen [110]. Intradermal vaccination of guinea pigs with 0.1 mg of this strain resulted in no antibody production, although other routes of immunization did elicit an antibody response.

Im et al. constructed an rBCG expressing the HIVA immunogen localized to the mycobacterial cell wall with the 19 kDa lipoprotein signal under the control of the α-antigen promoter [91]. HIVA is a synthetic gene containing ~73% of gag fused to a multi-CTL epitope from the Gag, Pol, Nef, and Env proteins [179]. The investigators obtained greater expression with an extrachromosomal plasmid compared with an integrated plasmid and used the higher expressing strain for all of their animal studies. A balanced lethal system was used to apply selective pressure on the rBCG strain to maintain the plasmid (a BCG Δ*lysA* lysine auxotroph was complemented with a functional copy of *lysA* on the plasmid). This strategy was quite successful in maintaining the plasmid as 10 of 10 colonies isolated from the spleens of mice 15 weeks after immunization still maintained the kanamycin resistance marker on the plasmid and 2 of 2 were positive for the HIVA gene by PCR. Unfortunately, the authors did not go one step further and check the isolated colonies for continued expression of HIVA. BALB/c mice were vaccinated

intraperitoneally with 10^6 CFU of rBCG for most experiments and 10^3–10^7 CFU for a dose-titration study. BCG–HIVA induced little or no CD8+ T-cell responses alone, but enabled enhanced responses when used to prime a subsequent boost with MVA–HIVA (given at 102 d). In a dose–response experiment, a high priming dose of BCG–HIVA was determined to be important for eliciting a broader T-cell response. Mice that were primed with a DNA–HIVA vaccine, boosted with BCG–HIVA, and challenged with a surrogate replication-competent vaccinia virus expressing HIVA had significantly increased levels of bifunctional CD4+ T-cells.

Yu et al. constructed rBCG vaccines expressing HIV-1 Env as a surface, intracellular, or secreted protein [114]. For surface expression, the 19 kDa lipoprotein signal was used and for secretion, the α-antigen signal was used. All constructs were under the control of the α-antigen promoter, a moderately strong mycobacterial promoter. In an attempt to address the very difficult challenge of HIV genetic diversity, the authors used two artificial consensus env genes (CON6 gp120 or CON6 gp140CF). BALB/c mice were vaccinated twice (at 0 and 8 weeks) intraperitoneally with 10^6, 10^7, or 10^8 CFU of the different rBCG strains, and antigen-specific T-cell responses were measured by IFNγ ELISPOT assays on lymphocytes isolated from spleens. Vaccines secreting Env yielded the strongest response. Interestingly, little or no response was obtained with a single dose of rBCG demonstrating a clear boosting effect of rBCG in this assay. Elevated Env-specific T-cell responses were also obtained with lymphocytes isolated from the lung and female reproductive tract after two doses of rBCG. The antigen-specific T-cell response was primarily due to CD4+ T-cells. rBCG did not elicit anti-Env antibodies on its own, but did prime an antibody response when followed by a boost of recombinant HIV-1 Env oligomer in RiBi adjuvant.

11.2 Other Viral Diseases

Many other viruses besides HIV have been targeted by recombinant BCG vaccines (Table 2). Very good protective efficacy has been achieved against respiratory syncytial virus (RSV) and encephalomyocarditis virus (EMCV) [130, 131]. Good protection against cottontail rabbit papillomavirus (CRPV) has been achieved [122, 123], and immune responses to hepatitis C virus (HCV) have provided good protection against a surrogate challenge with recombinant vaccinia virus expressing an HCV antigen [127, 128].

11.3 Parasitic Diseases

Nearly 20 studies on recombinant BCG vaccines targeting malaria, leishmaniasis, schistosomiasis, and toxoplasmosis are cataloged in Table 3. Several studies that examined protective efficacy of the rBCG vaccines will be highlighted here. Matsumoto et al. obtained very good protection against rodent malaria (*P. yoelii*)

with a recombinant BCG secreting the 15 kDa C-terminal region of merozoite surface protein 1 (MSP1) fused to the *M. kansasii* α-antigen (six of seven rBCG vaccinated mice survived versus 0% survival for controls) [135]. Protection was significantly better than that achieved with recombinant MSP1 protein with adjuvant. C3H/He mice were immunized intravenously with 10^6 CFU rBCG, boosted 1 month later intraperitoneally with 10^6 CFU rBCG, and challenged 1 month after the second immunization with *P. yoelii*. As neither route can be used to immunize humans and the route of immunization is known to affect the immune response to rBCG in small animals, the results should be interpreted cautiously. In a follow-up study, the authors found that protective immunity had waned significantly by 4 and 9 months postimmunization (only 4 of 9 and 3 of 9 mice survived at 4 and 9 months, respectively), but still was better than after immunization with recombinant MSP1 protein with adjuvant [136].

Connell et al. constructed an rBCG expressing the *Leishmania major* gp63 surface proteinase intracellularly under the control of a strong promoter (P_{hsp60}) [88]. BALB/c and CBA/J mice were vaccinated intravenously (10^4 or 10^5 CFU) or subcutaneously (10^6 CFU) and challenged 10 weeks later. Good protection from cutaneous leishmaniasis was obtained when the mice were challenged with *L. mexicana* (promastigotes and amastigotes) but not against *L. major* promastigotes. Abdelhak et al. also developed rBCG strains expressing *L. major* gp63 [139]. Mice were vaccinated twice at a 1-month interval (10^6 CFU intravenously; 10^7 CFU subcutaneously) and challenged with *L. major* amastigotes 1 month after the boost. Partial protection was observed with the rBCG expressing gp63 fused to the N-terminal portion of β-lactamase (a secreted protein) but not with the rBCG expressing gp63 intracellularly.

rBCG expressing the Sm14 antigen from *Schistosoma mansoni* have delivered partial protection from challenge with *S. mansoni* cercaria in two studies [145, 146]. In the first study, Varaldo et al. expressed Sm14 fused with β-lactamase under the control of a strong promoter (pBlaF*) with the fusion protein localized to the cell wall [145]. A single dose of rBCG (10^6 CFU subcutaneously) was as effective as a three-dose regimen of rSm14 protein in alum, with ~50% reduction in worm burden in outbred Swiss mice. Protective efficacy could not be boosted by a second dose of rBCG or by rSm14 protein. In a follow-up study, Varaldo et al. constructed a mycobacterial codon-optimized Sm14 gene and obtained fourfold greater expression with rBCG expressing the codon-optimized gene compared with their earlier construct [146]. However, this did not translate into an increased immune response (IFNγ secretion by splenocytes stimulated in vitro with rSm14) or protective efficacy. This contrasts with an earlier report in which an rBCG strain expressing codon-optimized HIV Gag was more immunogenic than an rBCG expressing wild-type Gag (~40-fold increase in expression for the codon-optimized Gag) [99].

11.4 Bacterial Diseases

Some of the earliest rBCG vaccines targeting bacterial pathogens produced very promising results [5, 150, 153]. Stover et al. developed an rBCG vaccine against

Lyme borreliosis by expressing the outer surface protein A (OspA) of *Borrelia burgdorferi* as a membrane-anchored lipoprotein [5]. Using this construct, they obtained protective antibody responses in inbred and outbred mouse strains that were 100–1,000 times greater than those obtained with rBCG strains expressing OspA intracellularly or as a secreted protein. In protection experiments, inbred and outbred mouse strains were immunized intraperitoneally with 10^6 CFU rBCG, boosted 17 weeks later with an identical dose, and challenged intraperitoneally or intradermally 5 weeks after the booster dose. Excellent protective efficacy was obtained against both challenge routes. Likewise, a single intranasal dose of this rBCG strain provided complete protection against intradermal challenge 13 weeks postvaccination [150]. This group also obtained very good humoral immune responses in mice immunized with rBCG strains expressing pneumococcal surface protein A (PspA) [153]. Interestingly, protective immunity was only induced in mice vaccinated with rBCG secreting PspA or expressing PspA as a membrane-anchored lipoprotein. Despite inducing a good humoral immune response, no protective efficacy was obtained with rBCG expressing intracellular PspA. The rBCG vaccine against Lyme disease was eventually tested for safety and immunogenicity in the first phase I clinical trial of an rBCG vaccine (and still the only human trial of an rBCG vaccine expressing a foreign antigen) [151]. Unfortunately, in stark contrast to the results obtained in mice, none of the 24 human volunteers vaccinated intradermally with 2×10^4–2×10^7 CFU rBCG developed a humoral immune response.

Similar to the studies with OspA and PspA above, Grode et al. constructed rBCG vaccines expressing secreted, membrane anchored, and intracellular *Listeria monocytogenes* p60 (a major secreted antigen) under the control of a strong promoter (P_{hsp60}) [156]. BALB/c mice were vaccinated intravenously with 10^6 CFU of rBCG and challenged 120 days later. Excellent protective efficacy (80–100% survival at 10 days postchallenge) was obtained with the rBCG strains expressing membrane anchored or secreted p60, but not with the rBCG strain expressing intracellular p60. Interestingly, only CD4+ T-cells were needed for protective efficacy in mice immunized with rBCG secreting p60, but both CD4+ and CD8+ T-cells were required in mice immunized with rBCG expressing membrane-anchored p60. The authors suggest that this could be due to decreased access of membrane-anchored p60 to the MHC class II loading compartment leading to less stimulation of CD4+ T-cells.

Recombinant BCG vaccines expressing bacterial toxins have produced good protection in several studies as well [7, 159, 160, 162].

12 Conclusions

As a vaccine vector for a recombinant TB vaccine, BCG is an obvious choice since it shares so many antigens with *M. tuberculosis*, and it has efficacy by itself. The emphases of current efforts aimed at an improved TB vaccine are on expanding the

repertoire of overexpressed immunoprotective *M. tuberculosis* antigens and improving the processing and presentation of both vector and recombinant antigens by altering the intracellular lifestyle of the vector, endowing the vector with immunomodulatory cytokines, enhancing apoptosis to promote cross-presentation of antigens, etc. Some of these modifications also show promise for improving BCG as a therapeutic against bladder cancer, the only other approved use of BCG vaccine aside from the prevention of TB.

Increasingly, primarily because of its safety record and high immunogenicity, BCG has been chosen as a vaccine vector to express foreign antigens, particularly where no convenient or safe alternative vector homologous to the target exists, e.g., in the case of parasites, cancer, and allergic disease. Recombinant BCG expressing HIV antigens are being intensely studied including in nonhuman primates. Some of these vaccines have induced strong immune responses against key HIV antigens, particularly when used as part of a heterologous prime-boost vaccination strategy.

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Part III
Manipulating Host-Pathogen Interactions to Make Vaccines

Basic Science Paves the Way to Novel Safe and Effective Pestivirus Vaccines

Norbert Tautz and Gregor Meyers

Abstract Pestiviruses are among the economically most important pathogens of livestock. Except for culling, vaccination represents the only feasible way to control pestiviruses. Therefore, a considerable number of pestivirus vaccines have been developed and put on the market. However, these vaccines still have disadvantages that should be eliminated in future approaches, some of which are based on recent findings and will be outlined in this chapter. One of the most important features of ruminant pestiviruses is their extraordinary tendency to establish lifelong persistence as the outcome of intrauterine infection. As a result, 1–2% of cattle worldwide are persistently infected with bovine viral diarrhea virus. The constant dissemination of the virus by these animals is central for maintenance of this pathogen in its host population; therefore, future vaccines must address this highly relevant problem. Elucidation of the molecular features of pestiviruses that are required for the establishment and maintenance of persistent infection has made significant progress, and the present knowledge on this topic is summarized in this chapter. These features include a unique strategy to restrict virus genome replication by a limiting host factor and viral virulence factors N^{pro} and E^{rns} interfering with the innate immune response of the host. Accordingly, a framework of viral functions is involved in the establishment and maintenance of persistence. On the basis of this knowledge, specific mutations in the recently identified virulence factors have resulted in the generation of attenuated viruses, building a perfect basis for future vaccine design.

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1 Introduction

Pestiviruses cause economically important diseases of livestock [1]. Moreover, because they are closely related to hepatitis C virus (HCV), pestiviruses are a widely used surrogate system for this human pathogen. Classical swine fever virus (CSFV), two types of bovine viral diarrhea virus (BVDV-1 and BVDV-2), and border disease virus of sheep (BDV) belong to this group of viruses, and are classified as one genus within the virus family *Flaviviridae* [2].

For pestiviruses, both cytopathic and noncytopathic viruses can be discriminated in cell culture [3]. The biotype of the virus is very relevant, because only noncytopathic viruses are able to establish persistent infections in their natural host. Persistence is a key feature for maintenance of pestiviruses in their host population. Persistent infections are achieved by diaplacental infection of fetuses in pregnant animals. Fetal infection, depending on the time of gestation at which infection occurs, can also lead to abortion or fetal malformation [4].

Killed and live attenuated vaccines against BVDV and CSFV are currently commercially available. Both types of vaccines have distinctive disadvantages. The established live virus vaccines were obtained via repeated cell culture passages of field isolates. This leads to an unpredictable risk of reversion to virulence, because the basis of attenuation of these viruses is undefined. Attempts to identify the molecular markers for attenuation in the viral genomes have not provided final conclusions [5–7]. Even more important than possible reversion to virulence is the fact that currently available live pestivirus vaccines are not safe in pregnant animals because they can be transmitted to the fetus and induce damage, trigger abortion, or establish persistent infection, depending on the vaccine virus [8]. Therefore, despite the outstanding efficacy of live pestivirus vaccines, their use is hampered by safety concerns. As an alternative, a variety of nonlive vaccines have been developed that contain killed viruses or heterologously expressed structural components of the viruses (several such vaccines are commercially available). These vaccine formulations are safe but considerably less efficient in inducing protective immunity in host animals, especially when efficacy is measured with respect to prevention of diaplacental infection of fetuses in pregnant animals. Sometimes a booster vaccination is necessary to achieve protective immunity. In addition to standard two-step immunization schemes using the same vaccine for both steps, prime–boost regimes utilizing a killed virus vaccine as the prime vaccination and a live virus vaccine for booster immunization have been developed to achieve improved protection.

Based on this background, there is an obvious need for improved pestivirus vaccines that are safe in young, adult, and, especially, pregnant animals. Vaccination should efficiently block transplacental transmission of field viruses to the fetus in pregnant animals and should prevent development of clinical symptoms as well as virus shedding in challenged animals. Recently obtained data on the biological functions of pestiviruses have significantly improved our understanding of the prerequisites for virus persistence and can be used for a rational design of attenuated recombinant pestivirus vaccines.

2 Pestivirus Molecular Biology

Pestivirus particles are enveloped and have a diameter of 40–60 nm. The virions contain four structural proteins. Within the virion, the viral positive-sense single-stranded RNA genome is found. The genomic RNA, with a length of about 12.3 kb, contains one long open reading frame (ORF), coding for all known viral proteins [9, 10]. In the genomic RNA the ORF is flanked by untranslated regions (UTRs), which contain RNA sequences and structures with crucial importance for viral RNA replication. The 5′ UTR also contains a complex RNA structure, termed an "internal ribosome entry site" (IRES). The IRES mediates initiation of protein translation in the absence of a 5′ cap structure [11]. Protein expression occurs via translation of a polyprotein that is co- and posttranslationally processed by viral and cellular proteases into the mature virus proteins. Within the polyprotein, the individual gene products are arranged in the order NH_2–N^{pro}/C/E^{rns}/E1/E2/p7/NS2/NS3/NS4A/NS4B/NS5A/NS5B–COOH [1] (Fig. 1).

Capsid protein C and the glycoproteins E^{rns}, E1, and E2 are structural components of the enveloped pestivirus virion [12]. E2 and, to a lesser extent, E^{rns} are targets for antibody neutralization [13–17]. E2 interacts specifically with the surface protein CD46, which functions as a pestivirus receptor. CD46 is necessary but not sufficient to mediate infection [18, 19].

E^{rns} is the second pestivirus protein that is accessible on the surface of virus particles [14]. It interacts with carbohydrate structures on the surface of target cells [20–22]. E^{rns} lacks a typical transmembrane sequence or another known type of membrane anchor and is secreted in considerable amounts from infected cells [23]. Recent analyses have shown that the C-terminal part of the protein functions as a novel type of membrane anchor [24, 25]. Notably, E^{rns} exhibits RNase activity [26–28].

The nonstructural proteins include the autoprotease N^{pro} (the first protein encoded by the long ORF) and the seven proteins located in the polyprotein

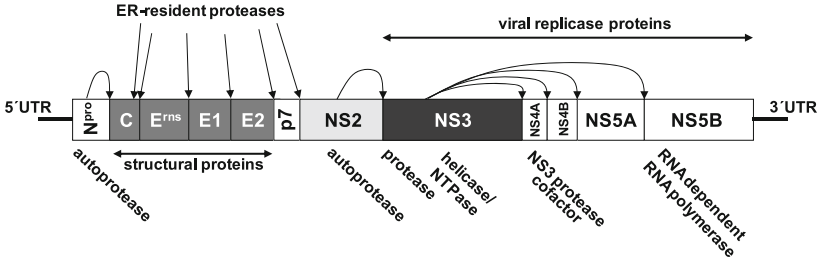

Fig. 1 Genome organization of a pestivirus. The 5′ and 3′ untranslated regions are indicated by *black lines*, and the single long open reading frame (ORF) is indicated by a *box*. The location of the regions of the ORF coding for the individual viral proteins is indicated, together with the basic organization of the genome into regions coding for structural and nonstructural proteins. Below the ORF, the currently known functions of the encoded proteins are given. The processing scheme of the polyprotein encoded by the ORF is indicated by *arrows*

downstream of E2. The small hydrophobic p7 is required for virion formation. In the related HCV system it has been shown that the ortholog of p7 is a so-called viroporin and forms an ion channel [29–32]. Host cell proteases resident in the endoplasmic reticulum catalyze all cleavages required for the release of the viral glycoproteins and p7, and virus encoded enzymes mediate processing of the nonstructural proteins NS3 to NS5B. The generation of the N terminus of NS3 by an autoprotease in NS2 is essential for viral RNA replication because NS3, but not uncleaved NS2-3, is an essential component of the viral replicase [33, 34]. In contrast, uncleaved NS2-3 is required for virion morphogenesis [34, 35]. NS3 is a multifunctional protein with protease, helicase, and NTPase activities. To gain full activity, the NS3 serine protease requires NS4A as a cofactor. This NS3/NS4A protease complex generates the C terminus of NS3 and catalyzes all downstream cleavages of the polyprotein [36–38]. No specific functions have been attributed to NS4B and the Zn-binding phosphoprotein NS5A so far [39]. NS5B is an RNA-dependent RNA polymerase, which catalyzes the replication of viral RNA, in concert with viral proteins NS3, NS4A, NS4B, and NS5A and an unknown number of host factors [40].

Cloning a complementary DNA copy of the entire viral genome into bacterial plasmids allows the manipulation of the viral sequence and the generation of viral genome copies in vitro. Upon transfection of these RNAs into cultured cells, autonomous replication of the viral genome occurs, and infectious progeny are released into the culture supernatant [41, 42]. This technique is the basis for the establishment of recombinant pestiviruses with properties desired for a vaccine.

3 Prerequisites for Pestivirus Persistence

Establishment of persistent infection is the strategy mainly responsible for the maintenance of pestiviruses in their host population. Persistence is best studied for BVDV, which achieves lifelong constant spread by persistently infected animals [4]. Any successful vaccination campaign must break this vicious cycle.

Viral persistence requires a delicate balance between the host immune system and viral factors and strategies that protect the virus against elimination. Intrauterine infection of a fetus in the first trimester (between days 40 and 120 of pregnancy) by noncytopathic BVDV strains may lead to viral persistence, which is accompanied by an acquired immunotolerance with strict specificity for the infecting virus strain [43, 44] (Fig. 2). It is generally believed that self-reactive elements of the adaptive immune system, including those directed against the persisting virus, are inactivated during this developmental stage of the infected fetus. Thus, the developing animals do not mount an adaptive immune response against the persisting virus strain during their lifetime. Furthermore, the virus is protected against the immune response of the mother cow by the fact that antibodies cannot cross the bovine placenta. Consequently, viral clearance in pregnant cows with preexisting antibodies against BVDV must occur prior to infection of the fetus. Otherwise, depending on the transmitted virus, persistently infected animals will develop, or

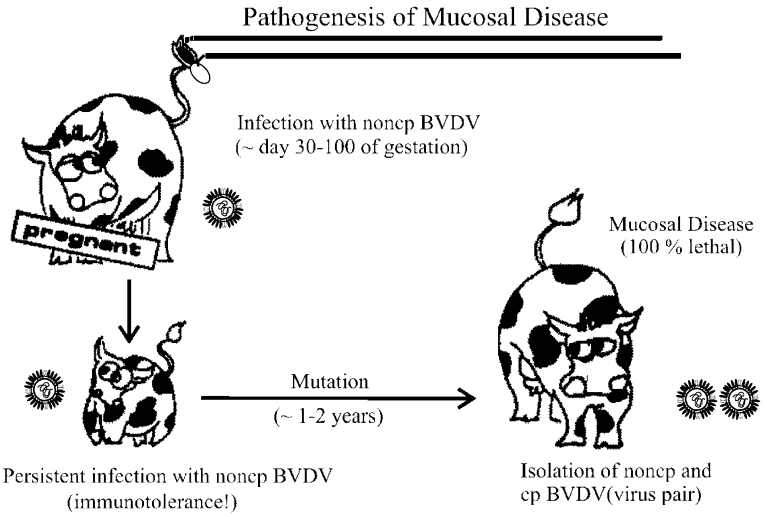

Fig. 2 The principal pathway to virus-specific acquired immunotolerance and establishment of persistent infection by bovine viral diarrhea virus (BVDV)

abortion of the fetus will occur. Accordingly, fetal safety requires a stringent level of immunoprotection against BVDV. Such protection is further hampered by a high level of antigenic variability within and between the subtypes BVDV-1 and BVDV-2.

To maintain a persistent infection for years, BVDV must also evade the host's innate immune system [45]. Because innate immunity is crucially important to control viral infections, all viruses seem to encode antagonists counteracting this system [46]. For BVDV at least three strategies or factors have been identified that work together to counteract the host's innate immune response (see below).

Although persistence is somewhat different in other pestiviruses, transplacental infection of fetuses is a general theme. On the basis of the facts described above, effective novel live pestivirus vaccine strains (1) must protect a pregnant vaccinee from even transient viral replication and (2) must not infect the fetus itself or at least must not establish a persistent fetal infection.

Taking these considerations into account, we should design improved live pestivirus vaccines on the basis of recently obtained knowledge about virulence factors and requirements for viral persistence. The description that follows will focus mainly on BVDV because the available data provide a rather complete picture of the processes leading to persistence.

3.1 Time Point of Infection, Biotype, and Beyond

As outlined already, infection of pregnant cattle with noncytopathic BVDV in the first trimester of gestation may lead to infection of the not yet immunocompetent

fetus and the development of persistently infected offspring with an acquired immunotolerance against the infecting BVDV strain [47]. Infections in the earlier time phases of pregnancy may have severe consequences for the developing animal, such as abortion, stillbirth, or malformations. When the fetus is infected in the late phase of pregnancy, the development of the immune system has already proceeded to the point where the virus infection may be cleared, but abortion can still occur [48]. Importantly, the capacity to establish persistent infections is restricted to the noncytopathic biotype of BVDV. Persistently infected animals can be retarded in growth or might appear completely healthy. However, all these animals eventually come down with the so-called mucosal disease. This disease is characterized by severe ulcerations of the mucosa throughout the gastrointestinal tract and the destruction of Peyer's patches, resulting in bloody diarrhea and the death of the animal. Onset of mucosal disease is correlated with the appearance of a cytopathic BVDV strain in the animal already persistently infected with a noncytopathic BVDV strain [43]. These cytopathic BVDV strains mostly arise from the persisting noncytopathic virus by mutation, often through RNA recombination [49]. The genomic changes associated with the switch in the viral biotype result in an uncontrolled release of NS3 from the viral polyprotein, which in turn causes a large increase in viral RNA replication. Mutations found in those cytopathic genomes are deletions, accumulated point mutations, or insertions of viral or cell-derived sequences, sometimes together with large sequence duplications [50]. In the recombinant genomes, fragments of cellular messenger RNAs are sometimes found upstream of the NS3-coding region. The cell-derived sequences encode substrates of cellular proteases such as ubiquitin, ubiquitin-like proteins, or proteins with ubiquitin-like folds [51]. Because the cellular proteases can recognize their cognate substrates in the context of the viral polyproteins, they generate the authentic N terminus of NS3 and thereby release this protein [52]. Moreover, duplicated versions of N^{pro} located upstream of NS3 in the polyproteins of cytopathic BVDV can also mediate processing at the N terminus of NS3 [53]. Other cytopathic BVDV strains are characterized by highly efficient NS2-3 processing via a deregulated NS2 autoprotease [33] (also see below). As a result, a large amount of free NS3 is present in cells infected with cytopathic BVDV and correlates with highly efficient viral RNA replication as well as viral cytopathogenicity and interferon induction [33, 54–56]. Studies of the different viral biotypes have revealed a unique control mechanism for viral RNA replication in cells infected with noncytopathic BVDV-1: the autoprotease in NS2 requires a cellular protein as a cofactor for catalysis of NS2-3 cleavage [57]. This cellular chaperone protein, termed "Jiv" (for J-domain protein interacting with viral protein), which stably interacts with two domains in NS2, is required in stoichiometric amounts for the activation of the NS2 protease and thus for NS3 release [58]. Because the endogenous level and turnover rate of Jiv seem to be very low, the number of Jiv molecules present in the cell at the time of infection only allows efficient cleavage of the NS2-3 molecules translated in the first few hours after infection (Fig. 3). After this time, mainly uncleaved NS2-3 is produced because, for most protease molecules, no cofactor is available [57]. Since NS3 is an essential component of the viral RNA

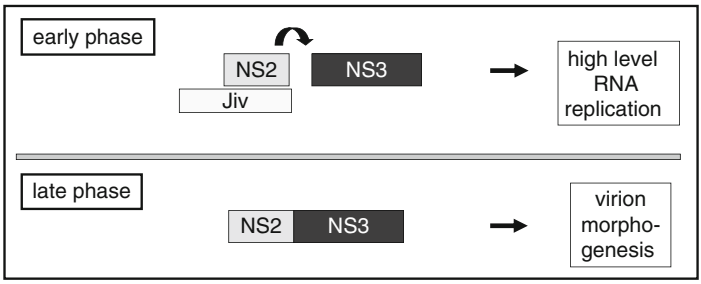

Fig. 3 The Jiv-dependent control mechanism of noncytopathic BVDV genome replication and its connection with different stages of virus replication. NS3 is an essential factor for RNA replication, and uncleaved NS2-3 is required for virion morphogenesis. A switch between these stages is mediated by the consumption of the cellular Jiv pool

replication complex and cannot be functionally replaced by NS2-3, the efficiency of viral RNA replication drops with the consumption of the endogenous Jiv pool and the concomitant decrease in NS3 release. Thus, low endogenous amounts of Jiv restrict viral replication. This restriction is crucial for the maintenance of the noncytopathic biotype. Accordingly, Jiv insertions in BVDV and CSFV strains render those viruses cytopathogenic [55, 59, 60]. Similarly, the regulated overexpression of Jiv induces a switch of the viral biotype from noncytopathic to cytopathic [61]. Most interestingly, whereas noncytopathic BVDV seems to be capable of suppressing the interferon response upon persistent infection via the action of N^{pro} and E^{rns}, cytopathic BVDV has obviously lost this capacity [62]. For CSFV, the change of the viral biotype from noncytopathic to cytopathic induced by a Jiv insertion correlated with an attenuation of the virus in its natural host and the induction of an interferon-induced gene [59]. This again emphasizes the importance of the Jiv-mediated regulation of viral RNA replication for the control of the innate immune system (also see later).

It is tempting to speculate that triggering of the interferon response together with the induction of apoptosis in the infected cell is causative for the failure of cytopathic BVDV to establish persistence.

3.2 Mutation of Viral Factors Interfering with the Innate Immune Response as a Strategy for Pestivirus Attenuation

3.2.1 How Can Innate Immune Reactions Be Reduced to a Tolerable Level?

Despite the control of RNA replication in noncytopathic BVDV, a minimal amount of viral RNA must be present within the infected cell, inevitably leading to the presence of double-stranded RNA, a very potent trigger of the type 1 interferon response. Viral persistence in an organism with huge numbers of infected cells

could hardly be imagined if infection resulted in a constantly high level of type 1 interferon production, with all the consequences for cellular translation, RNA degradation, induction of apoptosis, and inflammatory responses. In fact, persistently infected calves do not have increased serum levels of type 1 interferon (Bryan Charleston, personal communication). To avoid triggering the interferon response, with all its downstream antiviral effects, pestiviruses not only restrict viral RNA replication, but also express two proteins involved in controlling the type 1 interferon response.

3.2.2 N^{pro}

For both BVDV and CSFV, N^{pro}, the first protein encoded by the long ORF, is responsible for blocking the type 1 interferon response in pestivirus-infected cells. N^{pro} is an unusual cysteine protease [63], which cleaves at its own C terminus and thus generates the N terminus of the capsid protein. There is no other substrate known for this protease. Moreover, the N^{pro}-coding region can be deleted from the viral genome without dramatically influencing the growth characteristics of the virus [64]. Nevertheless, the N^{pro} deletion attenuates the virus [65, 66]. It was first reported for CSFV that deleting the N^{pro}-coding region results in a mutant virus that induces an interferon response in infected cells; therefore, N^{pro} was proposed to interfere with the innate immune system in CSFV-infected cells [67–69]. Loss of repression of interferon induction was also reported for BVDV N^{pro} deletion mutants [70, 71].

The observed repression of an interferon response by noncytopathic BVDV was independent of the proteolytic activity of N^{pro} [70] and correlated with the absence of activation of gene expression by interferon regulatory factor 3 (IRF3) [72]. It was reported recently that N^{pro} induces degradation of IRF3 via the proteasome [71, 73–75]. For this degradation the Zn^{2+}-binding site of N^{pro} is essential, indicating that the structural integrity of the protein must be maintained [76].

It has been a matter of debate whether N^{pro} deletion results in considerable attenuation of CSFV in piglets or adult animals. The first published data indicated that the deletion of N^{pro} in different CSFV isolates leads to attenuation [66]. In contrast, results obtained in the laboratory of one of the authors gave no indication that N^{pro} deletion causes considerable attenuation (G.M., unpublished data). More recent findings from Szymanski et al. showed that the observed attenuating effect is not due to loss of the N^{pro}-induced repression of the type 1 interferon response, but most likely results from reduced growth rates probably due to less translation of the mutated genomic RNAs [77]. This finding indicates that the N^{pro} type 1 interferon repressing function is not a virulence factor in piglets or adult animals.

3.2.3 E^{rns}

E^{rns} is another fascinating pestiviral protein. Not only is it an essential structural component of the virus particle, but it also exhibits enzymatic activity [26, 27, 78, 79].

E^{rns} contains sequence motifs typical of the T2 superfamily of RNases and hydrolyzes single- and double-stranded RNA upstream of U residues [28, 80–82]. The enzymatic function of the protein is not necessary for virus growth in tissue culture; RNase-negative virus mutants replicate in cultured cells as rapidly as wild-type viruses [83–85]. However, abrogation of the RNase activity by mutation of the active-site histidines in the conserved T2 RNase motifs results in considerable attenuation of both CSFV and BVDV [84–86].

The function of the RNase is still obscure, but involvement in pestivirus immune evasion seems most plausible. In fact, like N^{pro}, the E^{rns} RNase also can prevent induction of a type 1 interferon response [81, 82, 87]. Since the RNase efficiently degrades viral RNA [28], a function of the active RNase in the cytoplasm of an infected cell seems to be highly unlikely. Addition of purified E^{rns} or secretion of E^{rns} from cells expressing the protein is sufficient to prevent type 1 interferon induction when extracellular double-stranded and single-stranded synthetic or viral RNAs are used as triggers [81, 82, 87]. This effect is dependent on RNase activity and correlates with a double-stranded RNA binding feature of the protein [81]. E^{rns} cannot prevent a type 1 interferon response when the triggering RNA is introduced into the cells by transfection. Therefore, it can be concluded that the RNase does not repress the type 1 interferon response in the cytoplasm of infected cells and thus has a role that clearly differs from that of N^{pro}.

As already mentioned, E^{rns} is secreted in substantial amounts from infected or transfected cells. This feature of the protein plays a central role in hypotheses on the function of its intrinsic RNase activity. N^{pro} seems to function as an intracellular repressor of type 1 interferon induction in the cytoplasm; E^{rns} probably finds its target outside the infected cell. Therefore, secretion of E^{rns} could be directly connected to its function as a virulence factor. The amount of E^{rns} present in the serum of infected animals has been measured as approximately 50 ng/ml, an amount sufficient to exert a biological effect [82]. The mechanism leading to release of E^{rns} from the cell in which it is synthesized is not clear. E^{rns} is a membrane-bound protein [24] that is not usually found on the surface of the infected cell [88]. Accordingly, both the membrane anchoring and the intracellular retention signal of the protein must be circumvented to allow secretion. It was shown recently that E^{rns} is bound to membranes via a long amphipathic helix, composed of approximately 40 C-terminal amino acids [24, 25]. E^{rns} is the first surface protein shown to be anchored in this way. It seems likely that this unusual membrane anchor plays an important role in E^{rns} secretion and the tuning of the equilibrium between release and retention. On the basis of the considerations outlined above, this membrane anchor would also be important for the function of the RNase.

E^{rns} contains eight cysteines that form intramolecular disulfide bonds [89] and are conserved in all pestiviruses analyzed to date. A ninth cysteine is found rather close to the C terminus of the protein in the overwhelming majority of pestivirus isolates. This cysteine, E^{rns} residue 171, is engaged in forming E^{rns} dimers via disulfide bonds between two monomers. These homodimers are found in both infected cells and virus particles [12]. There are very few pestivirus strains, most

notably a biologically cloned virus of the BVDV-1 prototype strain NADL, that lack C171. The existence of such naturally occurring viruses and of several engineered virus mutants that lack C171 [90, 91] proves that formation of E^{rns} homodimers linked via C171 is not essential for pestivirus viability. Since E^{rns} proteins lacking C171 do no establish dimers stable enough to allow purification and analysis under mild conditions, the formation of stable dimers does not appear necessary for pestivirus viability [91]. Nevertheless, E^{rns} dimerization must have a distinct advantage, because C171 is conserved among the overwhelming majority of pestiviruses. Indeed, the growth in tissue culture of CSFV and BVDV lacking C171 is retarded by approximately 1 order of magnitude. More important, two different CSFV mutants with exchange or deletion of C171 are very significantly attenuated in animals. Therefore, dimer formation seems to be connected with virulence [91].

Taken together, the data suggest more than the ability to hydrolyze RNA is necessary for E^{rns} virulence factor activity. There is evidence for the involvement of both secretion and dimer formation in this interesting biological function.

3.2.4 N^{pro}, E^{rns}, and Persistence

The function of N^{pro} during infection of adult host animals is not clear. Only mild or even no attenuation has been observed for N^{pro}-negative mutants [77]. Defined mutants abrogating the type 1 interferon repressing function of the protein do not necessarily lead to significant attenuation, so a connection between N^{pro}-induced repression of the innate immune system and general pestivirus virulence is rather unlikely [77]. Attenuation due to N^{pro} deletion seems, therefore, to be a secondary effect, presumably a consequence of reduced viral protein translation. Similarly, the detailed function of the E^{rns} RNase remains obscure. A clear disadvantage of RNase-negative viruses was detected when the natural host was inoculated with them: the virus load in the infected animals was much lower, as indicated by the absence of virus transmission to contact animals. Despite the demonstration that secreted E^{rns} can block type 1 interferon induction by extracellular RNA, the mechanism underlying the RNase effect is not yet known. The presence of significantly increased amounts of extracellular RNA within infected animals is not established, so the mechanism and target of the E^{rns} RNase activity remain obscure.

Interestingly, an adequate interferon response was observed in immunocompetent cattle after acute infection with noncytopathic BVDV [45]. In contrast, absence of a type 1 interferon response is typical in animals persistently infected with noncytopathic BVDV and was proposed to play an important role in establishment and maintenance of persistent infection [92]. Therefore, the real functions of N^{pro} and the E^{rns} RNase may be to assist in the latter processes in the fetus. If so, these functions cannot be investigated in the adult animal. In fact, N^{pro} and the E^{rns} RNase activity are connected with the establishment of persistent BVDV infections [93]. When fetuses in pregnant heifers were directly infected with different BVDV mutants, as reported previously [92], a noncytopathic wild-type BVDV did not

induce a type 1 interferon response, but a corresponding cytopathic virus induced type 1 interferon synthesis. Both E^{rns}-RNase-negative and N^{pro}-deletion mutants of the same noncytopathic virus also induced the expression of type 1 interferon in the fetus, at levels similar to those induced by the cytopathic BVDV variant. The combination of these two changes in a double mutant resulted in an extremely elevated type 1 interferon response 7 days after infection [93].

Although induction of an interferon response in the infected fetus is not directly connected to prevention of virus persistence, there is at least some correlation. The RNase-negative or N^{pro}-deletion single mutants of BVDV could establish persistent infection, as documented by isolation of infectious virus from fetuses 2 months after infection of pregnant heifers [93]. In contrast, double mutants, with a combination of N^{pro} deletion and E^{rns} RNase inactivation, were never found in the fetus in these experiments. When such mutants were introduced directly into the fetus, abortion within the first 3–7 weeks after infection always occurred in all animals. Therefore, it can be concluded that the double mutant cannot establish persistence, presumably because it induces a strong type 1 interferon response, leading to severe innate immune reactions. The ongoing secretion of type 1 interferon and the resulting downstream effects should lead to apoptosis of cells, ultimately causing massive damage to the fetus.

3.3 Textbook of BVDV Persistence and Lessons for Vaccine Approaches

From currently available data, establishment of BVDV persistence relies on a set of prerequisites. The first prerequisite is the correct time of infection of a pregnant cow, so that antigens of the transplacentally transmitted virus are accepted by the fetus as "self," preventing adaptive immune responses. The second prerequisite is the need for a noncytopathic biotype that prevents cell damage by strict control of viral RNA replication to limit expression of danger signals. N^{pro} and the E^{rns} RNase are needed to repress innate immune reactions triggered by the presence of viral double-stranded RNA within the infected cell and at currently unknown additional sites.

One might expect that loss of either N^{pro} or the E^{rns} RNase function would prevent establishment of persistence, because both are hypothesized to exert quite different activities that block the innate immune response. However, because both seem to counteract the induction of type 1 interferon expression, one could also imagine a certain degree of redundancy. Alternatively, it may be that the type 1 interferon response must only be reduced below a threshold level, with just one of the two viral counteracting activities being sufficient to reach this level. In fact, experiments have shown that the presence of only one of the two functions is sufficient to allow establishment of persistence. However, it can be hypothesized that a reduction of the incidence and duration of persistence would be observed if a

sufficiently high number of pregnant animals could be challenged with viruses lacking an active RNase or N^{pro}. It is also important to note that efficient control of viral replication is absolutely necessary, because cytopathic viruses cannot prevent a type 1 interferon response, even in the presence of normal amounts of functional N^{pro} and active E^{rns} RNase.

Modern BVDV live vaccines should be designed to induce protective immunity against transplacental infection of the fetus in pregnant animals and to be safe in pregnant animals. For safety, a live vaccine virus should not establish persistent infection itself and should not cause any other adverse effects in the heifer or fetus. Available data indicate that this goal can be achieved by interfering with the viral repressors of the innate immune response. We have demonstrated that a noncytopathic double mutant, with deletion of the N^{pro}-coding sequence and elimination of a histidine residue in the active center of the E^{rns} RNase, is a promising vaccine candidate that meets the above-described criteria. When a pregnant heifer was inoculated with this mutant, the mutant did not detectably cross the placenta but did induce protective immunity that prevented fetal infection upon challenge. Further efforts will be necessary to elucidate the function of the E^{rns} RNase in detail and define the mechanism underlying the attenuation of RNase-negative viruses.

As mentioned already, two species of BVDV are found in the host population, designated BVDV-1 and BVDV-2. All vaccines currently available are derived from BVDV-1. Published data prove that these vaccines do not protect sufficiently against a BVDV-2 challenge. Therefore, full protection of cows against BVDV requires vaccines that most likely must include both BVDV-1 and BVDV-2 or at least structural proteins from both species.

4 Pestiviruses Other Than BVDV

The development of vaccines against pestiviruses other than BVDV is less advanced. The authors are not aware of ongoing efforts to produce BDV vaccines, even though border disease also has a significant economic impact. In contrast, discussions about novel CSFV vaccines have been reactivated because classical swine fever outbreaks in the last few decades have required culling campaigns, which are associated with serious ethical concerns and enormous costs. As for BVDV, safe and effective modified live vaccines with defined attenuating mutations would be reasonable for CSFV. Recently, a cytopathic strain of CSFV was established by the insertion of Jiv-coding sequences into the viral genome [59]. This virus showed more efficient NS2-3 processing and upregulation of viral RNA synthesis, as observed previously for cytopathic BVDV strains with Jiv insertions. In cell culture experiments the cytopathic CSFV mutant induced the expression of the interferon-regulated gene Mx, but the parental noncytopathic CSFV strain did not. Accordingly, increased replication of the cytopathic virus correlated with induction of an innate immune response. Interestingly, the cytopathic virus was

greatly attenuated in its natural host. Because the cytopathic CSFV mutant induced high levels of neutralizing antibodies, it is a potential vaccine candidate.

Not only must the vaccine prevent disease, horizontal virus spread, and vertical virus spread, but the vaccine virus itself must also be safe in pregnant animals. Because fetal infection and persistence is somewhat different for CSFV, it cannot be foreseen whether the same mutations used in the live attenuated BVDV vaccine will be appropriate. This point must be analyzed soon.

5 Alternative Approaches to Virus Attenuation

The attenuation of pestiviruses for vaccine approaches can, of course, also be achieved by mutations that do not affect the functions involved in controlling the innate immune response and establishing persistent infection. As a general rule, reduction of virus fitness by mutations reducing the efficiency of viral replication will most likely result in attenuation. In this context, mutations affecting genome replication, viral gene expression, the efficiency of target cell infection, or the tropism of the virus in its natural host can lead to live attenuated vaccines.

Different mutations reducing the efficacy of pestivirus gene expression have been identified. One of these changes is directly connected with deletion of N^{pro}. As mentioned already, the deletion of this nonessential protein usually results in a certain degree of virus growth retardation. This effect does not seem to be connected with the loss of interference with the type 1 interferon response, but has been hypothesized to result from less efficient translation of the viral RNA [77]. Pestiviruses recruit ribosomes for translation of their genome via the IRES located in the 5′ UTR. Mapping of the IRES revealed that, at least in several pestiviruses, the sequence relevant for efficient translation initiation extends into the region downstream of the IRES. The importance of the downstream sequences has been proposed to correlate with secondary structure constraints [94–97]. Thus, deletion of N^{pro} not only eliminates part of the ability of pestiviruses to interfere with the innate immunity of the host, but it also impairs IRES function and thereby reduces the efficiency of virus propagation.

IRES function can also be affected by other mutations. When different alterations were introduced into the UTRs of BVDV to reduce virus replication, a variety of different mutants were recovered that were viable and stable. These viruses showed reduced fitness in cell culture and were found also to be attenuated in their animal host. Such mutants with alterations in the IRES also represent potential vaccine candidates [98, 99].

Similarly, alterations in the 3′ UTR, which contains important *cis*-acting elements for virus replication, can attenuate pestiviruses. A 12-nucleotide insertion, first identified in a lapinized CSFV vaccine strain recovered after consecutive passage of pathogenic virus in rabbit cells, conferred attenuation when introduced into the highly pathogenic strain Shimen [100]. The alteration retarded the growth of the mutant virus in tissue culture by nearly 2 logs. This considerable growth

retardation is the most probable explanation for the observed attenuation in vivo. The stability of attenuation resulting from such inserted sequences is an important unanswered question. In theory, an insertion that hampers virus growth can easily be lost by recombination, restoring virulence.

Interference with virus fitness at the level of infection of a target cell has been achieved by introducing mutations into structural protein-coding regions. In one approach, a linear epitope in E2 used to differentiate CSFV from ruminant pestiviruses was changed in highly virulent CSFV Brescia in different ways to resemble the corresponding sequence of BVDV strain NADL. Two of the resulting viruses showed considerable growth retardation and proved to be attenuated in pigs [5, 6]. In addition, a 19 amino acid insertion introduced into the C-terminal region of E1 via transposon linker insertion mutagenesis resulted in an attenuated virus that had a growth rate equivalent to that of the wild type in tissue culture [101].

Other approaches to attenuation of CSFV by mutations in structural protein-coding regions rely on elimination of N-glycosylation sites. This was done for E2, E^{rns}, and E1 [6, 102, 103]. In all cases, the mechanism underlying the observed attenuation is not fully understood and the long-term stability of the mutations has not yet been demonstrated.

Virus fitness can also be impaired in chimeras that contain sequences from two different strains of one pestivirus species or even from members of two different species. Several chimeras in which sequences from the CSFV vaccine viruses "C-strain" and "CS-strain" were introduced into the background of the highly pathogenic wild-type virus Brescia are attenuated in pigs [5–7].

Similarly, chimeras that combine sequences from CSFV and BVDV have been established. In several cases, a CSFV background was used, and specific fragments, such as the E^{rns}-coding region or part of the E2-coding sequence, were replaced by the corresponding BVDV sequences [104, 105]. These chimeras have acceptable or even high growth rates in porcine cells. The chimeric viruses are not pathogenic in pigs, as is expected because the mutants have a CSFV vaccine strain background. Importantly, vaccination with the chimera protects against a stringent wild-type CSFV challenge.

A different approach was chosen to construct the chimeric virus CP7_E2alf. In this case, a BVDV background (stain CP7) was used, and the E2-coding region was replaced by the corresponding sequence from CSFV strain Alfort 187. After passage, this virus replicated in porcine tissue culture cells to rather high titers. Importantly, the chimera propagated in bovine cells to only very low titers. Vaccination of pigs with this BVDV-based recombinant virus induced protective immunity against a stringent challenge. A theoretical disadvantage of this approach is the lower complexity of the CSFV-specific immune response owing to the absence of CSFV E^{rns} as a second target for neutralizing antibodies [14] and the absence of a complete set of T-cell epitopes in viral structural and nonstructural proteins [106]. Owing to these considerations, a less effective cross-protection would be anticipated after vaccination with this mutant. The same disadvantage, though less relevant owing to the much smaller size of the exchanged fragments,

would also be intrinsic to other interspecies chimeras. It remains to be determined whether this theoretical flaw is of practical relevance.

In general, vaccination with interspecies chimeras bears an additional risk, because new types of viruses are created and spread in the field. The combination of sequences from different virus species might result in unforeseeable changes in tropism or virulence in "nontarget species." Risk assessment is difficult, because only a limited number of experiments can be conducted. If the most likely target species have tested negative for adverse affects from a chimeric virus infection, the risk can be hypothesized to be low. In the end, society must decide whether or not this risk is necessary and justified.

Additional alternative approaches to new pestivirus vaccines cross the border between live attenuated viruses and killed virus vaccines. These approaches are based on establishing autonomous replicons by deleting sequences from viral genomes. Several such putative replicon vaccines have been established for BVDV and CSFV [78, 107–110]. In all of these recombinant viruses, essential sequences were deleted from the genomes so that the vaccine candidates can only be propagated when the proteins encoded by the deleted sequences are provided in *trans*. An advantage of such replicons is certainly their safety, because infection of a cell is a dead-end, with no infectious virus released. In contrast to killed virus vaccines, the replicons express genes and viral proteins are synthesized de novo within the infected cells. Intracellular production of viral proteins allows presentation of viral peptides on MHC, resulting in a T-cell immune response in addition to a humoral response. Care must be taken during propagation of the deletion mutants in complementing cell lines so that fully replication competent viruses are not restored by recombination between the replicon genome and RNA coding for the complementing proteins.

Vaccination efficiency is a general question with the replicon approach. Because, even with a high vaccine dose, only a very limited number of cells are infected and produce viral proteins, the trigger for the immune system is not very prominent. Nevertheless, induction of protective immunity has been successfully demonstrated in stringent challenge models in several cases. It is not yet clear whether a single vaccination with a replicon can consistently prevent fetal infection in a pregnant animal.

6 Marker Vaccines

Modern approaches to eradication of viruses often rely on a combination of vaccination and culling of (persistently) infected animals. Marker vaccines that allow the differentiation of vaccinated from field-virus-infected animals by serological techniques have been successfully used for such projects. In the case of pestiviruses, the benefit of a marker in the vaccine virus is a matter of debate. The main reason for this debate is the absence of antibodies in persistently infected animals because of the special way in which these viruses establish persistence. It is

generally accepted in the field that the most important step to control pestiviruses is eliminating persistently infected animals. In contrast to, for example, animals infected with herpesvirus, animals persistently infected with pestiviruses cannot be identified by serological means. A vaccine would have to be designed so that, although the presence of vaccine virus in persistently infected animals could not be detected serologically, it could be detected by other means, such as a simple reverse transcriptase PCR test. In any case, such immunized but nevertheless persistently infected animals would have to be eliminated.

Despite the problems outlined above, pestivirus marker vaccines could certainly have advantages for control strategies; therefore, this aim is pursued by different groups (recently reviewed for CSFV in [111]). Because of the high degree of mutation and recombination, a positive marker is likely to be lost rather quickly if it is not combined with a selective marker. A negative marker introduced by a genomic deletion would be preferable. As a nonessential protein with immune evasion function, N^{pro} would be a perfect marker; however, N^{pro} does not seem to be immunogenic enough to induce the required levels of specific antibodies. Other ideas for establishing marker vaccines are based on the chimeric pestiviruses (described earlier) that have genomes combined, for example, from BVDV and CSFV sequences. Other approaches rely on the already mentioned replicons. After vaccination with such chimeras or deletion mutants, the animals could be serologically differentiated from animals that had been infected with the corresponding field viruses. As mentioned already, a general problem of such vaccines could be a lower degree of acceptance owing to difficult risk assessment or lower vaccination efficiency.

In summary, the search for a feasible marker for pestivirus vaccines remains an ongoing process waiting for novel ideas.

7 Conclusion

The importance of pestiviruses as livestock pathogens with enormous economic impact and a significant effect on food production demands the development of modern, safe, and effective vaccines. Detailed investigation of the molecular features of these viruses and their interactions with host animals and the establishment of infectious complementary DNA constructs for several pestiviruses provide a solid basis for approaches toward novel vaccines. Because of the need to protect fetuses in pregnant animals, live attenuated viruses, with their high immunogenic potential, seem to be most appropriate. The near future will show which types of putative vaccine viruses are promising enough for development of commercial products. Because the elucidation of pestivirus molecular biology is still in progress and frequently provides new surprises, there is also a good chance that there will be further interesting vaccine approaches in the future.

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Live Attenuated Influenza Virus Vaccines: NS1 Truncation as an Approach to Virus Attenuation

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Abstract Influenza virus causes significant morbidity and mortality worldwide. Vaccination, usually involving the inactivated type of vaccine, is the primary mechanism of influenza virus prevention. Live attenuated influenza viruses (LAIV), however, are also available for the prevention of disease. These vaccines have been shown to stimulate a robust cellular response, induce IgA and IgG antibodies, and can provide heterosubtypic protection. Cold-adaptation and temperature sensitivity are two mechanisms of influenza virus attenuation, yielding viruses that are both safe and immunogenic. At present, novel attenuation strategies, including the manipulation of viral gene sequences and proteins, are being developed in the hopes of providing new LAIV vaccines. One promising strategy involves the truncation of the NS1 protein of influenza virus, limiting the interferon antagonist capabilities of the influenza pathogen. Experimental vaccines that exploit this mode of attenuation have been tested in several animal models; as summarized herein, high efficacy in reducing mortality, morbidity, and transmission of influenza viruses has been observed.

1 Influenza Virus Vaccines: The Current Standard

Vaccination has proven to be the most cost-effective medical intervention targeting seasonal influenza [1]. Nonetheless, the very young, the elderly, and the immunocompromised – population groups at high risk of suffering severe complications of influenza [2–4] – do not respond optimally to vaccination. Thus, there is an identified need for more effective vaccination strategies eliciting greater protective

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immunity in at-risk populations [5, 6], as well as vaccines to better protect the general population.

1.1 Inactivated Influenza Vaccines

The most widely available human influenza vaccines are of the inactivated (killed) type. Typically, these vaccine preparations derive from reassortants possessing at least the hemagglutinin (HA) and neuraminidase (NA) genes from the presently circulating strains and the remaining genes from the master donor strain A/Puerto Rico/8/34 (A/PR/8/34) virus. This gene constellation confers high-yield viral growth of a vaccine preparation [7], while retaining the antigenic characteristics of the circulating strains. Influenza B virus vaccine preparation does not involve the use of a high-growth strain to improve titers in egg-based systems. Instead, bulk vaccine stocks derived from circulating influenza B vaccines are serially passaged and amplified in embryonated chicken eggs, followed by inactivation [8]. Trivalent inactivated vaccines (TIVs), however, are of limited efficacy in elderly and pediatric populations [3, 4]. In addition, it is possible that large or multiple doses of inactivated vaccines would be needed to provide protection against certain strains, including antigenically novel influenza viruses [9].

2 Live Attenuated Cold-Adapted Influenza Vaccine

Early attempts to create live attenuated influenza virus vaccines aimed to exploit physiological differences in the human respiratory tract; the temperature of the upper respiratory tract is approximately 32–34°C, while that of the lower respiratory tract is 37°C [10]. Cold-adapted viruses would theoretically replicate to levels capable of triggering an immune response in the upper respiratory tract, while keeping virus titers in the lower respiratory tract low enough to not cause adverse effects in the patient.

Early strategies to exploit these temperature differences included the selection of temperature-sensitive mutants that were grown in the presence of a mutagen, 5-fluorouracil. Temperature-sensitive mutants were attenuated in animal models [10, 11], but were overattenuated in human subjects [12]. In addition, the temperature sensitivity was a result of only one or two genetic mutations [13], making these viruses less genetically stable and more prone to reversion.

Via serial passage at low temperatures, A/Ann Arbor/6/60 (H2N2) (A/AA/6/60) [14–16] and B/Ann Arbor/1/66 (B/AA/1/66) viruses [16] were cold-adapted by the Maassab laboratory in the Department of Epidemiology at the University of Michigan School of Public Health, United States. These attenuated viruses displayed markers of cold-adaptation [17], thought to be a result of multiple genetic mutations [18, 19]. Specifically, five amino acid changes in polymerase basic 1 protein, polymerase basic 2 protein, and nucleoprotein (NP) have been implicated in the cold-adapted phenotype of A/AA/6/60 [20]. These mutations are thought to

reduce levels of M1 protein and vRNA expression and inhibit the export of vRNAs to the cytoplasm [20]. Mutations in NP, polymerase acid, and matrix (M) proteins are thought to cause attenuation of B/AA/1/66 virus, affecting polymerase function as well as efficient virus assembly and budding [21]. The multiple mutations found in these viruses are thought to contribute to their genetic stability, making them ideal for vaccine use.

Prior to the advent of reverse genetics, reassortants were created via coinfection to create vaccine viruses that contained six internal genes from the influenza A or B Ann Arbor strains and the HA and NA of influenza virus strains circulating in the community [22]. This coinfection technique for the creation of reassortants, whereby the master donor strains would be A/AA/6/60 virus or B/AA/1/66 virus, was therefore an attractive method of creating LAIV vaccines for newly circulating viruses in a timely fashion.

The advent of reverse genetics allowed for the creation of temperature-sensitive, cold-adapted reassortants to be used as vaccine viruses, creating "6 + 2" viruses (HA and NA of circulating strain and the remaining 6 segments from either A/AA/6/60 virus or B/AA/1/66 virus) via a plasmid rescue system. This method is of particular value when creating vaccine viruses for highly pathogenic avian influenza (HPAI) viruses that are normally lethal to eggs due to of the presence of a polybasic cleavage site.

It is possible that avian influenza viruses could contribute to an antigenically novel reassortant pandemic virus, as was seen in 1957 and 1968 [23]. HPAI viruses that could cause systemic infections are of particular concern. HPAI viruses contain a polybasic cleavage site in the HA molecule that contributes to the pathogenicity seen in these strains [24]. Ubiquitous proteases can cleave the HA molecule at this site, activating the HA molecule in a variety of tissue types [25, 26]. Because these highly pathogenic avian viruses are lethal in eggs, the multibasic cleavage site of these high pathogenic strains must first be modified by reverse genetics techniques so that stocks can be grown to high titer in this substrate [27, 28].

Inactivated HPAI virus vaccines are weakly immunogenic in human volunteers or require adjuvant to have an immunogenic effect [29–31]. "6 + 2" reassortants in the cold-adapted Ann Arbor background, however, show promise in animal studies. Suguitan et al. engineered vaccines on the cold-adapted background containing HA molecules from 1997, 2003, and 2004, with the multibasic cleavage site deleted. These vaccines also possessed an avian NA. A single dose of 10^6 $TCID_{50}$ of vaccines with HA from 1997, 2003, and 2004 H5 viruses resulted in 100% survival in mice following challenge with 50, 500, or 5,000 LD_{50} of A/Hong Kong/491/1997 and A/Viet Nam/1203/04 H5N1 viruses [28]. Recently, a cold-adapted live attenuated vaccine with a modified HA made against A/Anhui/2/2005 (H5N1) was also shown to be attenuated in chickens and mice. This vaccine virus also stimulated neutralizing antibodies and HA-specific CD4+ cells in rhesus macaques. Furthermore, macaques that received two doses of the vaccine did not experience any weight loss or temperature changes in the 15 days following challenge with 10^6 EID_{50} wild-type A/Anhui/2/2005 or A/bar-headed goose/Qinghai/2/05 viruses (H5N1) [32].

Vaccine viruses on the background of A/AA/6/60 are also safe and protective from wild-type H6 [33] and H9 [34] viruses in mice. However, note that cold-adapted virus vaccines generated to provide protection against avian influenza strains appear to be more attenuated than cold-adapted viruses for seasonal influenza in humans [35, 36]. Alternative vaccine strategies against avian influenza viruses may still be required.

Although the cold-adapted Ann Arbor strains are the present master donor strains for live attenuated vaccines available in the United States, other countries have explored the use of alternative virus strains. The former USSR has a long history in the use of live attenuated vaccine viruses; most influenza vaccination in Russia today is almost exclusively of the live attenuated type. The main master donor strains, A/Leningrad/134/57 (H2N2) and B/USSR/60/69, were attenuated by repeated passage in embryonated eggs at successively lower temperatures [37]. For decades, these viruses have shown to be effective.

2.1 Molecular Mechanisms of Protection

Cold-adapted LAIV vaccines stimulate increased levels of IgA in the upper respiratory tract [38, 39]. Increased IgA levels were seen in young children when compared with those immunized with TIV. These levels correlate well with decreased viral shedding in the nasal passageway following challenge with the parent virus 12 months postvaccination, compared with children vaccinated with TIV or children who were not vaccinated [39]. Because viral shedding correlates with severity of illness [40], IgA induction is most likely important to protection against influenza virus morbidity.

Following vaccination, robust cellular responses are induced [41], and systemic IgG levels are increased [42]. Interferon-γ, an important antiviral cytokine, is expressed following administration of the cold-adapted virus [43]. It is also possible that cold-adapted LAIV vaccines could protect patients from infection that occurs shortly after vaccination by replicative interference [44, 45]. It is thought that the M protein [46] interferes with vRNA replication of a noncold-adapted strain [47].

2.2 Trivalent Live-Attenuated Influenza Virus Vaccines: Protection for a Variety of Groups

2.2.1 Protection in Children

The trivalent cold-adapted influenza virus vaccine (CAIV-T) was licensed for use in healthy children as young as 5 years old in 2003 [48]. Its use has since been expanded to include children aged 2–4 [49].

In initial trials, cold-adapted influenza virus vaccine administered at a dose of 10^6 $TCID_{50}$ was safe and immunogenic in children. Response to the vaccine was age-dependent, however, with lower antibody titers in children 6 months or younger [50]. In a large, multicenter, double-blind, placebo-controlled study, administration of a CAIV-T to 1,602 children aged 15–71 months was safe, immunogenic, and protective against influenza A (H3N2) and B viruses circulating during the 1996–1997 monitoring season [51]. In this study, the genetic stability of CAIV-T following vaccination was also demonstrated [52].

Eighty-five percent of children vaccinated in year 1 returned for revaccination in year 2. This revaccination in 1997 proved to be immunogenic and provided protection against epidemic A/H3N2 and influenza B viruses in circulation during the 1997–1998 influenza season, including a variant strain A/Sydney/5/97 (H3N2). Protection from a virus not contained in the CAIV-T [53] demonstrated the ability of the vaccine to provide protection from heterologous challenge in children. Ninety-two percent vaccine efficacy was determined overall (95% CI: 0.89–0.94), with 89% vaccine efficacy seen in the second year (95% CI: 0.81–0.94), despite the presence of a strain variant not included in the trivalent vaccine [54]. In later studies, children 1.5–18 years of age were administered the CAIV-T against circulating H3N2, H1N1, and B virus strains. Those that received the vaccine in 1999 or 2000 were protected from the H1N1 and B virus strains circulating during the 2000–2001 influenza monitoring time [55]. Serum HA inhibitory antibody and IgA levels correlated well with the prevention of shedding [56].

Efficacy was also assessed when children were given CAIV-T annually. While higher antibody levels were seen after the first vaccination with CAIV-T, antibody titers were still high, particularly against H3N2 and B strains, in healthy children vaccinated for 4 consecutive years [57].

2.2.2 Protection in Adults

In early clinical trials, CAIV was well tolerated and had a greater estimated protective efficacy compared with trivalent, inactivated virus vaccine (85% versus 71%) [58]. In a larger, placebo-controlled, clinical trial, 4,561 working adults were enrolled. Vaccination caused a statistically significant reduction in severe febrile illness by 18.8% and febrile upper respiratory illness by 23.6% in those aged 18–49 relative to placebo. This correlated with a reduction in the number of days of illness, fewer days of lost work, and fewer health care provider visits [59]. A recent study of 1,952 healthy adults has suggested that TIV is more efficacious than CAIV-T in adults, however, resulting in a 50% reduction (95% confidence interval, 20–69) of influenza illness in those who received TIV as compared with those who received CAIV-T prior to the 2008 influenza monitoring time. Vaccine efficacy was also calculated with respect to influenza A and influenza B viruses. While greater vaccine efficacy was seen with TIV as compared with CAIV-T with respect to influenza A viruses, there were not enough culture positive influenza B cases to draw relative efficacy conclusions [60].

2.2.3 Protection in the Elderly

Cold-adapted vaccines enhance IgA levels in the upper respiratory tract in those 65 years and older [61] and cause both systemic and mucosal immune responses [62]. In many clinical trials, the superiority of CAIV-T over whole inactivated virus vaccine in inducing serum and secretory antibodies has not been demonstrated in the elderly [62, 63]. However, the safety of CAIV has been shown [64] and a randomized, double-blind trial studying nursing home patients over a 3 year period did confirm that additional protection could be provided if the CAIV (using A/AA/6/60 as the master donor strain) was administered in conjunction with TIV [65]. In a double-blind field trial involving nursing home occupants, combining these vaccines resulted in a 60% decrease of influenza A viral infections when compared to rates of infection seen in those vaccinated with only TIV [66].

2.2.4 Protection in the Immunocompromised

CAIV-T has not been shown to cause serious adverse effects in HIV positive, asymptomatic patients. Changes in HIV viral load and CD4+ cell numbers were not affected in an adult cohort following vaccination [67]. Similar results were seen in trials involving HIV-positive children [68].

3 Live Attenuated Influenza Virus Vaccines Using Micro-RNA Technology

Micro-RNAs (miRNA) are endogenous RNA segments that are approximately 21 nucleotides in length and are involved in gene silencing [69]. miRNAs are derived from RNAs that fold on themselves to create hairpin structures called pri-miRNAs, primarily in the nucleus [70, 71]. It is believed that in mammalian cells the RNase III enzyme Drosha converts pri-miRNA into an approximately 70 nucleotide stem-loop pre-miRNA [72, 73]; this stem-loop is exported to the cytoplasm where it is cleaved by another RNase III enzyme, Dicer, into a mature, cytoplasmic miRNA [74–77]. This mature miRNA binds complementary mRNA in the cytoplasm, in association with members of the RNA-induced silencing complex [78], specifically with the RNase Argonaute [79–81]. It is by association with this complex that translational repression or mRNA degradation is executed by miRNAs [69].

Perez et al. sought to attenuate influenza virus by incorporating miRNA response elements (MREs) into viral genomic segments. To achieve species specificity, miR-93 was chosen to target viral transcripts. This miRNA is ubiquitously expressed in mice (*Mus musculus)* and humans (*Homo sapiens)*, but not in chickens (*Gallus gallus)*, allowing for robust growth of vaccine stocks in eggs. In order to cause attenuation, two MREs were introduced into the NP segment of the A/PR/8/34 strain.

The growth of this virus was attenuated in the human embryonic kidney cell line HEK293 and in mice, but grew to high titer *in ovo*. Administration of either an A/PR/8/34 or a H5N1 6:2 reassortant virus (6 segments from A/PR/8/34 virus: modified HA and unmodified NA from A/Vietnam/1203/04) containing MREs in the NP protein induced an antibody response and caused less mortality than viruses lacking these sites [82]. Vaccination with this LAIV vaccine is therefore an exciting new strategy of attenuation and holds promise as a novel vaccine mechanism.

4 Influenza Virus Immunity Through Other Viral Vectors

The replication deficient vaccinia virus Ankara (MVA) was attenuated via serial passage in chick embryo fibroblast culture [83]. Attenuation is thought to be a result of deletions in the virus genome following passage [84]. Although replication is unhindered in avian cells, MVA is replication deficient in mammalian cells, making it an attractive mammalian vaccine vector, which can be used to express both viral and recombinant genes [85, 86]. As a result, recombinant MVA viruses expressing foreign viral antigens have been developed to protect against a variety of human pathogens [86–89]. MVA is an ideal viral vector, based not only on its species specific growth patterns but also due to its safety profile and immunogenicity [87].

MVA viruses expressing the HA of influenza viruses are effective vaccine viruses [90]. Transfection of virally derived cDNA into MVA-infected cells [91] allows for the incorporation and expression of recombinant genes under the vaccinia virus-specific PsynII promoter [92]. Following two vaccinations of the MVA virus expressing HA from A/Vietnam/1194/04 (H5N1), mice were fully protected from signs of morbidity following challenge with 10^3 $TCID_{50}$ of the parental virus and A/Indonesia/5/05 (H5N1). In addition, little to no weight loss was seen following challenge with these viruses [90]. Using similar strategies, other groups have shown safety and antigenicity in mice and chickens [93, 94]. Recently, the efficacy and safety of a MVA-based vaccine against A/Vietnam/1194/04 (H5N1) [90] was tested in nonhuman primates [95]. Vaccination with this vaccine was well tolerated. Following challenge with A/Vietnam/1194/04 or A/Indonesia/5/05 viruses, mock-vaccinated animals displayed severe necrotizing bronchointerstitial pneumonia. Animals vaccinated with the MVA-based vaccine were protected, experiencing only mild bronchointerstitial pneumonia [95].

5 Novel Live Attenuated Virus Vaccines Based on Modifications of the M2 Ion Channel

The M viral genome segment expresses M1 and M2 proteins by virtue of alternatively spliced mRNA transcripts [96]. M2 acidifies endosomes following virus binding and cell entry, allowing for the release of viral ribonucleoprotein into the cytoplasm [96]. When the transmembrane and cytoplasmic domains of the M2

protein are ablated by the insertion of stop codons, the mutant virus (M2 knockout virus – M2KO) displays deficiencies in replication and lack of growth in mice [97], probably due to interruptions in the normal virus life cycle [98]. Because the M2KO virus displays an attenuated phenotype in cell culture and in vivo, its use as a vaccine virus holds promise. When an M2KO virus made in an A/PR/8/34 background was generated by reverse genetics, low levels of virus were detected in the lungs following vaccination with 3×10^6 and 3×10^5 pfu. No virus was recovered from the lungs, however, when lower doses were administered, except for one animal vaccinated with 3×10^4 pfu. Virus-specific antibodies correlated well with survival rates following lethal challenge with wild-type A/PR/8/34 virus [99].

Although attenuated, virus growth deficiencies can be overcome when grown in a stable cell line expressing M2 protein [99]. This could therefore be a strategy for growing large vaccine stocks. This method of attenuation creates an attenuated and safe live virus vaccine, though further studies with this vaccine construct are warranted.

6 Novel Live Attenuated Virus Vaccines Based on Modification of Viral Interferon Antagonists

Research in recent years on influenza virus, as well as many other viral pathogens, has led to the identification of viral gene products that antagonize mammalian antiviral responses by inhibiting the type I interferon (IFN) system [100]. The widespread presence of IFN antagonists in diverse virus families provides a rationale for the generation of a novel class of live attenuated vaccines [101]. By engineering viruses that have an impaired ability to inhibit the type I IFN system, it should be possible to generate vaccine strains that can grow in vitro in IFN-deficient substrates but will be attenuated in vivo by inducing the host IFN antiviral response (Fig. 1). As added value, the induction of type I IFN can result in increased adjuvancy and enhanced B and T cell responses [39, 102–104], so that mutant viruses may be intrinsically more immunogenic than wild-type viruses. In support of this concept, it has been demonstrated that NS1 mutant influenza viruses are potent activators of dendritic cells [105, 106] and potent immunostimulators [107].

6.1 The NS1 Protein of Influenza Viruses

Segment 8 of influenza A virus encodes two proteins through alternative splicing of its mRNA: the NS1 and NEP proteins [108]. NS1 is a polypeptide of 215–238 amino acids, depending on the viral strain, and is the most abundant viral nonstructural protein expressed in influenza virus-infected cells. The NS1 proteins of both influenza A and B viruses inhibit the type I IFN response [109, 110]. This inhibition is achieved through a block in the transcriptional induction of type I IFN. Antagonism of this innate cellular response contributes to the virulence of influenza viruses.

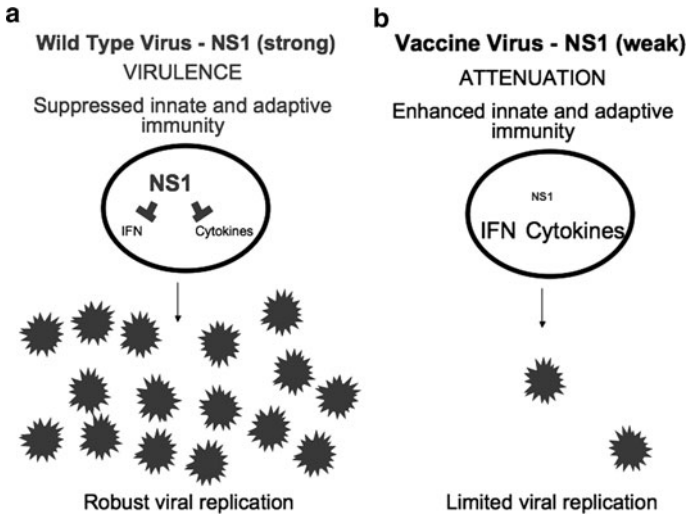

Fig. 1 Proposed rationale for live attenuated influenza virus vaccines based on NS1 modification. (**a**) Wild-type influenza viruses express the NS1 protein, which reduces induction of type I IFN and other related cytokines. This suppresses the innate and adaptive response of the infected cell, and virus is able to multiply unhindered. (**b**) Deletions in the NS1 gene interrupt the protein's interferon antagonist capability causing attenuation as a result of an enhancement of host innate and adaptive immunity. As a result, viral replication is hindered

Indeed, the growth of a mutant influenza virus based on the A/PR/8/34 strain but lacking the NS1 protein (delNS1) is highly restricted in interferon-competent substrates [109]. Poor replication and lack of disease following delNS1 virus infection was furthermore correlated to increased levels of IFN production by the host [111]. Thus, NS1 mutant influenza viruses induce higher levels of type I IFN than wild-type viruses. The induced type I IFN in turn limits further viral replication [111]. In contrast, NS1-deleted viruses replicate efficiently in IFN incompetent systems such as STAT1 knockout mice [109]. These initial findings supported the concept that NS1-mutated influenza viruses have potential as live attenuated vaccine candidates. However, it remained problematic that viruses carrying the delNS1 mutation may be too attenuated in animal hosts to constitute a viable live attenuated vaccine. Because of this potential limitation, mutations in NS1 that partially disrupt NS1 function were sought, with the aim of generating mutant viruses with intermediate attenuation characteristics between delNS1 and wild-type virus.

6.2 Mechanisms of NS1 Function

Early studies had demonstrated that the core region of the NS1 protein responsible for inhibition of type I IFN production lies within its N-terminal dsRNA-binding

domain [112]. This domain is a dimer of 73 amino acids, with exposed basic residues responsible for interaction with dsRNA [113]. These data suggested that NS1 inhibits the induction of IFN, at least in part, by sequestering dsRNA generated during viral infection, thereby preventing its interaction with the cellular sensor involved in triggering the IFN response, RIG-I. Although deletion of portions of the C-terminal region of the NS1 protein also decrease NS1 IFN-antagonistic functions, this is, in part, a result of destabilizing the dimer required for efficient dsRNA binding [112].

Subsequent studies have revealed that NS1 inhibits the production of type I IFN by inhibiting the activation of IRF-3, NF-κB, and AP-1 – three key transcription factors that coordinate the induction of IFN-β gene expression [111, 114, 115]. Considerable detail of the mechanism underlying NS1-mediated inhibition of type I IFN production has now been elucidated. A significant milestone involved the recognition of an interaction between NS1 and RIG-I, which leads to a block of downstream signaling from RIG-I to the MAVS/Cardif/IPS-1/VISA adaptor molecule and thereby prevents activation and nuclear translocation of the IFN-β enhanceosome [116]. The mechanism of NS1-mediated inhibition has since been further refined, with the finding that interaction of NS1 with the cellular ubiquitin ligase TRIM-25 blocks dimerization of TRIM-25 and subsequent ubiquitination of RIG-I. The lack of ubiquitination of RIG-I results in inhibition of signaling to MAVS and therefore to a downstream block in transcriptional activation of type I IFN [117].

This in-depth characterization of NS1 function suggested that viruses with impaired, but not entirely abrogated, type I IFN antagonistic properties were obtainable, and might prove to be ideal live attenuated vaccine strains. Toward the realization of this concept, several studies aimed at developing LAIV vaccines based on modification of the NS1 protein by reverse genetics have been performed in recent years and key findings are summarized here.

7 Testing the Concept: Vaccination Studies with NS1-Truncated Viruses

7.1 Studies in Mice

Initial proof of principle experiments were conducted using the mouse-adapted strain of influenza virus, A/PR/8/34. Thus, modification of NS1 as an attenuation strategy was tested in mice using the A/PR/8/34 NS1-99 virus. This virus encodes a truncated NS1 protein that contains only the N-terminal 99 amino acids and possesses reduced, but not completely absent, IFN antagonist activity, intermediate between that of wt A/PR/8/34 virus and the A/PR/8/34 delNS1 virus.

The results [101] are summarized in Table 1. Eighty percent of mice receiving 2×10^3 pfu of wild-type A/PR/8/34 virus died from infection. By contrast, intranasal infection with 1×10^6 pfu of NS1-99 virus led to the death of only two of ten

Table 1 Survival of mice immunized with NS1 attenuated influenza A viruses and challenged with wild-type influenza A/PR/8/34 virus

Group	Vaccine virus[1]	Dose (pfu)	Survivors postvaccination	Survivors postchallenge[2] 1.0×10^5 pfu	Survivors postchallenge[2] 5.0×10^6 pfu
A	A/delNS1	1.0×10^6	9/9	5/5	4/4
B	A/delNS1	3.3×10^4	5/5	0/5	ND
C	A/NS1-99	1.0×10^6	8/10	5/5	3/3
D	A/NS1-99	3.3×10^4	9/10	3/5	4/4
E	A/PR8	2.0×10^3	1/5	ND[3]	1/1
F	PBS	0	6/6	0/6	ND

Adapted from [118]
[1]Refers to the abbreviated description of the genotype used to identify each virus.
[2]Mice were either challenged with 1.0×10^5 pfu or 5.0×10^6 pfu of wild-type A/PR/8/34 virus
[3]ND = not determined

animals. When vaccinated animals were challenged, most mice immunized with either high or intermediate doses of NS1-99 virus were protected, indicating that the virus is immunogenic *in vivo* despite its significant attenuation. It was observed that the delNS1 virus required high-dose vaccination in order to provide protection against challenge. Protection was correlated with the induction of efficient humoral and cellular immune responses [101]. Although these results were encouraging, they were obtained using a mouse-adapted influenza A virus strain (A/PR/8/34), and it remained to be determined whether they would extend to wild-type isolates of influenza A virus infecting their natural hosts. An initial indication that observations with A/PR/8/34 could be generalized to other strains of influenza virus came with experiments by Talon et al., who showed that vaccination with an NS1-truncated variant of B/Yamagata/1/73 conferred sterilizing immunity against challenge of mice with human influenza B virus, as assayed by viral growth in the lungs [118]. In this instance, viral growth in the lungs was used as a correlate of protection, due to the lack of disease signs normally seen in mice infected with human influenza B virus strains.

Studies of NS1-truncated influenza B virus vaccines were recently extended by Hai et al. [119], who exploited a PKR knockout mouse model to study protection from disease. Three variants of B/Yamagata/16/88 virus were generated; Yam/88/NS1-80, Yam/88/NS1-110, and Yam/88/del-NS1. In vitro, each NS1-mutated virus generated an increased type I IFN response relative to wild-type and, in vivo, none of the mutants induced signs of disease in either PKR-/- or wt C57BL/6 mice. Furthermore, the NS1-truncated viruses grew to less than 10^4 FFU/ml in lungs of PKR-/- mice, a greater than 100-fold reduction from wild-type virus levels. Mice vaccinated with Yam/88/NS1-80, Yam/88/NS1-110, and Yam/88/del-NS1 were protected from disease or weight loss upon challenge from 5×10^5 pfu of the homologous strain. BALB/c mice vaccinated with either Yam/88/NS1-80 or Yam/88/NS1-110 virus were furthermore protected from death and weight loss upon challenge with the significantly drifted heterologous influenza virus B/Lee/40, highlighting the breadth of the immune response induced by the NS1-truncated LAIV vaccines.

7.2 Studies in Pigs

To address whether NS1-truncated virus vaccines could be effective in natural hosts, a study was conducted using a swine influenza virus isolate, A/swine/Texas/4199-2/98 (H3N2) (TX/98). Through reverse genetics, three TX/98-based mutant viruses expressing truncated NS1 proteins of 73, 99, and 126 amino acids in place of the 219 amino acid protein of the wild-type virus were generated [120]. Growth properties, induction of IFN in cell culture, and virulence-attenuation in pigs of the NS1 mutant TX/98 viruses were analyzed and compared to those of the recombinant wild-type TX/98 virus. All mutant viruses were impaired in their ability to prevent induction of type I IFN in swine epithelial cells. Perhaps surprisingly, the NS1-126 virus induced more type I IFN than the shorter NS1 mutant viruses. Examination of NS1 levels revealed that the 1–126 NS1 protein levels expressed in virus-infected cells were very low compared to levels of the wild-type or the 1–73 and 1–99 mutant proteins. Thus, it appeared that different truncations affect NS1 expression to differing degrees, resulting in viruses with varying abilities to block induction of type I IFN and, consequently, varying levels of attenuation.

In intratracheally inoculated pigs, both NS1 mutant viruses were attenuated relative to the wild-type, with the TX/98/NS1-126 virus exhibiting the greatest degree of attenuation [120]. All infected animals developed specific humoral responses characterized by the presence of HA neutralizing (HI) antibodies in sera by day 8 postinoculation. Sera from animals infected with wild-type viruses had neutralizing titers approximately twofold higher than sera from those immunized with mutant viruses.

The protective efficacy of the attenuated TX/98/NS1-126 mutant was tested in a swine vaccination study. The TX/98/NS1-126 virus was administered in two doses, separated by a 3-week interval, using an inoculum of 2×10^5 pfu per pig. This regimen conferred protective immunity against challenge with 2×10^5 pfu of a homologous wild-type virus isolate (H3N2 A/swine/Texas/4199-2/98). Furthermore, and remarkably, when vaccinated pigs were challenged with 2×10^5 pfu of an H1N1 subtype swine virus (A/swine/MN/37866/99), significantly fewer lesions in lung tissues and a lower viral load in lung lavage compared to the nonvaccinated controls at day 5 postinoculation were observed [121]. These data demonstrate that the attenuated TX/98/NS1-126 mutant has significant potential as a live attenuated virus vaccine, inducing immunity against homologous challenge, as well as generating considerable protection against heterosubtypic challenge.

However, as intratracheal inoculation is not a practical route for commercial vaccination, a further vaccination challenge study involving the TX/98/NS1-126 virus was performed in which intramuscular and intranasal routes of inoculation were compared [122]. Intranasal inoculation was found to result in a superior immune priming of the local mucosa, based on the detection of swine influenza virus-specific antibody in the respiratory tract by ELISA.

For the challenge experiments, the vaccine regimen consisted of two doses of 2×10^6 $TCID_{50}$ per pig. Three challenge viruses were employed, H3N2 A/swine/Texas/4199-2/98, H3N2 A/swine/CO/23619/99, and H1N1 A/swine/IA/00239/04. Similar results to the intratracheal vaccination were obtained using intranasal vaccination. Complete, sterilizing protection from challenge with the homologous virus was observed, while the titers of the drifted A/swine/CO/23619/99 virus in nasal swabs and lung lavage samples were reduced by around 100,000-fold, to approximately 0.5 $\log_{10}$ pfu. Animals challenged with the H1N1 virus strain also had statistically significant reduction in fever and virus titers.

Thus, in the vaccination challenge study, live virus vaccination through the intranasal route produced an immune response that provided effective and broad protection, including limited heterosubtypic protection, similar to the results seen with intratracheal vaccination.

7.3 Studies in Horses

Studies characterizing the growth of equine influenza viruses containing truncated NS1 proteins in equine hosts have also been performed, to assess the suitability of NS1-based vaccines in horses. The results from experiments using the reverse genetics derived influenza A/eq/KY/5/02 strains KY/02/NS1-126, KY/02/NS1-99, and KY/02/NS1-73 broadly mirror results obtained with the swine influenza viruses in cell culture and in a mouse model, with attenuated growth of NS1-truncated viruses observed relative to the wild-type virus [123]. In mouse lung, titers of the NS1-truncated viruses were reduced by 50-fold (KY/02/NS1-73) to greater than 100,000-fold (KY/02/NS1-126), relative to wild-type virus. Each of the three NS1-truncated viruses was administered intranasally to horses to ascertain safety and immunogenicity. No animals developed any clinical signs of infection, while KY/02/NS1-126 and KY/02/NS1-73 virus-infected animals seroconverted against influenza virus by 2 weeks postvaccination, as assayed by single radial hemolysis.

Subsequently, a vaccine challenge study was performed in horses. The study demonstrated that, following homologous wild-type challenge, the KY/02/NS1-126 vaccine strain significantly reduced clinical signs of infection, conferred protection from febrile response, reduced peak virus shedding by at least 100-fold, and reduced duration of shedding [124]. These results confirmed the findings of the vaccine studies performed in pigs and extended the results to a second natural host of influenza virus.

7.4 Studies in Birds

Because of their extreme virulence, highly pathogenic H5N1 influenza viruses have caused significant problems for the poultry industry, and have occasionally caused severe disease in mammals [125, 126]. The potential of NS1-truncated LAIV to

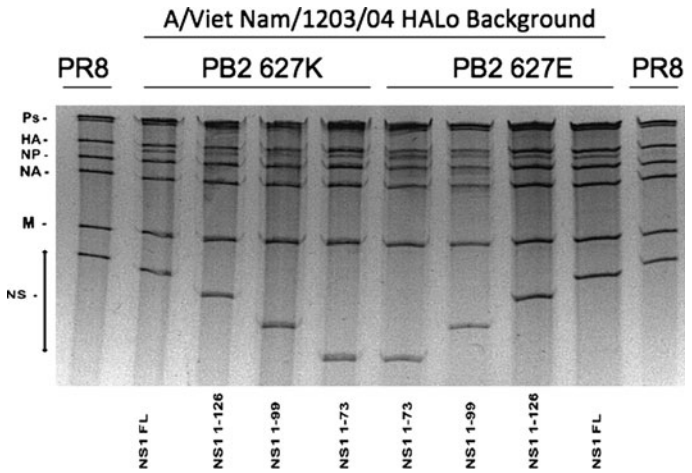

Fig. 2 vRNAs of purified A/Viet Nam/1203/04 recombinants. RNA was extracted from A/PR/8/34 and A/VN/1203/04 recombinant viruses and then separated on a polyacrylamide gel, followed by silver staining. The viral RNA gene segments are labeled: polymerase (Ps), HA (PR8), HALo (A/VN/1203/04), NP, NA, M, and NS (of varying lengths). Recombinant viruses contained either lysine or glutamic acid at amino acid position 627 in PB2. Adapted from [127]

mitigate this problem was examined by testing a panel of eight candidate live attenuated influenza vaccine viruses for poultry. The viruses were based on the strain A/VN/1203/04 and had truncated NS1 proteins. In addition to modification of the NS1, two further alterations to the viral genome were introduced: removal of the polybasic cleavage site in the HA protein of each candidate vaccine and alteration of the amino acid at position 627 of polymerase basic 2 protein from lysine to glutamic acid (to attenuate viruses in mammalian systems). The viruses were generated by reverse genetics, and their genotypes were initially confirmed through in vitro characterization (Fig. 2) [127]. Similar to results of previous studies with NS1-truncated viruses, growth was attenuated and the viruses induced high levels of interferon in mammalian substrates; nevertheless, each recombinant virus grew to high titer in embryonated chicken eggs [127]. All eight vaccine candidates were found to be markedly attenuated in a mouse model and to provide protection against lethal challenge following a single vaccination dose (Table 2).

A single vaccine that was selected for testing in chickens also protected the chickens against stringent challenge (100 CLD_{50}) with HPAI (H5N1) viruses. One hundred percent protection from death was observed with homologous challenge, and 88% protection was conferred against challenge with a heterologous H5N1 strain (Table 3).

In a separate study using an avian influenza virus with a naturally truncated NS1 protein (A/turkey/Oregon/71-delNS1 (H7N3)), Wang et al. [128], observed that chickens inoculated with the NS1-truncated virus showed no signs of disease and evidence of only extremely limited virus replication. Nevertheless, the birds were protected against a high-dose challenge (10^6 EID_{50}) with a heterologous H7N2

Table 2 Summary of the genotype and phenotype in mice of candidate vaccine viruses

Virus name[1]	Genotype[2]	MDT[3] (h)	MLD_{50}[4]	Maximum weight loss[5]	Lowest protective dose (EID_{50})[6]
VN HALo/627K/NS FL	PB2 627K, PB1, PA, HALo, NP, NA, M, NS	64, 61	$>10^6$	18% (4)	10^3
VN HALo/627K/NS 1-126	PB2 627K, PB1, PA, HALo, NP, NA, M, NS 1-126	45, 54	$>10^6$	15% (3)	10^4
VN HALo/627K/NS 1-99	PB2 627K, PB1, PA, HALo, NP, NA, M, NS 1-99	54, 49	$>10^6$	nd	10^4
VN HALo/627K/NS 1-73	PB2 627K, PB1, PA, HALo, NP, NA, M, NS 1-73	62, 36	$>10^6$	19% (8)	10^4
VN HALo/627E/NS FL	PB2 627E, PB1, PA, HALo, NP, NA, M, NS	58, 61	$>10^6$	2% (9)	10^4
VN HALo/627E/NS 1-126	PB2 627E, PB1, PA, HALo, NP, NA, M, NS 1-126	51, 54	$>10^6$	nd	10^6
VN HALo/627E/NS 1-99	PB2 627E, PB1, PA, HALo, NP, NA, M, NS 1-99	52, 47	$>10^6$	nd	10^5
VN HALo/627E/NS 1-73	PB2 627E, PB1, PA, HALo, NP, NA, M, NS 1-73	57, 52	$>10^6$	nd	10^6

Adapted from [127]

[1]Refers to the abbreviated description of the genotype used to identify each virus. Numbers following "NS" refer to the number of amino acids present in the NS1 protein starting from the amino terminal methionine. "FL" indicates full length

[2]All segments are derived from the A/Viet Nam/1203/04 (H5N1) virus. HALo refers to the HA segment of A/Viet Nam/1203/04 with the polybasic cleavage site removed, as described in Fig. 2. Numbering following the PB2 segment refers to the identity of the amino acid residue 627 in the PB2 protein

[3]Mean time to death of eggs infected with VN1203 viruses. The results of two independent experiments are shown

[4]The number of EID_{50} units required to kill 50% of groups of 6- to 8-week-old C57BL/6 mice ($n = 4$)

[5]The maximum average weight loss of groups of mice ($n = 4$) upon vaccination with 10^6 EID_{50} of virus. nd = no weight loss detected. Values in brackets represent standard deviation from the mean

[6]The lowest dose of vaccination virus that subsequently conferred 100% protection from death following inoculation with 1,000 MLD_{50} of challenge virus

virus. Viral load and duration of shedding were significantly reduced relative to mock vaccinated control animals. The results obtained in this study, as well as those of Steel et al. [127], support the idea that NS1-truncated virus vaccines are broadly applicable against avian influenza.

In addition to high protective efficacy, NS1-modified LAIV offers the advantage of allowing differentiation between vaccinated and naturally infected chickens

Table 3 Serological, clinical, and virological outcomes of chicken vaccination and challenge

Group	Shedding of vaccine virus in trachea (EID_{50}/ml) (day 3, day 5)	HI/SN titer[1]		Protection from death	Virus shedding after challenge (Maximum $TCID_{50}$/mL)	
		Homologous	Heterologous		Tracheal	Cloacal
VN HALo/627E/NS 1-99/VN1203 challenge	<10–4.8×10^3, <10	20–640/<40–320	<20–320/ND–320	6/6	<50	<50
VN HALo/627E/NS 1-99/Egret06 challenge	<10–480, <10–48	20–640/<40–320	80–640/ND–160	5/6[2]	<50	<50
Mock/VN1203 challenge	<10, <10	<20/<40	<20/<40–40	0/4	2.32×10^4–1.08×10^5	5.00×10^5–5.00×10^6
Mock/Egret06 challenge	<10–20, <10	<20–20/<50	<20/<40	2/4	2–1.08×10^6	1.08×10^4–1.58×10^6

[1]Hemagglutination inhibition (HI) and serum neutralizing (SN) titers are given as the reciprocal of the highest dilution of serum that showed activity. Homologous refers to activity against A/Viet Nam/1203/04 virus, and heterologous refers to activity against A/egret/Egypt/01/06 virus. ND = not determined

[2]One bird was alert only when personnel were nearby by 7 days postchallenge (dpc). Disease progressed thereafter, culminating in the development of neurological signs by 11 dpc when the animal was euthanized

using serological analyses. DIVA (differentiate infected from vaccinated animals) compatibility is a highly desirable feature of vaccines to be used in agricultural species because many countries restrict the import of livestock testing positive for HPAI. Although this concept remains to be rigorously tested, the humoral response to NS1-modified LAIV should differ from that induced by wild-type virus in that the former would not include antibodies to the C-terminus of the NS1 protein.

7.5 Studies in Macaques

To gain a better understanding of the cellular mechanisms underpinning protection conferred by live attenuated virus vaccination, Baskin et al. [129] compared the protective immune response provoked by inactivated influenza vaccine and an NS1-truncated LAIV in a macaque model. The authors analyzed both the serological and functional genomic immune responses following (1) vaccination and (2) challenge with the human influenza virus strain, A/Texas/36/91 (H1N1). Three treatment groups were used in the study: (1) intranasally vaccinated with a single dose of live vaccine virus, (2) intraperitoneally vaccinated with a single dose of inactivated vaccine, or (3) unvaccinated [129].

Initial analyses demonstrated that no clinical signs of disease were observed following vaccination with 6×10^7 pfu of the live virus vaccine TX/91/NS1-126 (H1N1), and no significant lung pathology was observed (on day 4) postvaccination, suggesting that the NS1-truncated virus is safe and suitable as a live attenuated vaccine. Interestingly, global analysis of gene transcription 2 days after vaccination, using a macaque oligonucleotide array, demonstrated a stronger induction of interferon-related genes in the lungs of the LAIV group, relative to the killed-vaccine and unvaccinated groups. This result indicated that a qualitatively different immune response was induced with the LAIV, most likely due to the growth of LAIV and interferon stimulation by live virus in the lung [129].

At 21 days postvaccination, all animals were challenged with 6×10^7 pfu of the human influenza virus A/Texas/36/91 (H1N1), and tissues were collected 4 days postchallenge. The frequency of lung lesions, the levels of viral mRNA present in those lesions, and the severity of inflammation in the upper respiratory tract were all lower in animals that received the LAIV than in control, unvaccinated, animals and in animals that received the inactivated virus vaccine. Analysis of postchallenge blood samples revealed that animals vaccinated with the LAIV had a sevenfold increase in the number of influenza-specific CD4+ T-cells, higher IgG levels, and higher HI titers in serum relative to the other groups [129].

Examination of global expression profiles revealed that the LAIV group had a less pronounced activation of innate immune genes at 4 days postchallenge than did the killed vaccine and control groups, most likely due to lower levels of replication of the challenge virus in the lungs at that time. Conversely, activation of cellular gene pathways associated with the induction of B and T cells was higher in the lungs of animals that received the LAIV. Thus, there appears to be a relationship between LAIV, a reduced innate immune response on challenge, and the presence

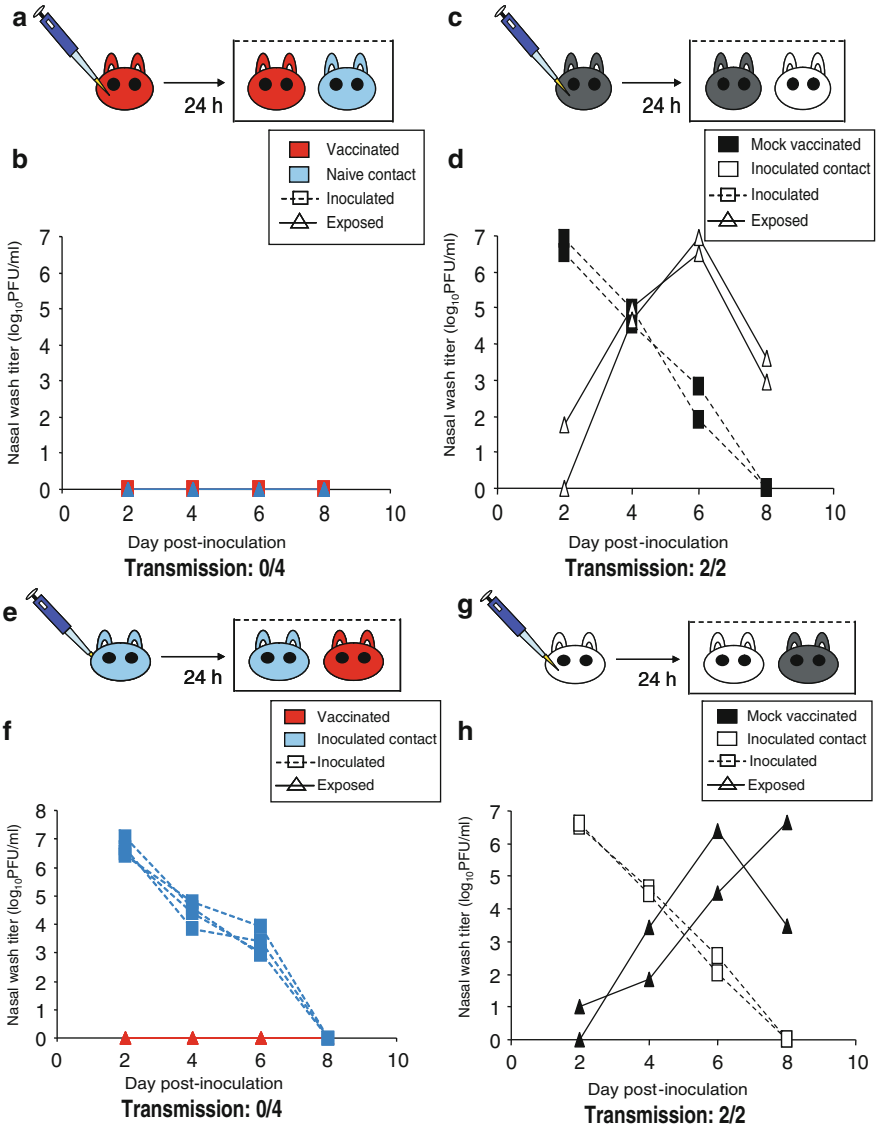

Fig. 3 Vaccination with an NS1-truncated LAIV provides 100% protection against transmission of the homologous strain and sterilizing immunity against homologous challenge. (**a**) Schematic representation of challenge by the intranasal route. Four guinea pigs previously vaccinated with LAIV (*red*) were challenged intranasally with 1,000 pfu of Pan/99 virus. At 24 h postinoculation, a naive contact animal (*blue*) was cocaged with each of the inoculated guinea pigs. (**b**) Results of homologous challenge by the intranasal route. No virus was detected in the nasal washings of challenged guinea pigs (*red squares with dashed lines*) or of the naive contact animals (*blue triangles with solid lines*). (**c**) Schematic representation of challenge of mock-vaccinated guinea pigs by the intranasal route. Two previously mock-vaccinated control animals (*black*) were inoculated intranasally with Pan/99 virus. At 24 h postinoculation, a naive contact guinea pig (*white*) was cocaged with each of the inoculated guinea pigs. (**d**) Results of Pan/99 challenge of

of a stronger, more mature adaptive immune response [129]. Whether this response is specifically stimulated by the high interferon-inducing NS1-truncated vaccine or is a property of LAIV in general is of great interest.

8 Blocking Influenza Transmission by Vaccination with NS1-Modified LAIV

The Centers for Disease Control and public health organizations in many countries recommend that household contacts of at-risk persons receive annual influenza vaccinations. This advice is based on the concept of vaccinating to block transmission and highlights the utility of the indirect effectiveness of immunization [1]. Clinical trials have confirmed the benefit of vaccination with the aim of preventing transmission [130, 131]. Nonetheless, efficacy in preventing disease is the primary measure in the evaluation of new influenza vaccines; the potential of a vaccine to disrupt the chain of transmission is seldom considered.

Using the guinea pig model, Lowen et al. evaluated immunization with an NS1-truncated LAIV in terms of its potential to reduce interhost transmission of influenza viruses [132]. Immunity from the NS1-truncated LAIV was compared with that obtained by natural infection and by vaccination with an inactivated influenza virus preparation. Both vaccines were applied in two doses, spaced 3 weeks apart. In each case, immunized animals acted as either donors or recipients in transmission; in this way, the efficacy of vaccination in blocking transmission from and to treated guinea pigs was tested. All three modes of immunization were found to reduce transmission to and/or from vaccinated animals. Natural infection was the most effective, providing sterilizing immunity against homologous challenge and heterologous challenge with a drift variant virus. The NS1-truncated LAIV also provided sterilizing immunity against homologous challenge and very good protection from transmission of the heterologous strain (Fig. 3). Although vaccination

Fig. 3 (continued) mock-vaccinated guinea pigs by the intranasal route. Mock-vaccinated guinea pigs were productively infected through inoculation (*black squares with dashed lines*) and transmitted efficiently to naive contact animals (*white triangles with solid lines*). (**e**) Schematic rep'resentation of challenge through exposure to an infected guinea pig. Four naive guinea pigs were inoculated intranasally with Pan/99 virus. At 24 h postinoculation, each acutely infected animal (*blue*) was placed into the same cage with one previously vaccinated guinea pig (*red*). (**f**) Results of homologous challenge through exposure to an infected guinea pig. Intranasally infected contact animals shed high titers of virus into nasal washes (*blue squares with dashed lines*); no virus was detected in nasal washes of the four vaccinated animals (*red triangles with solid lines*). (**g**) Schematic representation of challenge of mock-vaccinated animals through exposure to an infected guinea pig. Two naive contact animals were inoculated intranasally with Pan/99 virus. At 24 h postinoculation, two previously mock-vaccinated guinea pigs were each placed into the same cage with one infected animal. (**h**) Results of control challenge through contact with an infected guinea pig. Intranasally infected contact animals shed high titers of virus into nasal washes (*white squares with dashed lines*), and both mock-vaccinated guinea pigs (*black triangles with solid lines*) became infected through contact with the infected animals. Adapted from [132]

with the inactivated virus was found to induce high titers of HI antibodies (comparable to those obtained with natural infection and LAIV) and to reduce viral load in vaccinated guinea pigs, protection against transmission was moderate. Upon homologous challenge, transmission from guinea pigs that had received the inactivated vaccine was reduced only by 50%, and transmission to guinea pigs vaccinated with the killed virus was not reduced. Thus, similar to the situation following natural infection, intranasal vaccination with an NS1-truncated LAIV was found to be highly effective in blocking secondary transmission from and to animals that had received the vaccine. These findings support the use of live vaccines for influenza, and – if the results extend to humans – point to a simple and effective way to protect individuals who are less responsive to direct vaccination from contracting influenza virus infection.

9 Conclusions

LAIV vaccines confer effective protection against influenza virus infection. Despite the effectiveness of CAIV-T, other attenuation techniques could provide enhancements to the immunization options that are commercially available at present. Over the last 10 years, a considerable number of in vitro and in vivo studies have been conducted with NS1-truncated influenza viruses. The results have consistently indicated that viruses with C-terminal truncations in the NS1 protein are attenuated in growth in vitro and in vivo (in a number of animal model systems), do not generate signs of disease in animals, and stimulate the production of IFN in systems competent to do so. Increasing evidence from these studies suggests that one dose of vaccine may be sufficient to generate a strong, protective immune response in a number of host species, and that this response is most likely broader in terms of cross-protection amongst strains than conventional inactivated vaccines. The efficacy of NS1-truncated LAIV has been demonstrated to extend not only to disease prevention but also to prevent transmission between animals. It is therefore likely that NS1-truncated LAIV vaccines are safe and protective in humans.

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An Attenuated HSV-1 Live Virus Vaccine Candidate that is Replication Competent but Defective in Epithelial Cell-to-Cell and Neuronal Spread

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Abstract Live attenuated vaccines represent the most successful approach for the prevention of alphaherpesvirus infections, including varicella zoster virus, pseudorabies virus, and equine herpes virus 1. It is reasonable to consider that live virus vaccines may also be effective for the prevention of other alphaherpesviruses, such as herpes simplex virus 1 (HSV-1) and 2 (HSV-2). An HSV-1 mutant strain that is deleted in glycoprotein E (gE), NS-gEnull, is replication competent but is defective in spread from one epithelial cell to another, from epithelial cells to axons, and from the neuron cell body into axons. The defect in spread likely accounts for the favorable safety profile of the live virus vaccine candidate in mice. The NS-gEnull mutant is also defective in immunoglobulin G (IgG) Fc receptor binding, which is a process used by the virus to escape antibody attack. NS-gEnull when used as an immunogen is highly effective in providing protection against epidermal and vaginal challenge by wild-type (WT) HSV-1 and HSV-2. NS-gEnull represents a novel HSV-1 vaccine approach that retains replication competency while impairing virus spread at the inoculation site and in neurons. Only gE is deleted from the vaccine strain, ensuring that most viral antigens are presented to the host. This strategy is worth considering for prevention of HSV-1 and possibly HSV-2.

1 Introduction

Attenuated live virus vaccines are safe and effective for several members of the alphaherpesvirus family, including varicella zoster virus (VZV), pseudorabies virus (PRV), and equine herpes virus 1 (EHV-1) [1–4]. Nevertheless, no effective vaccine

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is available to protect humans against herpes simplex virus 1 (HSV-1) or 2 (HSV-2) infection. HSV-1 and HSV-2 cause significant morbidity and occasional mortality in humans with 58% of people in the United States ages 14–49 testing seropositive for HSV-1 and 17% for HSV-2 [5]. HSV-1 generally causes lesions on the vermillion border of the lip and HSV-2 typically causes genital ulcers.

An attenuated live virus vaccine that is replication defective is currently under development for prevention of HSV-2 infection (see chapter "Replication-defective Herpes Simplex Virus mutant strains as genital herpes vaccines and vaccine vectors" by D.M. Knipe) [6]. Another novel approach uses a replication competent, neuronal spread defective HSV-1 that is also defective in evasion of host antibody responses [7]. This attenuated HSV-1 strain has a large deletion within the unique short 8 (Us8) gene encoding glycoprotein E (gE) and is the focus of this chapter. The characterization of this virus revealed its potential utility as an attenuated vaccine [8].

2 Lifecycle of Alphaherpesviruses

A common feature shared by alphaherpesviruses is that they infect neurons of the peripheral nervous system (PNS). The neurons of the PNS are also the site where alphaherpesviruses establish life-long latent infection. In their natural host, these viruses periodically reactivate, replicate in the cell body of PNS neurons, and spread along axons to the mucosa or epithelial cells innervated by the neurons. Reactivation of VZV, HSV-1, and HSV-2 frequently causes itching and discomfort and produces virus-filled vesicles in the skin or at mucocutaneous borders.

3 Varicella Zoster Virus

VZV causes chickenpox and shingles. Chickenpox is no longer a common childhood illness in developed countries due to an attenuated vaccine, VZV Oka, derived from serial passage of a Japanese clinical isolate. VZV Oka contains a number of mutations throughout the genome and although lesions may develop at the inoculation site, the vaccine strain rarely causes serious sequelae [9]. VZV Oka is effective in preventing chickenpox in children, while vaccination of older individuals reduces the incidence of shingles. In those individuals who develop shingles despite immunization, the vaccine modifies the severity of postherpetic neuralgia, which is prolonged and often debilitating pain associated with shingles [10, 11]. The VZV vaccine thus represents an effective live attenuated virus vaccine against an alphaherpesvirus that is safe and effective in humans. This success supports efforts for a similar vaccine approach against two other human alphaherpesviruses, HSV-1 and HSV-2.

4 Pseudorabies Virus and Equine Herpes Virus 1

PRV is an alphaherpesvirus for which the natural host is the adult pig. Infection of adult pigs causes poor weight gain and abortion in pregnant sows. Infection of newborn pigs less than 1 month of age is virtually 100% lethal [12]. PRV infection of other secondary hosts such as cows, cats, dogs, rats, and mice is uniformly lethal, causing death within 2–3 days [12]. In many secondary hosts, clinical signs include a "mad itch" followed by neurological signs that precede death. Humans are not susceptible to PRV infection. Disease caused by PRV can be economically devastating to the pork industry; therefore, a vaccine was developed in 1961 by serial passage in tissue culture. The resulting attenuated vaccine, PRV-Bartha, has a large deletion within the Us coding region, encompassing several genes that are necessary for anterograde spread (defined as movement of virus from one cell to another that includes movement in axons away from the neuron cell body) [13]. These genes include Us7, Us8, and Us9 that encode the gI, gE, and Us9 proteins, respectively. PRV-Bartha is defective in anterograde spread, but retrograde spread (defined as movement of virus from one cell to another that includes movement in axons toward the neuron cell body) is intact. Therefore, this vaccine strain has been used experimentally as a retrograde neuronal tracing virus [14]. PRV-Bartha is highly effective in protecting swine from infection [15].

Attenuated vaccine approaches have also been successful in preventing infection of horses with the alphaherpesvirus EHV-1. One attenuated EHV vaccine candidate, Kentucky A (KyA), is notable for its similarity to the attenuated PRV vaccine [16]. KyA was produced by serial propagation in Syrian hamsters followed by passage in a murine cell line. KyA contains a large deletion in the Us region of the genome encompassing the genes encoding gE and gI. Another attenuated EHV-1 candidate vaccine strain is defective in gE alone [3]. These vaccine strains are highly attenuated in horses but only partially protect from respiratory symptoms after challenge [2, 3, 17, 18].

The attenuation of the PRV-Bartha and EHV-1 KyA strains was done by serial passage and did not specifically target particular genes; however, it is notable that both viruses contain deletions of genes required for efficient anterograde spread in neurons. Extensive similarities exist in genome organization and sequences of PRV, EHV-1, HSV-1, and HSV-2, and in requirements for neuronal spread as part of the virus lifecycle. Therefore, an approach of targeted attenuation of HSV-1 or HSV-2 by deletion of a gene or multiple genes required for neuronal spread represents a potentially effective means for vaccination of humans.

5 Characterization of HSV-1 gE

HSV-1 gE is a type-1 membrane glycoprotein that is incorporated into the virion envelope and is also expressed on the surface of infected cells. An HSV-1 gE deletion strain was constructed from a low passage clinical isolate (NS). The gE

deletion strain retains a portion of the Us8 sequence encoding the first 123 amino acids, but deletes amino acids 124–510, including the transmembrane domain that is replaced by a LacZ reporter gene [7]. The 3′ region of Us8 encoding amino acids 511–552 is not deleted but is not in frame, and therefore should not be expressed. This HSV-1 gE deletion virus, referred to as NS-gEnull, was evaluated as an attenuated HSV-1 vaccine candidate. A number of linker scanning mutants within the Us8 gene were also constructed within the HSV-1 NS background to further characterize specific gE functions, as discussed later in this chapter.

NS-gEnull does not produce a gE protein, while adjacent genes Us7 and Us9 are not affected, as assessed by Western blot [19]. NS-gEnull has intact single-step growth kinetics in Vero cells. When evaluated for cell-to-cell spread in human epidermal (HaCaT) cells, NS-gEnull forms plaques that are approximately fourfold smaller than WT virus at 48 h postinfection (hpi) [20, 21]. Repair of the Us8 deletion in NS-gEnull restores the WT plaque phenotype, indicating that gE is required for efficient cell-to-cell spread. As a vaccine candidate, the ability of NS-gEnull to replicate normally may provide an advantage in priming the host immune system compared with replication defective strains. The cell-to-cell spread defect of NS-gEnull is an important safety feature of the live virus vaccine.

6 Defining the Role of HSV-1 gE in Anterograde and Retrograde Spread

The mouse flank scarification model is useful for HSV pathogenesis studies. Five- to six-week-old female BALB/c mice were anesthetized and the hair removed from their right flanks by shaving followed by application of depilatory cream that is then rinsed off with water. The next day, mice were again anesthetized and a 10-μl droplet containing virus at the desired titer was applied to the surface of the denuded flank skin. Thirty to forty gentle scratches of approximately 0.5 cm in length were made in different directions through the droplet using a 26 5/8-gauge needle [7].

Following flank scarification, HSV-1 replicated at the inoculation site forming a lesion by 3 days postinfection (dpi). During that time, HSV-1 spreads cell to cell in the epithelial cell layer of the skin and enters local sensory nerves that innervate the skin. Virus enters the axon terminus and spreads to the cell body of the neuron located in the dorsal root ganglia (DRG). HSV-1 replicates in neuron cell bodies, infects adjacent neurons in the DRG, and spreads to the axon terminus and then to epidermal cells in the skin. Replication occurs in the skin resulting in lesions by 4–5 dpi that are confined to the dermatome innervated by the DRG (zosteriform lesions) [22]. Therefore, zosteriform disease requires both retrograde and anterograde spread.

Mice infected with WT HSV-1 typically die 8–10 dpi; however, infection with NS-gEnull results in no death or zosteriform disease, while inoculation site disease

is no different than after mock scarification [23]. Therefore, the lack of virulence of NS-gEnull as a potential vaccine candidate is notable. Virus titers performed at the inoculation site demonstrate $5\log_{10}$ less virus in mice infected with NS-gEnull than WT virus by 3 dpi. Additionally, less viral antigen is observed by immunohistochemistry of sectioned skin. These in vivo results can be explained by the cell-to-cell spread defect observed in cultured cells. NS-gEnull titers in DRG are negative at 1, 3, 6, and 8 dpi compared with peak WT virus titers of $4\log_{10}$ at 3 dpi. The negative NS-gEnull DRG titers can also be explained by a cell-to-cell spread defect in the epidermal cells or by a defect in spread from epidermal cells to innervating neurons [23]. The failure of NS-gEnull to infect DRG adds an important safety feature to the candidate vaccine, since DRG are the site of latency for the virus. Since no virus reaches the DRG, the flank model cannot be used to assess the spread of NS-gEnull from DRG to skin (anterograde).

We chose the mouse retina infection model to assess NS-gEnull anterograde spread. Five- to eight-week-old BALB/c mice were anesthetized and a small cut was made in the sclera with a 30-gauge needle [8]. This needle was then used to puncture the vitreous body of the eye. A Hamilton syringe was used to inject virus into the vitreous body through the same puncture hole. The ganglion cell neurons comprise the innermost layer of the retina and are the first neurons to become infected. The axons from these neurons form the optic nerve. Three to five dpi with WT virus (NS) or NS-gEnull, eyes, optic nerves, and brains were removed, sectioned, and stained for HSV-1 antigen. This staining revealed a robust infection of the retina with WT and NS-gEnull strains, although more antigen was detected in retina infected with WT virus, which is consistent with in vitro results demonstrating a cell-to-cell spread defect for NS-gEnull.

Optic nerve sections from mice injected with NS-gEnull showed no HSV-1 antigen when stained with an anti-HSV-1 polyclonal antibody, compared with abundant antigen seen after infection with WT virus or the rescue strain, rNS-gEnull [8]. These results indicate that HSV-1 gE is required for spread of virus from the retina ganglion cell neurons to the optic nerve. Antigen staining for HSV-1 envelope glycoproteins gB, gC, and gD revealed that none of these envelope proteins was present in the optic nerves of NS-gEnull-infected mice. Additionally, the tegument protein, VP22, and capsid protein, VP5, were not detected in the optic nerve after retina infection with NS-gEnull. Therefore, HSV-1 gE is required for spread of HSV-1 envelope, tegument, and capsid proteins from the cell bodies of retina ganglion cell neurons into their axon fibers in the optic nerve. This spread defect is another safety feature of NS-gEnull as a vaccine candidate.

Further support for the requirement of HSV-1 gE for spread in vivo comes from an analysis of brain sections from WT or NS-gEnull-infected mice [8]. After retina infection with WT virus, brains contained antigens in the optic tract, dorsal, and ventral lateral geniculate nuclei and superior colliculus, which represent regions of the brain reached by anterograde spread. Additionally, antigens were detected in brain nuclei reached by retrograde spread of virus from structures in the eye or orbit, including the intergeniculate leaflet of the lateral geniculate nucleus, Edinger–Westphal, and oculomotor nuclei. No viral antigens were detected in

any region of the brain following NS-gEnull infection, indicating defects in both anterograde and retrograde spread. The failure of NS-gEnull to reach nuclei in the brain by routes involving retrograde spread is consistent with the observation in the flank model that the virus failed to infect DRG.

An in vitro neuronal culture system was employed to further assess the neuronal spread properties of NS-gEnull [24]. In this system, 17-day embryos were removed from pregnant Sprague-Dawley rats and sympathetic motor neurons were established in Campenot chamber cultures by dissecting the superior cervical ganglia (SCG) [20]. Campenot cultures contain three separate chambers, the Soma (S), Middle (M), and Neurite (N) compartments (Fig. 1a). Dissociated SCG neurons are plated in the S chamber and allowed to differentiate for approximately 2 weeks. Axons (neurites) sprout from these neuronal cell bodies and grow through a silicone grease barrier that separates the S and M chambers. The M chamber is filled with 1% methylcellulose to prevent the diffusion of virus between chambers. By 2–3 weeks, neurites grow into the N chamber through a silicone grease barrier that separates the M and N chambers.

NS-gEnull was added to the S chamber neurons and epithelial cells were added on top of neurites in the N chamber to evaluate virus spread from neuron cell bodies to epithelial cells (Fig. 1c). NS-gEnull replicated to levels comparable to WT or rescue (rNS-gEnull) virus in the S chamber, as determined by titering the contents of the S chamber at 24 and 48 hpi. In contrast, no NS-gEnull was detected in the N chamber, compared with 4-5$\log_{10}$ WT or rescue virus at 48 hpi, indicating an impressive spread defect of NS-gEnull from the neurons in the S chamber to epithelial cells in the N chamber [20]. Immunofluorescent studies of SCG neurons

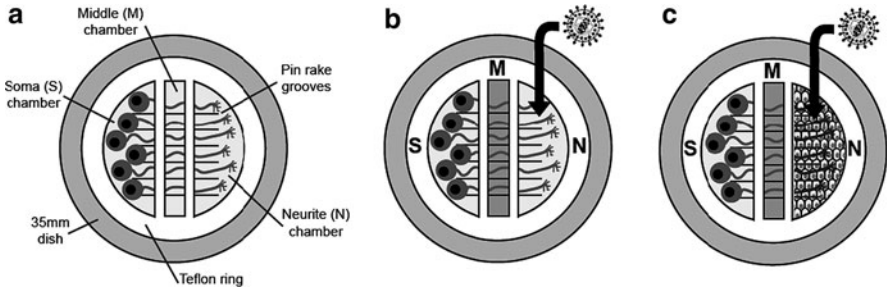

Fig. 1 (**a**). Campenot chamber consists of a Teflon ring that divides the culture dish into three compartments. SCG neurons are placed in the Soma (S) chamber. Over time, the neurons extend their axons (neurites) along pin rake groves that are made in the culture dish to guide the growth of the neurites, which penetrate into the Middle (M) chamber and then Neurite (N) chamber. Prior to infection of S chamber neurons, the M chamber is filled with methylcellulose to prevent virus leaking between chambers. (**b**). For some experiments, virus is added to the N chamber and neurons are harvested in the S chamber to determine virus transport from axons to neuron cell bodies. (**c**). For some experiments, epithelial cells are added to the N chamber prior to infection of neurons in the S chamber. Contents of the N chamber are harvested to determine spread of virus from epithelial cells in the N chamber to neurons in the S chamber. Figure is reprinted with permission of the editor of the *Journal of Virology*

infected with NS-gEnull demonstrated viral antigens in the cell body but not in the axons, supporting a role for gE in targeting viral antigens from the neuron cell body into axons [8]. This in vitro result is consistent with the in vivo observation in the mouse retina infection model that failed to detect NS-gEnull antigens in the optic nerve. The mechanism by which HSV-1 gE mediates axonal localization is unknown and currently under evaluation.

Further experiments were performed to evaluate the contribution of gE to retrograde spread. WT virus or NS-gEnull was added directly to axons in the N chamber and virus transport was assessed by titering the contents of the S chamber (Fig. 1b) [20]. No differences were detected comparing WT and NS-gEnull virus titers in the S chamber. Therefore, gE is dispensable for virus transport from the axon terminus to the neuron cell body. The Campenot chamber system was then modified to test the ability of WT virus or NS-gEnull to spread from epithelial cells to neurites by seeding HaCaT cells over the neurites in the N chamber. Virus was added to the HaCaT cells in the N chamber and assayed for spread to the S chamber. One-hundred-fold less NS-gEnull than WT virus was detected in the S chamber. Therefore, the defect in NS-gEnull spread stems from the contribution of gE to virus spread from epithelial cells to axons, which is consistent with a cell-to-cell spread defect noted in epithelial cells [25]. Table 1 summarizes replication and spread properties of the HSV-1 gE deletion mutant, NS-gEnull.

The mechanism by which gE contributes to spread from epithelial cells to axons may relate to the observation that gE is required to target virus to the basolateral surface of polarized epithelial cells [26]. HSV-1 gE mutant virus is transported

Table 1 Replication and spread phenotypes of NS-gEnull

Replication that requires a single growth cycle in Vero cells or SCG neurons	Similar to WT virus
Replication that requires virus spread from cell to cell as measured by plaque size or skin titers in the mouse flank	Greatly reduced relative to WT virus
Spread from one cell to another that requires virus transport away from the neuron cell body (anterograde) in Campenot chambers (from S to N chamber) or in the mouse retina infection model (from retina to specific nuclei in the brain such as the superior colliculus nucleus)	Greatly reduced relative to WT virus
Spread from one cell to another that requires virus transport in axons toward the neuron cell body (retrograde) in Campenot cultures (from epithelial cells in the N chamber to SCG neurons in the S chamber), mouse flank (from skin to DRG), or mouse retina model (from eye to specific nuclei in the brain such as the Edinger–Westphal or oculomotor nuclei)	Greatly reduced relative to WT virus
Targeting of viral antigens from the neuron cell body into axons in SCG cultures (infect neurons and observe for viral antigens in axons) or mouse retina model (infect retina and observe for viral antigens in the optic nerve)	Greatly reduced relative to WT virus
Targeting of viral antigens from the axon terminus to the neuron cell body in SCG cultures (infect N chamber axons and titer virus in S chamber neurons)	Similar to WT virus

primarily to the apical surface of polarized epithelial cells, which may explain the cell-to-cell spread defect of gE defective virus. An elucidation of the cellular binding partners of gE in epithelial and neuronal cells may help clarify mechanisms of gE-mediated virus spread.

7 Defining the Role of HSV-1 gE in IgG Fc Binding Activity

Another important function of HSV-1 gE is its role as an IgG Fc receptor (FcγR) in the immune evasion of host IgG antibody responses [27]. Earlier work identified gE and gI as heterodimers that bind the IgG Fc domain [28, 29]. HSV-1 gE forms a lower affinity receptor for Fc, whereas the gE–gI complex constitutes a higher affinity receptor [30]. The FcγR activity of gE was evaluated using a rosetting assay in which sheep erythrocytes were coated with anti-sheep erythrocyte IgG and then incubated with HSV-1-infected cells. WT virus-infected cells express gE at the cell surface and formed rosettes (defined as $\geq$4 erythrocytes per infected cell), whereas uninfected cells or those infected with NS-gEnull failed to form rosettes, indicating that WT virus expresses a receptor for IgG Fc [27].

Antibody bipolar bridging is a mechanism that explains the immune evasion activity mediated by the HSV-1 FcγR [27]. The term "antibody bipolar bridging" stems from studies indicating the HSV-1 FcγR preferentially binds the Fc domain of anti-HSV IgG compared with nonimmune IgG. This observation led to the hypothesis that as the Fab domain of an anti-HSV IgG antibody binds to an antigen on the infected cell surface, the Fc portion of the same antibody molecule binds to the HSV-1 FcγR to form an "antibody bridge". By binding the IgG Fc domain, the FcγR blocks downstream effector functions mediated by this domain, including complement-enhanced antibody neutralization, antibody-dependent cellular cytotoxicity, and attachment of granulocytes [31, 32]. The hypothesis of antibody bipolar bridging is supported by the crystal structure of the HSV-1 FcγR bound to IgG Fc [33].

Interestingly, the HSV-1 FcγR binds the Fc domain of human IgG, but not murine or guinea pig IgG [34]. The failure of the HSV-1 FcγR to bind murine IgG makes murine models useful for studying its activity. Mice were passively immunized with human anti-HSV IgG and then infected with either WT or NS-gE339, which is an HSV-1 gE mutant strain that has an insertion of four amino acids after gE residue 339 [7]. NS-gE339 is defective in FcγR activity but is much less impaired in cell-to-cell spread than NS-gEnull, making NS-gE339 a useful mutant strain to evaluate FcγR function independent of spread activity [25]. Mice infected with WT virus developed severe inoculation site disease when passively immunized with human anti-HSV IgG; however, the disease was greatly reduced in mice infected with NS-gE339. Nonimmune human IgG had no effect on disease caused by WT virus or NS-gE339, while murine anti-HSV IgG modified both viruses to a comparable degree. These results indicate that the HSV-1 FcγR mediates evasion from human anti-HSV antibodies, and suggest that another safety feature of NS-gEnull as a vaccine candidate is its inability to evade host IgG antibody responses.

The gE domains that mediate FcγR activity, cell-to-cell spread and neuronal spread have been partially characterized through linker scanning mutagenesis of the Us8 gene [8, 25, 35, 36]. Some mutant strains are defective in spread and others in FcγR activity, suggesting that these functions are mediated by different domains on the protein. Of note is the fact that all regions responsible for these activities are absent in NS-gEnull.

8 NS-gEnull as an Attenuated Vaccine Candidate

NS-gEnull has intact single-step replication kinetics but impaired spread from one epithelial cell to another, from epithelial cells to axons, and from neuron cell bodies to axons. NS-gEnull is greatly attenuated in the mouse flank infection model causing no inoculation site disease and no zosteriform site disease or death. These features make NS-gEnull a novel candidate for a safe live-virus vaccine.

To address immunogenicity and efficacy of the vaccine candidate, the mouse flank model was again employed. Mice were immunized by flank scarification with NS-gEnull, or mock immunized, and 28 days later challenged by flank infection on the opposite flank with 10^5 PFU of WT virus, HSV-1 NS [23]. One hundred percent of mock-immunized mice died, while 100% of NS-gEnull-immunized mice survived. The NS-gEnull-immunized mice had only mild inoculation site disease and no zosteriform lesions, in contrast to the severe inoculation and zosteriform disease that developed in mock-immunized mice. Titers of skin at the inoculation site and DRG following challenge were striking in that no WT virus was recovered from NS-gEnull-immunized mice, while 4–5$\log_{10}$ PFU were detected in the mock group. By real-time quantitative PCR, low levels of viral DNA were detected in DRG of vaccinated mice 1, 3, 6, and 8 dpi, while DNA copy number was 3–4$\log_{10}$ higher in mock-immunized mice; however, no determinations were performed to distinguish vaccine from WT virus DNA in the DRG. The results indicate that immunization with NS-gEnull protects mice from moderate or severe inoculation site disease, entirely prevents zosteriform disease and death, and results in greatly reduced titers of challenge virus reaching the DRG.

DRG explant cocultures were performed to assess the efficacy of NS-gEnull vaccination in preventing the establishment of latency by WT virus challenge [23]. Mice were either mock vaccinated or vaccinated with NS-gEnull by flank scarification and challenged by infection of the opposite flank with HSV-1 KOS. The advantage of KOS over some other HSV-1 strains is that KOS causes severe zosteriform disease but rarely leads to death when inoculated by the flank route. Zosteriform disease indicates that the virus has reached the DRG (site of latency) and returned to the skin. Survival of the animal enables DRG to be harvested after infection once latency is established. One-hundred percent of mock-vaccinated mice challenged with KOS developed severe inoculation site and zosteriform site disease. In contrast, NS-gEnull-vaccinated mice had only mild inoculation site disease and no zosteriform lesions. Twenty-eight days after KOS challenge, all

mock-immunized mice had recovered and several weeks had passed since the last signs of disease. DRG were removed and explant cocultures were performed with Vero cells. The explant cocultures were observed for 20 days for cytopathic effects as an indication of virus reactivation from latency. Importantly, 100% of DRG from mock-vaccinated mice reactivated KOS virus compared with 10% of DRG from NS-gEnull-vaccinated mice. Therefore, NS-gEnull vaccination was highly effective in protecting the DRG.

To evaluate whether NS-gEnull-vaccinated mice were protected against different HSV-1 isolates, mice were challenged by flank scarification with HSV-1 strains F and 17 [23]. NS-gEnull-vaccinated mice were protected against death and moderate or severe inoculation site disease. One mouse challenged with F strain developed mild zosteriform disease, which indicates that the virus reached the DRG. These challenge experiments support the KOS explant coculture results in that DRG were greatly, but not totally protected against challenge virus.

Prior HSV-1 infection appears to provide some degree of cross-protection against HSV-2 [37]. Therefore, an attenuated vaccine for HSV-1 may provide protection against HSV-2; however, cross-protection may also be problematic if prior HSV-2 infection reduces the immunogenicity of an HSV-1 vaccine. Note that a clinical trial of an HSV-2 gD subunit vaccine showed protection of HSV-1 seronegative women but not HSV-1 seropositive women [37]. The explanation for this result is either that the vaccine failed to improve the cross-protection already provided by HSV-1 infection or that prior HSV-1 infection reduced the ability of the HSV-2 gD vaccine to elicit a robust immune response.

NS-gEnull-or mock-vaccinated mice were challenged by flank scarification with HSV-2 strain 2.12, a low-passage clinical isolate [23, 38]. One-hundred percent of mock-vaccinated mice developed severe inoculation site and zosteriform site disease, and 80% died. In contrast, none of the mice vaccinated with NS-gEnull died. These mice developed mild inoculation site disease and no zosteriform lesions. DRG explant cocultures were performed on survivors 1 year after challenge and revealed no reactivation of the challenge virus. Therefore, NS-gEnull provides robust protection against HSV-2 infection and the establishment of latency.

The finding that NS-gEnull cross-protects against HSV-2 has important implications. An attenuated HSV-1 vaccine would likely be given at a young age, since HSV-1 infection is typically acquired early in life. An HSV-2 vaccine would likely be given in early adolescence. Therefore, robust cross-protection by an attenuated HSV-1 vaccine against HSV-2 infection may protect young children against HSV-1 while also providing early protection against HSV-2.

VZV immunity wanes after vaccination, which led to a recommendation to revaccinate children during adolescence. Immunity to natural infection with VZV also wanes over time, which is the rationale behind vaccinating adults over the age of 60 years to prevent shingles [10]. The duration of protection after immunization with NS-gEnull was evaluated by flank challenge 1 year after vaccination. Mice were protected against death, zosteriform disease, and severe inoculation site disease [23].

NS-gEnull protected against WT virus challenge when immunization was given by skin scarification, intramuscular (IM), or subcutaneous (SubQ) routes [23].

Table 2 Neutralizing antibody responses to NS-gEnull immunization and efficacy against challenge by WT virus

Neutralizing antibody responses	Highest when NS-gEnull given IM compared with SubQ or flank scarification
Protection against death after flank challenge by WT (parental) strain	No death at 10^5 PFU challenge
Protection against inoculation site disease by WT (parental) strain	Almost total protection
Protection against zosteriform disease by WT (parental) strain	No disease detected
Protection of DRG against WT (parental) strain at 1, 3, 6, and 8 dpi	No WT virus isolated
Protection against death after flank challenge by WT isolates other than parental virus	No death at 10^5 PFU challenge
Protection against inoculation site disease by WT isolates other than parental virus	Mild disease detected
Protection against zosteriform disease by WT isolates other than parental virus	Mild disease detected
Protection of DRG against WT isolates other than parental virus assessed by attempts to reactivate latent virus (explant coculture)	Substantial, but not total protection
Protection against HSV-1 vaginal challenge	No death, no disease, and very low vaginal titers compared with WT virus
Protection against HSV-2 flank challenge	No death, mild inoculation site disease, no zosteriform disease, and no reactivation of latent virus from DRG

Neutralizing antibody titers were highest after IM and lowest after SubQ immunization. Both scarification and IM vaccination resulted in complete protection against death, severe inoculation site disease, and zosteriform disease when challenged by the WT strain that was used to derive the vaccine virus. IM is a more acceptable immunization route than skin scarification, since IM inoculation is used for many vaccines.

Genital infection caused by HSV-1 rather than HSV-2 comprises 30–40% of first time genital herpes virus infections [39]. Therefore, NS-gEnull immunization was evaluated for protection against WT HSV-1 vaginal challenge [23]. All mock-vaccinated mice developed vaginal disease and had high virus titers in vaginal swabs until 8 dpi. Mortality was 60%. In contrast, NS-gEnull-vaccinated mice all survived and had no observable vaginal disease. Vaginal swab titers were 2–4$\log_{10}$ lower than in mock-vaccinated mice and were undetectable by 3 dpi. Table 2 summarizes neutralizing antibody responses and the efficacy of protection provided by NS-gEnull immunization.

9 Conclusions

Studies using NS-gEnull, an HSV-1 gE deletion mutant, demonstrate that it is replication competent, but defective in epithelial cell-to-cell spread, epithelial cell to axon spread, and spread from neuron cell bodies into their axons. The vaccine

candidate is also deficient in IgG Fc-mediated immune evasion. These characteristics represent important safety features for this attenuated live virus vaccine candidate. In mouse models, the vaccine provides protection against challenge by skin and mucosal routes and offers cross-protection against HSV-2 challenge. In addition, the protection provided by NS-gEnull immunization is long-lasting. A similar approach may be effective for HSV-2 since the HSV-2 gE protein shares 72% amino acid homology with HSV-1 gE.

Additional safety features may be considered for this live virus vaccine approach. Along with gE, Us9 and gI are involved in anterograde spread in PRV and HSV-1 [19, 40–42]. PRV-Bartha has a deletion encompassing the genes encoding gI, gE, and Us9. A similar deletion in HSV-1 or HSV-2 may provide added safety by further impairing the spread features of the vaccine strain; however, excessive attenuation may hamper growth kinetics and impair antigen presentation to the immune system.

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Live Attenuated Vaccines for Respiratory Syncytial Virus

Michael N. Teng

Abstract In the five decades since the identification of respiratory syncytial virus (RSV) as an important pediatric pathogen, no effective vaccine has been developed. Previous attempts to develop inactivated RSV vaccines resulted in vaccine-enhanced disease, resulting in a greater focus on the generation of live attenuated RSV vaccines. However, identifying a live attenuated vaccine candidate that is appropriately attenuated and sufficiently immunogenic has proven to be difficult. Recently, reverse genetics systems have been developed for RSV, allowing researchers to introduce specific mutations into the genomes of recombinant vaccine candidates. These systems provide a means of determining the effects of known attenuating mutations and identifying novel methods of attenuating the virus without decreasing immunogenicity. In addition, different mutations can be combined in a single genome to fine-tune the level of attenuation and immunogenicity to achieve the proper balance in a viable vaccine candidate. Current research into RSV attenuation includes investigation of point mutations responsible for temperature sensitivity, nontemperature-sensitive attenuating mutations, and deletion of nonessential viral genes that play roles in viral RNA synthesis and/or inhibition of innate immune responses. Development of an effective RSV vaccine will likely rely on using reverse genetics systems to optimize the attenuation and immunogenicity of a live vaccine candidate, while preserving viral replication in vitro.

Keywords Live attenuated vaccines · Paramyxoviruses · Pediatric vaccines · Recombinant vaccines · Respiratory infections · Vaccine development

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1 Respiratory Syncytial Virus

Respiratory syncytial virus (RSV) is the most important etiologic agent of pediatric viral respiratory infection and remains a major cause of morbidity and mortality among infants. Infection rates for RSV in infants have been found to be 68.8 per 100 children for the first year of life, reaching 82.6 per 100 children for the second year [1]. Lower respiratory tract illness (LRTI) is more common during year 1, though LRTI occurs frequently during year 2. Approximately half of all children are reinfected by age 2, but most children experience only 1 LRTI [1]. RSV infection accounts for between 70,000 and 120,000 hospitalizations in the United States of infants under 6 months of age and ~70% of hospitalizations due to bronchiolitis [2–5]. Severe RSV infection has been associated with long-term effects such as asthma and wheezing and can cause significant mortality in high-risk groups, such as premature infants or children with immunodeficiency, chronic pulmonary disease, or cardiovascular disease [6–9]. In addition, RSV infection is a serious complication in immunocompromised subjects, particularly bone marrow transplant patients, and the elderly [10].

Previously, RSV bronchiolitis was thought to be caused by an overactive antiviral immune response, similar to allergic asthma [11–13]. However, recent evidence indicates that severe RSV disease is likely due to virus-induced cell death and sloughing of apoptotic cells into the lumen of the bronchioles [14]. Examination of autopsy specimens from fatal cases of RSV bronchiolitis showed the presence of overwhelming RSV antigen and massive apoptotic sloughing of epithelial cells, but a relative dearth of infiltrating T cells. In addition, infants who suffered nonfatal cases of RSV showed decreased expression levels of cytokines, particularly IFN-γ, IL-17, IL-4, and IL-6, compared to infants infected by influenza [14, 15]. Cytokine expression levels in RSV-infected infants did not appear to correlate with the severity of RSV infection. However, viral replication levels directly correlated with the severity of RSV disease [14, 16]. Thus, severe RSV LRTI is likely due to high levels of RSV replication in ciliated and nonciliated airway cells, resulting in cell death and a large influx of neutrophils and macrophages. This hypothesis also fits with the time course of RSV infection and the observation that corticosteroids are ineffective in treating RSV bronchiolitis [17]. These results suggest that reducing viral replication levels by the induction of protective immune responses via vaccination is likely to reduce the morbidity and mortality due to RSV infection.

Infection by RSV causes severe disease in the very young (infants under 6 months of age) and the elderly [18]. One distinctive characteristic of RSV infection is that it does not induce long-lived immunity upon exposure, resulting in recurrent infection throughout life. Reinfections occur frequently throughout life, though the symptoms of subsequent infection are generally milder [18]. Thus, the target populations for RSV vaccines would be individuals at the extremes of age. In both populations, lung function is suboptimal due to relatively inelastic lungs, either due to developmental immaturity or loss of elasticity. Premature infants are particularly susceptible to severe RSV disease due to interrupted lung development, leading to

decreased lung function with reduced airway diameter and increased smooth muscle. In addition, both populations present challenges to vaccination because of deficiencies in their immune responses. For infants, there are two major hurdles to effective immunization: (1) developmental immaturity of the immune system and (2) presence of maternal antibodies. Neonatal immune responses are both quantitatively and qualitatively different from those in adults, and these differences persist throughout the first year of life. The neonatal immune system appears to be biased toward Th2-like responses, although Th1 responses can be induced in neonates with certain stimuli including certain microbes [19–21]. This effect is likely due in part to immaturity of dendritic and other accessory cell populations. Serum antibodies derived from the mother pose a challenge for vaccine take, as seen in the experience with the measles virus vaccine. In contrast, premature infants born before 28 weeks of gestation, when maternal antibody transfer occurs, have increased susceptibility to RSV. Premature infants born closer to full term are likely better protected, as maternal antibody levels are proportional to gestational age.

At the other end of the age spectrum, immunosenescence is a hurdle for RSV vaccination in the elderly population. Not only are adaptive immune responses blunted in the elderly, but innate immune function appears to be decreased as well [22–24]. Protection from RSV by vaccination will likely require the induction of both B- and T-cell responses in the elderly, similar to influenza vaccination [19, 25, 26]. Thus, a more complete understanding of the mechanisms responsible for immunosenescence is required to improve the efficacy of RSV vaccines in the elderly.

Immunologic protection from RSV infection requires induction of high-affinity neutralizing antibody responses. Both infants and the elderly show decreased B-cell responses compared with healthy adults [27–29]. Moreover, these two populations display a limited ability to generate diversity in their antibody responses to antigenic stimulation [27, 30]. The exact mechanisms for these defects are not well understood. However, increasing the diversity and affinity of the immunoglobulin response in vaccinees is essential for efficient protection.

2 Agent

RSV is an enveloped virus classified in the family Paramyxoviridae in the order Mononegavirales, and is the prototype member of the *Pneumovirus* genus. The nonsegmented, negative-sense RNA genome of RSV is 15,222 nucleotides long and contains 10 genes from which 11 proteins are translated (Fig. 1). The genome is encapsidated by the viral nucleocapsid (N) protein, and this ribonucleocapsid complex serves as the template for viral transcription and RNA replication. RSV enters cells by direct fusion of its envelope with the plasma membrane and replicates solely in the cytoplasm. RSV packages its own viral RNA-dependent RNA polymerase (RdRP), which is essential for the initial transcription of its

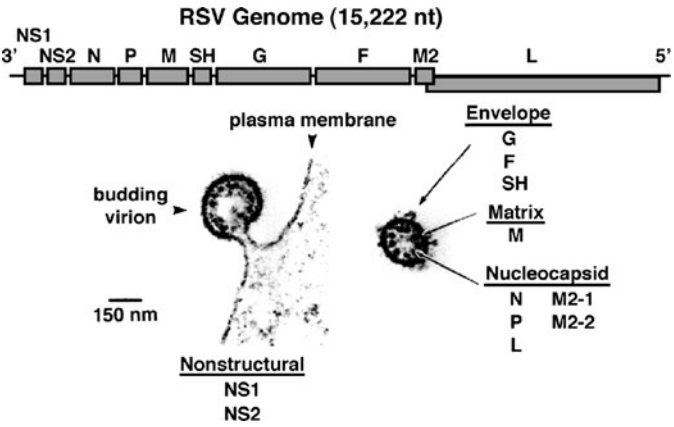

Fig. 1 RSV genome and virion structure. The M2 gene overlaps with the L gene. Photograph by Anthony Kalica (courtesy of Peter Collins, NIAID)

genome upon infection. The RdRP for RSV transcription is minimally composed of P, M2-1, and L. L encodes the large enzymatic subunit of the polymerase, and P is an essential cofactor for RNA synthesis. M2-1 is specific for the viral transcriptase and is an antitermination/processivity factor. The polymerase complex accesses the genome at a single promoter at the 3′ end of the genome and initiates transcription at the first gene (NS1). Each gene is bounded by conserved transcription initiation and termination signals and is separated from the adjacent genes by a variable length of intergenic sequence. The linear array of viral genes is transcribed sequentially in a start/stop fashion, resulting in a polar gradient of mRNA production, whereby genes proximal to the 3′ promoter are transcribed more efficiently than those that are promoter-distal. At a low frequency, the RdRP will fail to terminate, resulting in an oligocistronic or "readthrough" mRNA that is terminated at a subsequent transcription termination signal, or will fail to reinitiate, resulting in transcription attenuation and a gradient of expression inversely proportional to the distance from the 3′ end of the genome. After primary transcription has occurred, the polymerase complex begins replicating the viral genome, synthesizing a full-length copy of the vRNA called the antigenome (cRNA). The regulation of the switch from transcription to replication by RdRP is not clear; however, the M2-2 protein is thought to be involved in this process. The antigenome is also encapsidated by N protein and serves as a template for synthesis of more vRNA. In infected cells, there is more vRNA than cRNA [10]. Encapsidated vRNA interacts with the matrix (M) protein and traffics to the plasma membrane where the viral N interacts with the cytoplasmic tails of the attachment (G) and fusion (F) proteins. Virion morphogenesis occurs at lipid raft domains in the membrane where F is localized. In addition to G and F, the RSV viral envelope contains a small hydrophobic (SH) protein of unknown function. Importantly, G and F are the major neutralizing antigens for RSV. The two remaining RSV proteins, NS1 and NS2, are nonstructural proteins that have been

shown to inhibit IFN-β induction and signaling but are otherwise dispensable for viral replication in vitro [31, 32].

3 Treatment

Currently, there are no effective antiviral drugs to treat RSV infection. Ribavirin has been previously used to treat severe RSV disease, but the efficacy of this treatment is questionable and the cost is high [33–35]. Supportive care with supplemental oxygen is the most common treatment option, although treatment with corticosteroids and/or β-agonists has been tried with limited success [35]. Nebulized hypertonic saline with or without epinephrine has been found to decrease length of stay in infants hospitalized with viral bronchiolitis [36, 37]. Immunoprophylaxis has been the mainstay for the prevention of RSV infection in high-risk infants. Synagis (palivizumab), a recombinant humanized monoclonal antibody to the RSV F protein, has been shown to be effective in preventing infection in premature infants and children with underlying risk factors for severe RSV disease [38–40]. The recent development of a higher affinity monoclonal antibody to F has improved the efficacy profile of RSV immunoprophylaxis [41, 42]. However, Synagis treatment is not cost-effective in normal populations due to the need to administer the drug monthly during RSV season and the lower incidence of hospitalization for severe RSV bronchiolitis.

4 RSV Vaccines

Although RSV is the most important cause of viral lower respiratory tract disease in infants, initial attempts to develop an RSV vaccine by using inactivated virus met with failure. In the early 1960s, vaccination of infants with a formalin-inactivated (FI)-RSV vaccine not only failed to protect against RSV disease during the following RSV season but some vaccinees developed enhanced disease upon infection with RSV, resulting in increased rates of severe pneumonia and two deaths [43–45]. Studies on autopsy samples as well as in the mouse model suggested that the enhanced disease due to FI-RSV vaccination was due to an imbalanced T helper cell response, predisposing the vaccinees to a response resembling allergic asthma upon subsequent infection by RSV (reviewed in [46]). More recently, it has been determined that the FI-RSV vaccine has reduced the capacity for inducing high avidity antibodies, due to reduced TLR stimulation, likely resulting in the deposition of complement in the lungs [47, 48].

In the intervening years, a number of different approaches have been evaluated including subunit vaccines, vectored vaccines, and live attenuated vaccines; however, as of the writing of this chapter there remains no licensed RSV vaccine. Currently, the most promising vaccine candidates for RSV are live attenuated viruses. These

viruses have several benefits: (1) enhanced RSV disease has not been observed either after natural infection or vaccination with live attenuated viruses [49–53]; (2) administration of live attenuated RSV vaccines induces balanced immune responses that more closely match natural immunity compared with parenterally administered subunit (or inactivated) vaccines [54, 55]. Also, vaccination with live attenuated viruses intranasally would likely induce better local immunity compared with intramuscular injection of subunit or killed vaccines [56]. Live attenuated RSV vaccines have been in development for several decades, using a combination of cold passage (*cp*) and chemical mutagenesis to induce temperature sensitivity (*ts*) (reviewed in [57, 58]). The initial RSV vaccine candidates were either under- or over-attenuated, with reversion of one of the *ts* mutants in vaccinated children [50, 59–61]. However, children vaccinated with these live attenuated viruses did not show enhanced disease upon subsequent infection with RSV [62]. Therefore, further development of live attenuated vaccine candidates was performed, combining cold passage and chemical mutagenesis to generate temperature-sensitive RSV. A spectrum of *cpts*RSV vaccine candidates were produced by this method, with a range of temperature sensitivity in culture and attenuation in animal models (Fig. 2a) [53, 63–66]. Candidate vaccines from this method were immunogenic and protected against RSV challenge in both rodent and nonhuman primate models. Two candidate vaccines (*cpts*248/955 and *cpts*530/1009) were chosen for testing in the clinic [53]. These candidates induced protective immune responses in seronegative children; however, both candidates were underattenuated in this age group, precluding further analysis in infants (Table 1). One additional candidate, *cpts*248/404, was found to be sufficiently attenuated and immunogenic in seronegative children and was tested in 1- to 3-month-old infants [49]. However, *cpts*248/404 caused nasal congestion in these infants, an unacceptable adverse effect in this population [49].

Production of live attenuated RSV vaccine candidates by mutagenesis and screening for temperature sensitivity is a laborious and inefficient process. Therefore, it is essential to develop a method of systematically deriving *ts*RSV and identifying additional attenuating mutations that can be incorporated into RSV vaccine candidates. The recent advent of reverse genetics systems for RSV has allowed the development of live attenuated RSV vaccine candidates encoding specific attenuating mutations, rather than relying on random mutagenesis. The ability to generate recombinant RSV (rRSV) from cDNAs also allows the identification of novel viral targets for attenuation through the investigation of the virus–host interactions important for viral pathogenesis. Reverse genetics systems for RSV rely on the coexpression of the viral polymerase components (N, P, M2-1, and L) with a complete copy of the viral genome [67, 68]. Coexpression is achieved by transfection of plasmids encoding each of the viral polymerase genes and a plasmid encoding the full-length cDNA of the viral genome into cultured cells. Expression from the plasmids is driven by the bacteriophage T7 RNA polymerase, which is supplied exogenously. For the purposes of vaccine development, T7 RNA polymerase is expressed by cotransfection of an expression plasmid with the other plasmids into qualified Vero cells [69]. Upon expression of viral components,

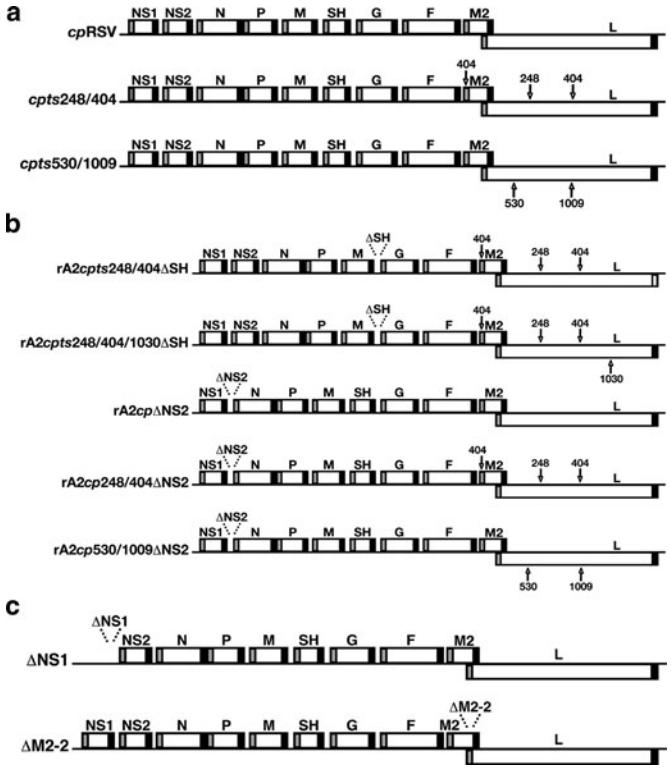

Fig. 2 RSV vaccine candidates. (**a**). Genomic organization of biologically derived, temperature-sensitive RSV vaccine candidates. *Arrows* indicate relative position of the attenuating mutations corresponding to the mutant, indicated on the *left*. (**b**). Recombinant RSV vaccine candidates. ts point mutations are identified as in (**a**). Deletions are indicated with *dashed lines*. (**c**). Potential recombinant RSV vaccine candidates. ts point mutations are identified as in (**a**). Deletions are indicated with *dashed lines*

transcription and replication of the viral genome initiates the RSV infectious cycle, resulting in the production of infectious rRSV. The cDNA copy of the viral genome can be mutated by standard molecular biology techniques in order to attenuate the resultant rRSV.

Initial studies using rRSV focused on two different means of attenuating RSV. The first method involved combining the known mutations from the *cpts*RSV isolates in rRSV strain A2 (rA2) to increase attenuation of the vaccine candidates. This resulted in the generation of rA2*cpts*248/404/1009 and rA2*cpts*248/404/1030, combining the *cpts*248/404 mutations with those of 530/1009 and 530/1030 [70]. These new mutants were more attenuated than the *cpts*248/404 parental virus, indicating that some mutations have additive effects in attenuation. However, these studies also showed that certain mutations are incompatible with others, as the rA2*cpts*248/404/530 could not be recovered, due to incompatibility of the 530

Table 1 Clinical trials on live attenuated RSV vaccine candidates

Vaccine candidate	Attenuation phenotype	Immunogenicity	References
Biologically derived			
*cp*RSV	Underattenuated in seropositive children	Mild (adults)	[53]
*cpts*248/955	Underattenuated in seronegative children	Good (seronegative children)	[53]
*cpts*530/1009	Underattenuated in seronegative children	Good (seronegative children)	[53]
*cpts*248/404	Underattenuated in infants (partial reversion)	Good (seronegative children) Mild (infants)	[49]
Recombinant			
rA2cpts248/404ΔSH	Underattenuated in seronegative children	Good (seronegative children)	[52]
rA2cpts248/404/1030ΔSH Ongoing trials	Sufficiently attenuated in infants (partial reversion)	Good (seronegative children) Poor (infants)	[52]
rA2cpΔNS2	Underattenuated in seropositive children	Mild (seropositive children)	[79]
rA2cp248/404ΔNS2	Underattenuated in seronegative children	Moderate (seronegative children)	[79]
rA2cp530/1009ΔNS2	Sufficiently attenuated in seronegative children	Poor (seronegative children)	[79]
Vectored			
MEDI-534 (rB/HPIV3-RSV-F) Ongoing trials	Attenuated in seropositive children	Poor (seropositive children)	[117]

mutation with, particularly, the 248 mutation [70]. Therefore, it would be desirable to have a panel of attenuating mutations from which to select to incorporate into rRSV vaccine candidates, so that the level of attenuation can be properly tuned. In order to increase the number of attenuating mutations that could potentially be combined in a vaccine candidate, specific viral proteins have been mutagenized to replace charged amino acids with a noncharged amino acid (e.g., alanine). This procedure has been employed to identify a number of mutations in both P and L that result in attenuation of RSV, both in culture and in rodents [71–73]. These mutations thus add to the panel of mutations available for inclusion in future vaccine candidates, either alone or in combination with the previously identified *cpts* L mutations.

Another avenue of attenuation for RSV has been the deletion of nonessential genes. Gene deletion should be more stable than the point mutations responsible for temperature sensitivity, reducing the risk of reversion to virulence of the vaccine candidate. rRSVs (rA2) lacking one or a combination of NS1, NS2, M2-2, and SH were generated and shown to be attenuated in preclinical trials [31, 74–76]. RSV lacking SH (rA2ΔSH) replicated similarly to wild-type (wt) RSV (rA2) in culture but showed a low level of attenuation in the respiratory tracts of rodents and nonhuman primates [77]. Because clinical trials indicated that rA2cpts248/404

was only slightly underattenuated, the SH gene deletion was incorporated into this vaccine candidate to increase the level of attenuation (Fig. 2b). However, this vaccine candidate (rA2cpts248/404ΔSH) was not further attenuated in adults, seropositive or seronegative children (Table 1) [52]. It was not possible to determine from these observations whether the SH deletion mutation confers attenuation to RSV in humans, even though rA2ΔSH was attenuated in mice and chimpanzees. These results indicate that attenuation of RSV by combining different mutations is not necessarily additive. However, subsequent addition of the 1030 mutation to rA2cpts248/404ΔSH resulted in a virus that was more *ts* and more attenuated in seronegative children [52]. Further trials in seronegative infants showed that rA2cpts248/404/1030ΔSH was well tolerated and appropriately attenuated (Table 1) [52]. Only a minority of vaccinees produced increased neutralizing antibody responses, even after a second dose of the vaccine virus. However, replication of the second dose of vaccine was significantly reduced, indicating that some protective immunity had been induced by the initial dose [52].

Preclinical testing of RSV lacking NS1 or NS2 (rA2ΔNS1 and rA2ΔNS2, respectively) showed that these viruses were deficient in replication in culture and also attenuated in rodents and nonhuman primates [31, 32, 76, 78]. In chimpanzees, rA2ΔNS2 displayed an attenuation phenotype similar to rA2cpts248/404, and rA2ΔNS1 was significantly more attenuated in both the upper and lower respiratory tracts [74, 75]. However, both deletion mutants induced levels of serum-neutralizing antibodies against RSV to levels comparable or slightly lower than wt RSV. In addition, chimpanzees immunized with rA2ΔNS2 were protected against subsequent challenge with RSV. Therefore, an NS2-deletion rA2 derivative was then tested in clinical trials as a vaccine for the elderly because it was less attenuated in chimpanzees than the *cpts*248/404 vaccine candidate (Fig. 2b) [79]. rA2cpΔNS2 was shown to be overattenuated in adults; however, it was also underattenuated in children, a contraindication for testing in infants (Table 1). The NS2 deletion virus was further attenuated by inclusion of the *ts* mutations 248/404 or 530/1009. These vaccine candidates were more attenuated than their parental strains and modestly immunogenic when tested in seronegative children [79].

5 Live Vectored RSV Vaccines

An alternative means of delivering RSV antigens in attenuated virus vaccines has been the use of heterologous viral vectors expressing RSV F and/or G. Early efforts focused on vaccinia viruses (VV) expressing RSV proteins. VV-F and VV-G together were immunogenic and protective in the mouse model of RSV; however, these VV recombinants did not induce protective immunity in chimpanzees [80–83]. In addition, VV is likely too virulent to use as a vector for current vaccine development. More recently, use of the attenuated modified vaccinia Ankara as a vector for RSV antigens has shown some efficacy, though a prime-boost strategy may be required to elicit sufficiently protective immunity [84, 85].

Adenovirus vectors were initially used to immunize against RSV F and G over 15 years ago and, with the advent of replication-deficient adenovirus vectors, have been further investigated more recently [86–90]. Adenovirus-vectored F and/or G have been shown to provide protection to RSV in mice and ferrets; however, this vaccine modality does not immunize chimpanzees against RSV, indicating that this strategy will likely not be clinically useful [88, 89]. Alphavirus replicons have also been tested for their ability to vaccinate against RSV [91–94]. Immunization via either the intranasal or intramuscular route with Venezuelan equine encephalitis virus replicons expressing RSV F induces balanced Th1/Th2 immunity, protects mice and cotton rats against RSV challenge, and induces serum antibodies in macaques [91, 92].

The recent proliferation of reverse genetics systems for the paramyxovirus family has provided the possibility that RSV antigens can be expressed in the context of a number of different paramyxoviruses, including Sendai virus, Newcastle disease virus (NDV), and human parainfluenza viruses (HPIV) 1, 2, and 3 (reviewed in [95–97]). Sendai virus and NDV are murine and avian viruses, respectively, and thus are naturally attenuated in humans due to host range restriction. NDV is a strong inducer of IFN-β and may therefore provide better stimulation of dendritic cell (DC) maturation and T-cell responses than RSV infection [98]. Both of these vector systems have been shown to be immunogenic and protective against RSV challenge in animal model systems [98–102].

An additional consideration is the possibility of combining vaccines against multiple pediatric viral pathogens into a single recombinant virus. Infection of children by HPIV1 and HPIV2 generally occurs later in life (approximately 6 months of age), so immunization would occur in older infants. Thus, an HPIV1- or HPIV2-vectored RSV vaccine may be useful as a booster to prevent secondary disease or as a vaccine in the elderly. In addition, attenuated HPIV1 and HPIV2 are being developed for use as vaccine candidates [103–109].

Because HPIV3 is also an important cause of pediatric respiratory tract disease, significant effort has been put into developing a live attenuated HPIV3 vaccine that could also be used as a vector for an RSV vaccine (Table 1). One candidate vaccine utilizes the bovine PIV3 (BPIV3) backbone, which has been shown to be safe and immunogenic in infants [110, 111]. In order to generate a bivalent HPIV3/RSV vaccine, the BPIV3 F and HN genes were replaced by their HPIV3 counterparts and RSV F was inserted into the B/HPIV3 chimera; thus, the resulting virus expresses both RSV and HPIV3 surface antigens. Recombinant B/HPIV3-RSV-F was slightly more attenuated than the parent virus, but remained immunogenic and was protective against both RSV and HPIV3 in animal model systems [112–115]. This vaccine candidate (MEDI-534) has recently been tested in clinical trials. Although the vaccine was attenuated and safe, it was minimally immunogenic in both adults and children, indicating that further modification may be required [116, 117]. However, the major advantage of this approach is that the viral vector is also a vaccine, thus providing protection against multiple pathogens. Because the RSV F protein is likely not incorporated into its viral envelope, RSV-specific antibodies were ineffective at neutralizing the chimeric virus [112], suggesting it could be also used as to boost anti-RSV immune responses.

6 Future Directions

There remain a number of challenges to the development of an efficacious RSV vaccine. First, it will be important to develop additional animal models for RSV challenge that more faithfully represent the target populations of infants and the elderly. Although nonhuman primate models have yielded important information on both vaccine safety and immunogenicity, these models also have not recapitulated some aspects of the replication of vaccine candidates in humans. For example, ΔNS2 was immunogenic in chimpanzees but not in seropositive children [75, 79]. In addition, the partial reversion of the *ts* phenotype seen with the 248/404/1030 mutations in infants was not detected in animal experiments [49, 52, 70, 118]. Defining the correlates of protection and attenuation in animal models will aid in the selection of vaccine candidates for clinical trials. In addition, a model that recapitulates stimulation of the immature immune system in the presence of maternal antibodies will be important for the development of a pediatric RSV vaccine.

Perhaps the most important challenge in the development of an effective RSV vaccine has been achieving the proper balance between immunogenicity and attenuation. The rA2cpts248/404/1030ΔSH vaccine candidate, which was appropriately attenuated in infants, was only mildly immunogenic [52]. It is possible to enhance immunogenicity of vaccines by increasing the dose or boosting with multiple inoculations. However, the target population of a pediatric RSV vaccine would be infants who are entering their first RSV season, thus shortening the window in which immunization would be effective. Therefore, a better understanding of the induction of immune responses in the target populations for RSV vaccines will be essential. Identifying signals (e.g., TLR agonists, cytokines) that can induce DC maturation and/or activate other antigen-presenting cell populations stimulate Th1 responses that can augment the immunogenicity of an RSV vaccine. For example, studies in mice suggest that deletion of NS1 results in a virus that has enhanced capacity to induce DC maturation, likely due to increased production of IFN-β [119]. In addition, NS1 appears to play a role in viral replication beyond IFN antagonism, indicating that deletion of this gene might be both attenuating and immunomodulatory [31].

An alternative method to enhancing immune responses that has been explored is the expression of cytokine genes as an additional transcription unit in rRSV [120–122]. Stable expression of additional gene products in the rRSV genome has been shown for a variety of genes [123]. rRSV encoding GM-CSF as an additional gene shows reduced replication in the respiratory tracts of mice with a concomitant increase in the number of pulmonary DCs and in the expression of IFN-γ and IL-12 [121]. By contrast, insertion of genes for the cytokines IL-4 and IFN-γ into rRSV results in viruses that caused increased pathogenesis after immunization and/or challenge [120]. Skewing of the T helper response can have adverse effects on secondary exposure and even to unrelated viruses [124]. Thus, significant care must be taken in identifying specific immunomodulators that will

increase the immunogenicity of an RSV vaccine candidate without causing enhanced disease.

One potential mechanism of improving B-cell responses to RSV is increasing the expression of the RSV F and G proteins, which serve as the major protection antigens [18]. Because of the linear nature of the RSV genome, the promoter-proximal genes are expressed to a greater extent than the promoter-distal genes [18]. Rearrangement of the gene order in the related vesicular stomatitis virus (VSV) has been shown to result in genome site-specific levels of expression for the viral genes [125]. These rearranged viruses displayed an attenuated phenotype both in vitro and in vivo and were able to vaccinate pigs against subsequent VSV infection [126, 127]. For RSV, rearrangement of the gene order in a recombinant virus, such that the F and/or G genes are the promoter-proximal, resulted in an approximately twofold increase in protein expression [128]. Unlike VSV, these viruses replicated slightly better than wt virus in culture and similarly to wt in the respiratory tracts of mice [128]. Thus, gene rearrangement alone in the context of RSV is not attenuating. In addition, shifting F to a promoter-proximal position resulted in an increase in anti-F serum antibody responses in mice, suggesting that increased F expression may be desirable in a vaccine candidate [128]. Expression of F and G might be further increased by optimizing the codon usage of these genes for translation [129]. Combining these relatively small increases in antigen expression might allow for an additive effect for vaccination. Studies with anti-RSV F antibody prophylaxis show significant increases in efficacy with even minor increases in antibody titer [130, 131]. Thus, increasing the amount of antigen available for presentation to the immune system may allow for a more robust anti-RSV response.

RSV G is unique among paramyxovirus attachment proteins in that it is produced in both a membrane-bound and a secreted form. Secreted G (sG) is produced from the G mRNA by alternative initiation from a downstream AUG [132, 133]. Ablation of this translation initiation codon in rRSV results in RSV that produces only membrane-bound G [134]. Studies have shown that the sG can act both as an antigenic decoy in vitro and as an immunomodulatory factor in mice [135]. Importantly, sG appeared to affect restriction of RSV replication in vivo by both anti-G and anti-F antibodies through a mechanism involving FcγR-bearing immune cells [135]. Thus, a vaccine candidate that does not express sG may have increased immunogenicity and may be more efficiently controlled by the immunity induced. In addition, sG showed proinflammatory functions in the lungs of mice, likely via its CX3C (fractalkine) motif [135]. Because pulmonary inflammation is associated with increased pathogenicity of RSV, removal of this factor may result in decreased reactogenicity. However, sG may be necessary for vaccine take in infants in the presence of maternal antibody. Further studies will clarify these disparate effects of sG on RSV pathogenesis and immunity. An alternative to ablating the expression of sG might be removal of the CX3C motif from G; studies have shown that mutagenesis or deletion of this sequence does not affect viral replication in vitro or in mice [136].

One important characteristic of vaccine candidates is genotypic and phenotypic stability. Genomic stability is important during the scaling up of production for the vaccine viruses, which undergo multiple rounds of replication and thus have a greater chance for mutation. In addition, phenotypic stability is essential during vaccination, during which reversion to virulence can cause increased pathogenicity and shedding. In this case, the attenuated phenotype is more important than specific genotype provided that immunity to the major protective antigens is achieved. Deletion of nonessential viral genes should provide the most stable attenuating mutations because genetic recombination of RSV is extremely rare and has only been observed in the laboratory under optimal conditions [137]. In addition to the NS2 and SH deletion viruses, RSVs lacking NS1 or M2-2 (Fig. 2c) are significantly attenuated and protective in animal models and are potentially good vaccine candidates [31, 74, 76, 78].

All of the *ts* mutations identified in the RSV vaccine candidates that have undergone clinical trials are point mutants. *ts*248, *ts*530, *ts*1009, and *ts*1030 are all missense mutations within the viral polymerase (or L protein), and *ts*404 is a point mutation in the M2 gene start sequence [65, 66, 70]. Characterization of virus shed from vaccinees has shown that these point mutations can readily revert, resulting in less *ts* RSV, in some cases despite the "stabilization" of these mutations in rRSV by changing two residues of the specific codon encoding the *ts* mutant. For example, analysis of nasal wash specimens from seronegative infants vaccinated with rA2cpts248/404/1030ΔSH showed that approximately one-third of the samples had lost a measure of their *ts* phenotype, displaying a 1–3°C increase in permissive temperature [52]. Sequencing of these clinical specimens identified reversion mutations at either the *ts*248 or the *ts*1030 mutation [52]. Although these partial revertants retained four of the five attenuating mutations and a measure of attenuation, these results demonstrate the difficulty of using point mutations to attenuate RNA viruses, which encode an error-prone viral polymerase. To counteract this problem, there are a number of possibilities to generate genotypically and phenotypically stable *ts* RSV vaccine candidates.

It is possible to generate phenotypically stable attenuated RSV viruses by introducing several *ts* point mutations in a variety of places in the RSV genome. The difficulty with this approach is that some combinations of mutations might increase the attenuation of the vaccine virus beyond the level required for inducing protective immunity. In addition, some *ts* mutations are not compatible with each other, resulting in a nonviable virus [70]. Thus, the spectrum of mutations that can be combined would have to be empirically defined. The benefit to this strategy is that reversion at any one site should be compensated by the presence of the additional attenuating mutations. However, as seen with rA2cpts248/404/1030ΔSH, particular mutations have a more prominent effect on attenuation of the vaccine virus and reversion at these sites may result in a significant loss in attenuation.

One method of preventing reversion is to "stabilize" existing *ts* mutations by altering the codon usage to require two mutation events in order for the mutant to revert to the wt phenotype. Theoretically, the viral polymerase would not be likely

to introduce two mutations at the same site. Recently, Luongo et al. have constructed rRSV that have mutations at position 831 of L (*ts*248) encoding every possible amino acid residue. Although most mutants could be recovered, only two mutants were found to confer temperature sensitivity (831I and 831F) to the rRSV in addition to the 831L mutation [138]. Furthermore, neither 831I nor 831F was as attenuated as 831L in the respiratory tracts of mice, suggesting that 831L has an attenuating function beyond temperature sensitivity. Interestingly, using the different codons for Leu resulted in different frequencies of reversion (to wt genotype) or pseudoreversion (to wt phenotype) [138]. These data suggest that careful selection of mutant codons may offer a strategy for increasing genotypic stability of attenuating point mutations. However, the genetic code precludes certain mutations from being "stabilized" by this method, as not all mutations can be made with two nucleotide differences from the wt assignment.

A novel potential mechanism of providing genotypic stability for point mutations is increasing the fidelity of the viral polymerase. Recent studies with poliovirus (PV) have shown that mutations that alter replication fidelity and/or replication speed of the PV RdRp produce attenuated viruses that protect mice transgenic for the PV receptor from a lethal challenge with wt PV [139–141]. Furthermore, mutation of a single amino acid residue that is conserved in all viral RdRps appears to control both replication speed and replication fidelity. This amino acid residue is a lysine that is present in conserved structural motif D of the RdRp [142, 143]. In the PV model, changes to this residue produce slow, high-fidelity RdRps [143]. Biochemical analysis shows that mutation of the homologous lysine in HIV RT and T7 RNA polymerase results in similar effects on polymerase speed and fidelity [143]. Thus, application of this technology to RSV could allow the identification of an additional attenuating mutation and could prevent or delay the emergence of more virulent variants of the vaccine candidates. Combinations of L mutations that increase polymerase fidelity and known attenuating mutations could allow for even finer tuning of vaccine efficacy and prevent outgrowth of more virulent viruses, which could then be spread to naive individuals.

7 Summary

Much progress has been made recently toward the development of an effective, live attenuated RSV vaccine; however, a number of hurdles remain. Most importantly, achieving the proper balance of attenuation and immunogenicity has been difficult because of the lack of animal models and immune correlates to investigate induction of immune responses in infants, a target population for RSV vaccines. Future studies into the molecular biology of the virus may lead to novel ways to address current difficulties in RSV vaccine development.

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Live Attenuated Cholera Vaccines: Flagella and Reactogenicity

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Abstract The rational design of attenuated *Vibrio cholerae* strains has been an attractive method for live cholera vaccine development because the major mechanisms of *V. cholerae* virulence are well defined and convalescence from cholera, the disease it causes, is a strongly immunizing process. After decades of effort to develop safe live attenuated cholera vaccines, however, the appearance of reactogenicity, defined as adverse symptoms in immunized volunteers, has precluded further development of most live vaccine candidates. We now know that *V. cholerae* flagellar motility is associated with human and animal reactogenicity in early live attenuated cholera vaccines, and recently developed nonflagellated *V. cholerae* mutant strains have shown great promise as live attenuated vaccines in volunteer studies. This chapter briefly summarizes our current understanding of *V. cholerae* pathogenesis and describes efforts to use this knowledge to design immunogenical and nonreactogenic live cholera vaccines.

1 *Vibrio cholerae* and Disease

Vibrio cholerae, a highly motile Gram-negative bacterium, is the etiological agent of cholera, a human diarrheal disease that kills an estimated 100,000–200,000 people annually [1]. Besides its traditional home in countries of the Ganges delta (i.e., India and Bangladesh), cholera in the last several years has extracted particularly high fatality rates in Africa including countries like Zimbabwe where the reported case fatality rate was nearly 5% in 2008 [2, 3]. One of the most rapidly fatal diseases known, cholera can kill its victims in as little as 10–18 h following initial symptoms due to severe dehydration and hypovolemic shock [4]. In endemic

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regions, cholera largely targets young children not previously exposed to the disease, but people of all ages are equally at risk in newly invaded areas during epidemic spread [5]. *V. cholerae* was originally identified as the cause of cholera by Filippo Pacini in 1854, but his observations were largely ignored until Robert Koch independently discovered the causal connection between the comma-shaped bacterium and voluminous diarrhea in 1884 [6]. Since then, great strides have been made in understanding the virulence mechanisms of *V. cholerae,* its ecology, and the nature of host immunity following convalescence.

2 Ecology of *V. cholerae*

V. cholerae is found in marine and brackish water and has historically caused epidemic disease throughout the world [6, 7], but improved sanitation and healthcare facilities in the developed world have largely confined cholera outbreaks to the coastal regions of southern Asia, Africa, and central America. Classified by the immunogenicity of lipopolysaccharide (LPS) O-antigen, over 200 serogroups of *V. cholerae* have been identified in the environment, but only specific "biotype" strains within the O1 and O139 serogroups are known to cause widespread disease [8]. O1 "classical" strains were likely responsible for at least six pandemics that spread throughout Asia, Europe, and the Americas in the nineteenth century [9], and O1 "El Tor" strains are responsible for the current seventh pandemic that began in Indonesia in 1961 [10]. In the early 1990s, seroconversion of an O1 El Tor strain to the O139 serogroup allowed it to quickly overtake the O1 El Tor strain as the primary cause of cholera in India and Bangladesh [11–13], likely due to its ability to circumvent acquired immunity in endemic communities against the O1 antigen [14]. In recent years, O1 El Tor strains have reemerged, and presently both the O1 and O139 strains cause recurrent disease. While these "toxigenic" O1 and O139 strains cause a vast majority of cholera disease, several non-O1, non-O139 strains are known to cause sporadic human disease [15, 16], often using alternative virulence mechanisms including type III and type VI secretion [17–19].

3 Virulence Mechanisms of *V. cholerae*

As a waterborne disease, cholera is acquired by ingestion of water or food contaminated with *V. cholerae*. A high-infectious dose of at least 10^8 bacteria is required to cause human disease due in large part to *V. cholerae* sensitivity to the stomach's gastric acid barrier [20]. Once in the small intestine, the bacterium uses its single polar flagellum and an extensive chemotaxis sensory network to target and then penetrate the mucus layer, a thick glycocalyx gel covering the small intestine epithelium [21–23]. Recent work suggests that *V. cholerae* loses its flagellum

shortly after entering the mucus layer but is still able to actively transit through the layer, possibly using an additional as-yet-unidentified motility system [24].

Upon reaching the epithelial layer, *V. cholerae* uses an intricate regulatory network known as the *toxR* regulon to induce expression of virulence genes including cholera toxin (CT), the AB_5 enterotoxin responsible for the bulk of the diarrheal response seen in the disease, and the toxin coregulated pilus (TCP), a type IV bundle forming pilus that is essential for *V. cholerae* intestinal colonization [25–27]. In the regulatory cascade, the inner membrane protein ToxR acts with its membrane partner ToxS and a second pair of membrane proteins TcpPH to induce expression of ToxT, an AraC family transcription factor that then activates transcription of the toxin genes *ctxAB* and the *tcp* pilus biosynthetic operon [28]. Further regulatory control is provided by the *V. cholerae* quorum-sensing system, which uses expression of the transcriptional regulators AphA and AphB to control TcpPH levels [29–31].

The host intestinal environment also plays an intricate role in this virulence gene cascade as both ToxT and ToxR are posttranscriptionally controlled by the components of bile, a heterogeneous mixture found at high concentration in the small intestine where it aids in digestion. Oleic acid and other unsaturated fatty acids (UFAs) in bile directly inhibit ToxT activity by inducing a "closed" ToxT conformation that is unable to activate *tcp* and *ctx* transcription [32, 33]. Since UFAs in bile exist at high concentration in the intestinal lumen but are readily absorbed by the small intestine epithelium, the resulting UFA concentration gradient provides *V. cholerae* with an ideal measuring stick to ensure that virulence gene expression occurs only at or near the intestine epithelial surface. Interestingly, the bile acids cholate and deoxycholate seem to function in an opposing manner: they activate ToxR to cause increased CT expression independent of ToxT activity [34]. Other intestinal stimuli including changes in pH and temperature are known to regulate virulence gene expression, but their specific modes of action remain unknown [35].

4 Cholera Disease Dynamics

In endemic regions, *V. cholerae* infection rates follow a distinct biannual seasonality in which a large outbreak of disease occurs following monsoon rains in the fall and a smaller outbreak occurs in the spring. This pattern of disease correlates strongly with the presence of culturable toxigenic *V. cholerae* in the environment, and several studies have linked this increase in *V. cholerae* abundance and the epidemic spread of disease to changes in water salinity, temperature, and zooplankton and phytoplankton blooms [6, 36]. During a cholera outbreak, as many as several trillion *V. cholerae* may be shed into the environment by a single symptomatic person [37], and these bacteria exist in a transient hyperinfectious state that reduces the bacterial load required to infect other people in the community [38].

These features of *V. cholerae* pathogenicity help to explain the explosive nature of cholera outbreaks, but equally interesting is the self-limiting nature of these

epidemics, which often end as suddenly as they begin. Recent work suggests that this may be due to lytic bacteriophage in the environment that specifically target O1 and O139 strains of *V. cholerae* [39]. In this phage-based model of cholera disease dynamics [40–42], the large number of *V. cholerae* in the environment (and even inside patients) during a cholera outbreak provide ample targets for lytic-phage infection and growth. The resulting high-phage predation rate serves to reduce the concentration of toxigenic *V. cholerae* in the environment, and the concomitant drop in new human infections reinforces the decline since fewer hyperinfectious bacteria are shed from cholera patients into the environment. In the subsequent interepidemic months when O1 and O139 strains are in low abundance, the lytic-phage population in the environment is reduced by dispersion and dilution, allowing for the cyclic reemergence of toxigenic *V. cholerae* strains.

Recent mechanistic modeling suggests that cholera epidemic dynamics are also heavily influenced by the high rate of asymptomatic cholera infections in humans [43, 44]. As discussed below, the resulting spike in transient protection from reinfection in endemic communities may play an important role in the cyclic decline and reemergence of cholera disease in these regions.

5 Immunity to Cholera

Convalescence from symptomatic cholera disease induces a durable immunity that is years in duration and perhaps life-long in some individuals [5, 45]. Because cholera is a mucosal, noninvasive disease, it has long been thought that elements of the mucosal immune system play the predominant role in protective immunity, with secretory immunoglobulin A (IgA) serving to aggregate *V. cholerae* in the intestinal lumen and neutralize its toxins and colonization factors [46]. Since direct measurement of IgA levels in the intestinal lumen and mucosal layer is cumbersome, serum vibriocidal antibody levels have traditionally been used as a correlate for immunity at the gut mucosal surface [47]. Serum vibriocidal antibody levels in patients typically rise rapidly following *V. cholerae* infection but usually drop down to near baseline levels within 6 months [48]. Serum vibriocidal levels in patients correlate with immunity to cholera [48], but are at best an incomplete predictor of protection because some patients with very high vibriocidal titers are still susceptible to disease [49]. In fact, serum IgG antibodies specific to *V. cholerae* LPS and the B subunit of CT (CTB) are abundantly found in convalescent patients, but serum IgG titers against either of these antigens do not correlate well with protection from reinfection [50].

Serum IgA levels, on the other hand, do correlate well with protection from *V. cholerae*. IgA titer against *V. cholerae* LPS, CTB, and the TCP pilin TcpA are all predictive of protection from the disease [51]. Intestinal lavage studies have shown that *V. cholerae*-specific IgA levels in the intestinal lumen are elevated shortly after infection but drop significantly within 4 weeks of convalescence [52]. Since the resulting resident mucosal IgA levels appear to be too low to prevent *V. cholerae*

infection in immune individuals, it is likely that a rapid anamnestic response by memory B and T cells in the gut-associated lymphoid tissue (GALT) is responsible for the observed protection [53]. Memory T cells specific to *V. cholerae* antigens are observed in patients as soon as 7 days after infection [54], and circulating memory B cells specific to *V. cholerae* LPS, CTB, and TcpA can be found in patients at least 1 year after infection [55]. It is still unclear, however, if these particular memory cells represent the gut lymphocyte population that is actually protective.

6 Killed Whole-Cell Cholera Vaccines: Parenteral and Oral Inoculation

The first cholera vaccines contained killed, whole-cell *V. cholerae* lysates that were parenterally administered, but broad field trials showed that they failed to elicit an adequate level of long-term immunity [56–58]. These vaccines elicited high serum IgG levels against *V. cholerae* antigens, but they induced only low levels of serum IgA compared to oral administration of the vaccine. It was eventually recognized that immune stimulation at the intestinal mucosa was required to induce strong immunological memory toward *V. cholerae* [52], and since then the field has largely focused on developing oral cholera vaccines that trigger an immune response in the small intestine mucosa.

In the 1980s, oral vaccination against cholera was explored in several volunteer studies using killed whole-cell *V. cholerae* strains [59], and Holmgren and colleagues were the first to test these vaccines for efficacy in a cholera-endemic country [52]. In the vaccine, they included a mixture of formalin-treated and heat-killed *V. cholerae* strains belonging to both the O1 El Tor and classical biotypes and added purified CTB to induce additional antitoxin immunity. Three doses of this vaccine produced 85% efficacy at 6 months and 50% efficacy lasting at least 3 years in a large-scale field trial in Bangladesh [60, 61], but long-term immunity was much more predominant in older age groups and tended to fall off in children under 5 years of age, the very group that is most susceptible to infection in endemic regions. The vaccine was subsequently approved for sale as Dukoral [62], and similar vaccines that include an O139 strain but are not dosed with CTB have been developed in Vietnam [63–65] and India [66].

Results from these field trials confirm that cholera vaccines can prevent disease in endemic countries, but it remains to be determined if killed oral vaccines are the most effective public health tool to control and ideally eliminate cholera in endemic settings. Important drawbacks of killed oral vaccines include the following: (1) multiple doses are required to induce significant immunity, (2) they are less effective in infants and children who carry a disproportionate share of the disease burden, and (3) their manufacture may be cumbersome compared to alternatives such as live attenuated vaccines.

7 Live Attenuated Cholera Vaccines

The concept of using live attenuated microbes as vaccines dates back to work on viruses such as polio, measles, mumps, and rubella where laboratory propagation of virulent strains led to attenuation. These attenuated strains serve as good vaccines because they retain the ability to infect people and induce an adaptive immune response targeted at actively replicating organisms, but their attenuation allows the host-immune response to overwhelm the virus before progression to symptomatic disease can occur. Over half a century ago, naturally attenuated strains of *V. cholerae* were also explored for cholera vaccination until molecular genetic techniques were developed to combine attenuating traits like auxotrophy and streptomycin-dependence into a single strain [67, 68].

An important shift in cholera vaccine design occurred in the early 1970s when it was recognized that virulence factors might be particularly good targets for attenuating *V. cholerae* vaccine strains. In particular, it was hoped that disruption of CT in a toxigenic *V. cholerae* strain would stop it from causing diarrheal disease but would not affect its ability to colonize the human intestine and elicit an adaptive immune response that normally leads to long-term immunity. Howard reported isolating CT mutants after chemical mutagenesis in 1971 [69] but these mutants were not further characterized or tested in volunteer studies. In 1979, Honda and Finkelstein reported the isolation of Texas Star, a chemically induced mutant of a *V. cholerae* O1 El Tor strain that did not produce the CT A subunit (CTA) but continued to produce the nontoxic B subunit (CTB) [70]. When tested in volunteer studies, Texas Star did not induce voluminous diarrhea and remained fully immunogenic as had been hoped, but the strain also elicited adverse side-effects in recipients including cramps, fever, malaise, and mild diarrhea [71]. These symptoms are not normally seen in clinical cholera patients, and their induction by *V. cholerae* strains has been termed "reactogenicity."

Advances in the molecular genetics of CT led to the development of attenuated *V. cholerae* mutants that had deletions in the CT genes *ctxAB* created by mutagenic phage or recombinant DNA techniques [72–74]. It was hoped that the precision associated with genetic engineering would lead to defined, stable, attenuated live vaccines that were free of reactogenicity; however, volunteer studies quickly established that while these strains were highly immunogenic and protective in experimental human challenge studies, they also remained significantly reactogenic [75].

The field as a whole entertained several theories that could explain this reactogenicity, including the possibility that it was caused by an additional undefined accessory toxin [76] or that it resulted from a local inflammatory response caused by colonization *per se* of the relatively bacteria-free upper small intestine. Indeed, volunteer experiments revealed that *V. cholerae* strains with defined deletions that caused a defect in intestinal colonization also caused less reactogenicity and immunogenicity in patients [27], suggesting that *V. cholerae* colonization, immunogenicity, and reactogenicity are tightly linked.

Eventually one mutant (CVD-103-HgR), derived from the poorly colonizing *V. cholerae* classical strain 569B, was found in volunteer studies to induce very little reactogenicity while eliciting enough immunogenicity to justify significant development efforts [77]. However, for reasons that to this day remain unknown, other mutants (CVD101 and CVD110) with a similar constellation of defects as CVD103-HgR but derived from different parental strains were found to be reactogenic [75]. Despite the poor understanding of the underlying mechanism of immunity and reactogenicity in CVD103-HgR, clinical development continued over the next decade [78, 79] and the vaccine was eventually licensed in Europe as Orochol, a single-dose oral travelers vaccine for cholera [80]. In 2000, however, the results of a large, placebo-controlled field trial of Orochol in Indonesia indicated that this vaccine provided protection from cholera in only 14% of subjects despite inducing adaptive immune responses in nearly 70% of vaccine recipients [81]. These and other considerations resulted in suspended manufacture of Orochol.

8 New Insights and New Approaches toward Stable Attenuation of *V. cholerae*

With the outbreak of cholera in Peru in 1991 after nearly a century-long hiatus in the Western hemisphere, new urgency was placed on developing highly immunogenic, live attenuated cholera vaccines based on the El Tor biotype strain that caused the outbreak. This was based in part on the recognition that El Tor strains expressed an antigenically distinct TcpA that was presumed to be a protective mucosal immunogen [82] and in part on the assumption that these strains would be more immunogenic because of their increased colonization capacity compared to classical strains. Mekalanos and colleagues at Harvard Medical School soon created *V. cholerae* O1 El Tor strains with deletions in *ctxAB* as well as several genes involved in the integration of the CTX genetic element, a large DNA segment believed necessary for the acquisition of *ctxAB* by nontoxigenic *V. cholerae* strains [83]. The CTX genetic element was also suggested to encode accessory toxins, but vaccine strains with deletions in these genes remained reactogenic [84, 85], and the putative toxin genes (named *zot* and *ace*) were later shown to be morphogenesis genes of the filamentous phage that encoded CT (see below), providing strong evidence that these putative toxin genes had no role in reactogenicity [86].

The whole concept of a genetically stable live attenuated cholera vaccine was brought into question by the work of Waldor and Mekalanos who reported in 1996 that the CTX genetic element corresponded to the genome of a filamentous bacteriophage termed CTXΦ [86]. This phage could efficiently transduce nontoxigenic *V. cholerae* strains using TCP as a receptor, and the specter of genetically engineered *V. cholerae* vaccine strains reverting to toxicity by simple phage transduction immobilized many vaccine developers during this period. It was subsequently recognized that CTXΦ requires a 17 bp site called attRS1 to integrate into the

V. cholerae large chromosome; *V. cholerae* strains deleted for attRS1 can be transduced by CTXΦ but the episomal CTXΦ genome is unstable and is easily lost because it cannot integrate into the chromosome [86]. Deletion of *recA* in vaccine strains was also explored as a stabilizing mutation because it was predicted to block reacquisition of the CTX phage through other pathways that require homologous recombination. Deletions in both *recA* and attRS1 have been incorporated into newly designed vaccine strains, and live cholera vaccine development has continued. While this approach has solved, in theory, the issue of CTXΦ-mediated reversion to toxigenicity, the deeper issue of reactogenicity, remained unsolved.

9 Development of the Concept: Evaluation of Motility Defective Vaccine Candidates

A breakthrough was achieved when the live vaccine strain Peru-14 was found to be highly immunogenic but also very well tolerated in volunteer studies [87]. Derived from an O1 El Tor clinical strain isolated in Peru in 1991, Peru-14 contained deletions in the entire CTXΦ genome and attRS1 site, and *recA* was replaced with a high-expression construct for *ctxB*, the gene that encodes the immunogenic but nontoxic cholera toxin B subunit CTB (Fig. 1). Importantly, Peru-14 also carried an undefined mutation that gave it a filamentous morphology; Peru-14 was motile when observed by light microscopy but did not penetrate soft agar in motility assays, so it was thought that Peru-14's low reactogenicity resulted from its filamentous morphology and not its motility defect *per se*. To further address this possibility, Peru-14's parental strain Peru-3 was screened for spontaneous mutations that rendered it nonmotile but left its cell morphology intact. One stable nonmotile mutant, Peru-15, was tested in initial volunteer studies and found to also be highly immunogenic and completely devoid of reactogenicity [88]. Peru-15 does not form a flagellum and as such represents the first aflagellar bacterial mutant to be evaluated in human volunteer studies as a live attenuated vaccine.

10 Peru-15: an Aflagellar, Nonreactogenic Cholera Vaccine

The initial positive results with Peru-15 prompted expanded study of its safety and immunogenicity in various buffers and in a lyophilized form. As expected, Peru-15 was least immunogenic in saline, which cannot neutralize stomach acid, but an oral dose of 1×10^8 bacteria in a buffer called CeraVacx induced seroconversion in all ten volunteers immunized [89]. A lyophilized version of Peru-15 was evaluated in a larger double-blind, placebo-controlled volunteer immunization/challenge trail by Cohen and colleagues [90]. In this study, 59 volunteers were randomly selected to receive either 2×10^8 colony-forming units (CFU) of lyophilized Peru-15 vaccine

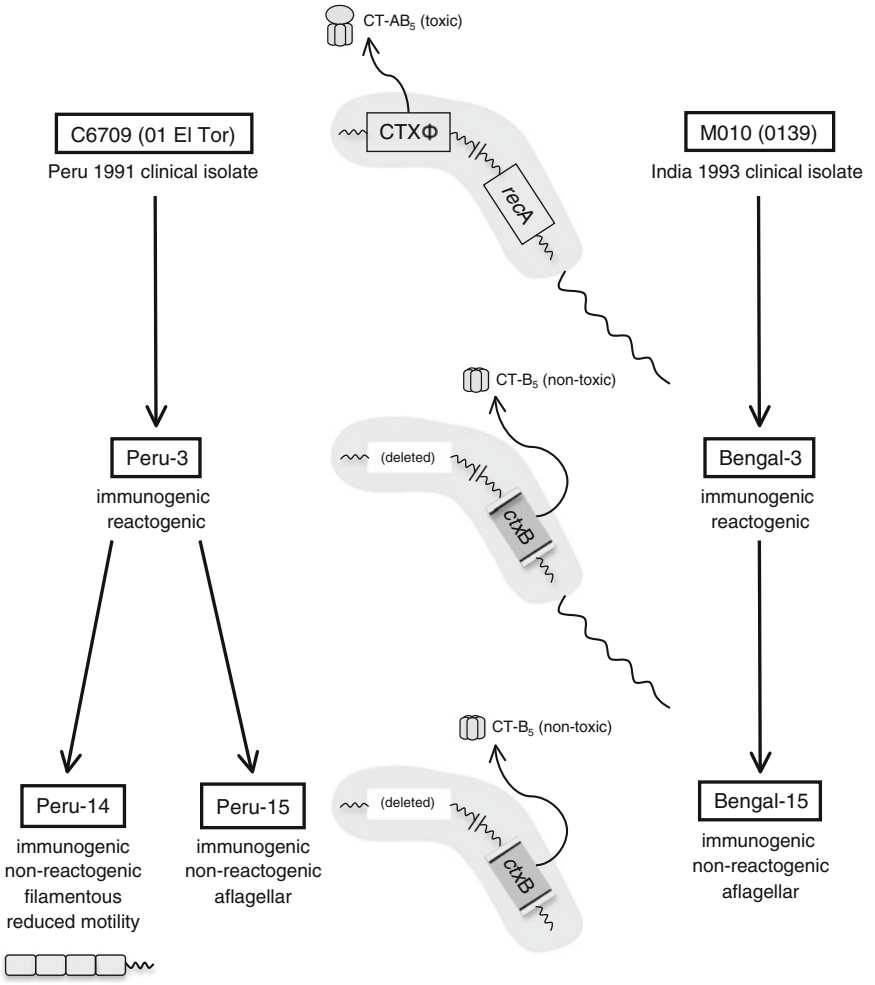

Fig. 1 Live cholera vaccine design. Peru-3 and Bengal-3 were created from *V. cholerae* clinical isolate strains C6709 and MO10 by deleting the CTXΦ genome and replacing *recA* with a *ctxB* expression construct. Peru-15 and Bengal-15 contain spontaneous mutations that produce aflagellar cells that retain immunogenicity but strongly reduce reactogenicity in vaccine recipients

reconstituted in CeraVacx buffer or a placebo containing only CeraVacx buffer. After unblinding, it was found that Peru-15 was well tolerated compared to buffer alone and impressively 98% of the Peru-15-immunized volunteers showed at least a fourfold rise in serum vibriocidal antibody. Three months after immunization, 36 volunteers were challenged with *V. cholerae* O1 El Tor strain N16961, a toxigenic clinical isolate from the current seventh pandemic. Remarkably, none of the 24 volunteers who received the vaccine developed even moderate diarrhea while 5 of the 12 placebo recipients (42%) developed either moderate or severe diarrhea.

11 Bengal-15 and More Evidence for the Link between Motility and Reactogenicity

In order to produce a live attenuated vaccine protective against O139 serogroup strains of *V. cholerae* and obtain more evidence for the role of flagellar motility in vaccine reactogenicity, another vaccine derivative called Bengal-15 was developed and tested in North American volunteers [91, 92]. Starting with the toxigenic O139 strain MO10, Bengal-15 was constructed to contain the same genetic "blueprint" as Peru-15 including its aflagellar phenotype (Fig. 1). Bengal-15 and its motile parental strain Bengal-3 were evaluated in a small volunteer study for their immunogenicity and reactogenicity after oral inoculation. At a dose of 1×10^8 bacteria, one of the four Bengal-3 recipients experienced diarrhea but none of the ten Bengal-15 recipients did, suggesting that the loss of flagella formation in Bengal-15 reduced its reactogenicity. Upon challenge with the toxigenic MO10 strain 1 month after vaccination, five of six patients who had received only the placebo vaccine developed severe diarrhea while only one of seven patients vaccinated with Bengal-15 developed mild diarrhea (protective efficacy 83%). It was concluded that the aflagellar strain Bengal-15 was a nonreactogenic vaccine candidate that was highly immunogenic and protective against cholera caused by O139 serogroup strains of *V. cholerae*.

12 Field Trials of Peru-15 in a Cholera-Endemic Region

Building on the impressive results obtained in North American volunteers immunized with the aflagellar Peru-15 strain, Qadri and colleagues sought to evaluate the vaccine in adults in Bangladesh, a region where cholera is endemic [93]. These studies were carried out by a team at the International Center for Diarrhea Disease Research, Bangladesh (ICDDR,B) in collaboration with the investigators at the International Vaccine Institute (IVI, Seoul, Korea), Avant Immunotherapeutics (now Celldex Therapeutics, Needham, Massachusetts), and Harvard Medical School, Boston, USA. In the double-blind, placebo-controlled study, no major adverse events were associated with the vaccine in adult volunteers, and despite the fact that Peru-15 was detected in stools of only one volunteer, 30 of the 40 vaccine recipients (75%) seroconverted for serum vibriocidal antibody and 35 (88%) displayed elevated levels of serum IgA antibodies directed against *V. cholerae* LPS O-antigen. Detectable immunogenicity against CTB, however, was lower in the Bangladeshi volunteers than had been previously observed in North Americans.

Demonstration of the safety and immunogenicity of Peru-15 in adults from endemic communities set the stage for further vaccine trials in Bangladeshi children in an age-descending study [94]. In a double-blind, randomized, placebo-controlled trial, 140 children aged 9 months to 5 years were given the vaccine, with a further 100 children receiving placebo. Two different doses were examined and children

were monitored for adverse reactions, excretion of the vaccine strain, and seroconversion by vibriocidal assay and IgA anti-LPS responses. Peru-15 was not associated with adverse events in this study and was isolated from only 8 of 140 recipients. However, 84% of toddlers (age 2–5 years old) and 70% of infants (9–23 months old) showed a serum vibriocidal response after receiving the higher dose of Peru-15 (2×10^8 CFU). Sixty percent of the vaccinated toddlers and 34% of the vaccinated infants also showed IgA responses against *V. cholerae* LPS-O antigen compared to 15% of toddlers and 12.5% of infants who received a placebo. Responses to CTB subunit were lower but also significant (46% of toddlers and 36% of infants). Thus, Peru-15 was clearly safe and immunogenical in Bangladeshi children when administered in a single dose. Further development of Peru-15, known commercially as CholeraGarde, awaits creation of a formulation suitable for large-scale manufacture followed by efficacy trials in a cholera-endemic setting [95].

13 Host-Innate Immunity and Flagellin Signaling

Development of the aflagellar vaccines Peru-15 and Bengal-15 in the mid 1990s actually predated the recognition that bacterial flagellins were key signaling molecules of the innate immune system and were capable of inducing proinflammatory responses. Early reports by Mizel and colleagues identified flagellin as a bacterial protein that could be recognized by high-affinity receptors on human monocytes in a process that mysteriously activated cytokine production [96, 97]. Eventually, Hayashi and colleagues reported that Toll-like receptor 5 (TLR5) recognizes a conserved amino acid sequence in the flagellin protein [98]. We now know that flagellin is one of many highly conserved components of bacterial cells such as lipopolysaccharide, peptidoglycan, lipoproteins, and DNA that contain pathogen-associated molecular patterns (PAMPs) that are recognized by host pattern recognition receptors (PRRs) [99]. Upon binding their cognate PAMP, PRRs like TLR5 then activate signal transduction pathways leading to proinflammatory cytokine production. In brief, TLR5 responds to flagellin binding by signaling through MyD88 and the serine/threonine kinase IRAK-4 to ultimately activate the mitogen-activated protein kinase p38 (p38 MAPK) and the transcription factor NF-κB, which go on to induce expression and secretion of the cytokines TNF-α, IL-8, and IL-1β (Fig. 2) [100]. These cytokines promote an influx of neutrophils and other inflammatory leukocytes and induce further cytokine expression and release by surrounding cells. IL-1β must be activated by caspase 1, found in an innate immune complex known as the inflammasome that can independently recognize flagellin [101]. Such inflammation in the gut could cause symptoms such as fever, cramps, and nausea that closely resemble the reactogenicity induced by flagellated live cholera vaccines.

TLR5 is expressed in mucosal tissues and can recognize flagellins produced by invasive pathogens [102] as well as extracellular organisms like *Pseudomonas aeruginosa* [103]. Intestinal epithelial cells (IECs) were originally thought to

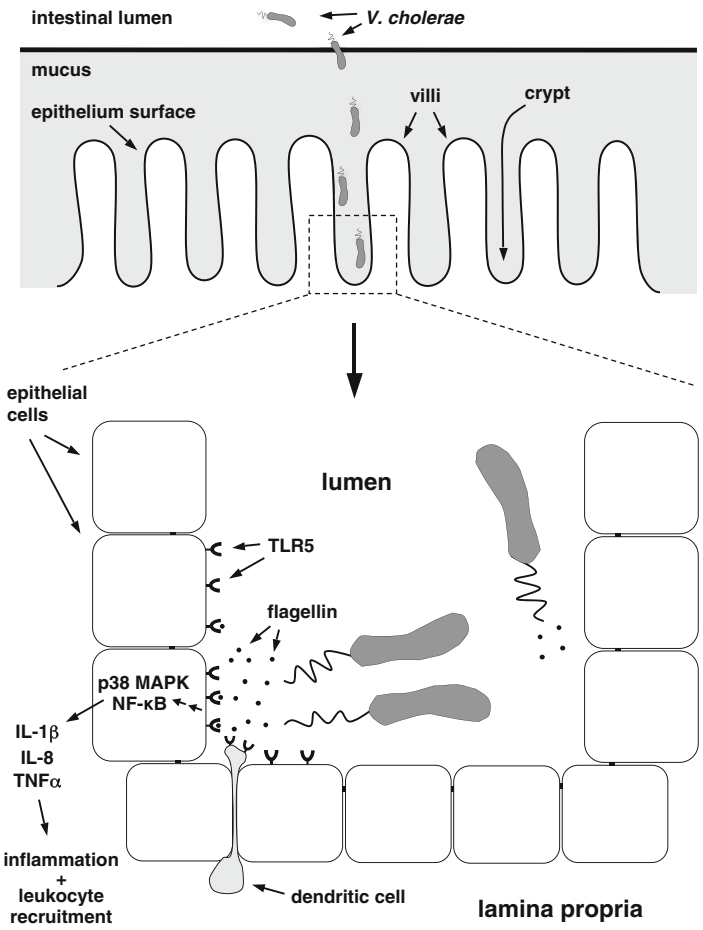

Fig. 2 *V. cholerae* flagellin-induced inflammation model. During infection, *V. cholerae* penetrates the small intestine mucus layer and colonizes the epithelium where membrane-bound TLR5 receptors of epithelial or dendritic cells recognize *V. cholerae* flagellin. TLR5-mediated innate immune signaling causes activation of NF-κB and p38 MAPK and subsequent expression and secretion of proinflammatory cytokines including IL-1β, IL-8, and TNF-α into the lamina propria

express TLR5 only on the basolateral membrane [104], but more recent work has shown that TLR5 is found on the apical surface as well and readily detects flagellin produced by noninvasive pathogens like *V. cholerae* [105]. To avoid detection by TLR5, some enteric pathogens such as *Campylobacter jejuni* and *Helicobacter pylori* have altered their flagellin peptide sequence [106]. *Salmonella*, on the other hand, may actually use flagellin to activate TLR5 signaling and induce inflammation as part of its infection process before downregulating flagellin expression after gaining intracellular access to intestinal enterocytes [107, 108]. *V. cholerae* produces a membranous sheath that covers the length of its flagellum and helps to

hide its flagellin components from TLR5 recognition [109]. Nevertheless, all five of the flagellins that *V. cholerae* produces are abundantly found in culture supernatants and all five are recognized by TLR5 [110].

14 Motility and Reactogenicity: The Infant Rabbit Model

The Peru-15 and Bengal-15 vaccine trials detailed above provided strong correlative evidence that flagellar motility plays an important role in live cholera vaccine reactogenicity, but the precise cause of these patient symptoms remained undefined because of the pleiotropic role that flagellar motility plays in *V. cholerae* pathogenesis. Flagellar motility is required for *V. cholerae* to efficiently colonize the small intestine in the infant mouse model of infection [111], and flagellar motility is thought to be necessary for the bacterium to traverse the thick mucus layer protecting the intestinal epithelium [23]. At a genetic level, the flagellar biosynthesis regulon in *V. cholerae* directly regulates the quorum sensing and virulence cascades *in vitro* [24, 112] and may serve as a mechanical sensor to induce virulence gene expression in response to mucus-induced flagellar shearing. Finally, the flagella itself could be the principle cause of reactogenicity since TLR5 recognition of its flagellin components may cause a local inflammatory response at the intestine epithelial surface.

As a result, the aflagellar vaccine strains may not induce reactogenic diarrhea because it mislocalizes in the intestine, is unable to initiate virulence gene expression, or fails to induce an inflammatory innate immune response. Finally, it is important to point out that Peru-15 and Bengal-15 were isolated in screens for spontaneous nonmotile mutants and may contain secondary mutations, so it is formally possible that the reduced reactogenicity of these strains is independent of their aflagellar morphology.

All these theories provide clear testable hypotheses, but until recently there was no adequate animal model of diarrheal disease for cholera. The infant mouse has long served as an accurate model for *V. cholerae* intestinal colonization and virulence gene induction, but the lack of a robust diarrheal phenotype negates its use in any comprehensive examination of diarrheal disease [113]. Rabbit models of cholera infection, including the ligated ileal loop and removable intestinal tie adult rabbit diarrhea (RITARD) models, have been developed to measure diarrheal disease following *V. cholerae* inoculation [114, 115], but these models rely on surgical intervention to block peristaltic flow through the intestine during *V. cholerae* infection, a severe nonphysiologic condition that limits their use in modeling natural cholera disease in people.

To address many unanswered questions about cholera pathophysiology, Richie et al. [116] have recently developed an infant rabbit model of cholera infection that closely resembles the diarrheal disease seen in human victims. When infant rabbits are pretreated with cimetidine to inhibit acid production in the stomach, orogastric inoculation of *V. cholerae* causes a voluminous watery diarrhea that resembles

cholera disease in people, and a large majority of the infected rabbits die 24–30 h after infection. Importantly, deletion of the *ctxAB* toxin genes in this strain strongly reduces the occurrence of watery diarrhea and enables the rabbits to survive infection, but most of the animals go on to exhibit a self-limiting noncholeric fecal diarrhea that resembles the reactogenic diarrhea observed in people inoculated with motile live cholera vaccines.

To directly determine the cause of reduced reactogenicity in the Peru-15 vaccine strain, Rui et al. [117] introduced defined mutations into flagellar genes of an O1 El Tor strain of *V. cholerae* that is deleted for *ctxAB* and is closely related to the parental strain of Peru-15. To separate any reactogenic phenotype associated with flagellar biosynthesis from that associated with swimming motility, they measured reactogenicity in rabbits infected with either a flagellar motor mutant that is flagellated but nonmotile or a flagellar filament mutant that lacks all five flagellin genes and is both aflagellate and nonmotile. The flagellated nonmotile mutant caused reactogenicity at nearly the same level as the parental strain but the aflagellar mutant caused very little reactogenicity in the animals. Disruption of all five flagellins was necessary to achieve the lowest reactogenicity levels, suggesting that all five of the flagellins are able to induce reactogenic diarrhea.

An intriguing observation in these rabbit studies was that the aflagellar mutant was able to penetrate the intestinal mucus layer and travel into the deep intestinal crypts, an area thought to be inaccessible to nonmotile strains [21, 23, 118]. The fact that motile and nonmotile *V. cholerae* strains show the same intestinal localization in these experiments may help to explain why the nonmotile Peru-15 strain is able to induce the same immunogenicity in vaccine recipients as its motile Peru-3 parent strain [88, 94], and it lends further weight to the recent suggestion that *V. cholerae* uses flagellar-independent motility to travel through the intestinal mucus layer [24]. In tandem with data showing that *V. cholerae* flagellins directly activate TLR5, these infant rabbit experiments strongly suggest that the reduced reactogenicity seen in Peru-15 is specifically due to reduced flagellin production.

15 Perspective on other Live Attenuated Vaccines vis-à-vis Flagellins and Reactogenicity

In conclusion, studies of flagellar defective *V. cholerae* vaccine candidates in volunteer subjects and new animal models for reactogenicity have now provided strong evidence that flagellin is a significant reactogenic factor elaborated by the organism. *V. cholerae* produces other potentially reactogenic factors including the MARTX toxin [119] and effectors of the newly described type VI secretion system [120], but in the context of human infection with CTXΦ-deleted vaccine candidates, it seems likely that flagellin production is the most important proinflammatory signal and is directly responsible for the reactogenicity seen in vaccine recipients. It is worth noting that other live attenuated bacterial vaccine candidates have suffered from the problem of reactogenicity in human subjects, and one might

speculate that these organisms produce flagellin *in vivo* that might elicit reactogenicity as well. For example, live attenuated *Shigella* vaccines are often reactogenic in volunteer studies [121, 122], and although *Shigella* are notoriously nonmotile, some strains have been reported to contain intact flagellar operons and to produce flagella [123]. Even the flagellin produced by commensal organisms such as *Escherichia coli* may be involved in pathology associated with inflammatory disease. In human disease, flagellin has been implicated as an elicitor in inflammatory bowel diseases such as Crohn's [124, 125] and mutations in TLR5 are associated with enhanced susceptibility to *Legionella* disease [126].

Clearly, recognition of flagellin by the innate immune system is an early host response that may affect the outcome of infection through induction of locally protective inflammatory responses. However, in the context of a live bacterial vaccine such a local immune response may cause adverse symptoms and could even block the development of long-term immunity in the vaccine recipient by controlling the infection before it can induce a strong adaptive immune response. Continued exploration of flagellin as a reactogenic factor in natural infection and experimental immunization seems warranted.

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Part IV
New Types of Replicating Vaccines

Replication-Defective Herpes Simplex Virus Mutant Strains as Genital Herpes Vaccines and Vaccine Vectors

David M. Knipe

Abstract Viral vaccines have traditionally been live, attenuated viruses, or inactivated virus/subunits. Herpes simplex virus (HSV) vaccine candidates based on inactivated viruses or subunits have not been effective thus far. In addition, attenuation of HSV to make a safe vaccine candidate has not allowed good immunogenicity to be retained. Therefore, novel vaccine strategies have been initiated, including replication-defective and single-cycle HSV strains. In this chapter, I will review the design and properties of these replication-defective virus vaccine candidates and the preclinical and clinical results that have been obtained using them.

1 Introduction

The herpes simplex viruses cause significant morbidity and mortality, including encephalitis, neonatal herpes, and keratitis [1]. Despite the existence of some good antiviral drugs, there is enormous need for a herpes vaccine, in particular for a genital herpes vaccine. Herpes simplex virus 1 (HSV-1) causes orofacial lesions including the common cold sores or fever blisters, but causes more serious encephalitis and keratitis in a limited number of cases. Herpes simplex virus 2 (HSV-2) causes most of the genital herpes infections but becomes life-threatening in neonates and immunocompromised individuals. Importantly, in terms of global public health, HSV-2 infection increases the susceptibility to human immunodeficiency virus (HIV) by 3–4-fold [2, 3]. This effect is possibly exerted through the herpetic lesions providing breaks in the epithelial mucosa that allow HIV to enter the epithelium and which also contain elevated numbers of $CD4^+$ T lymphocytes, the host cell for HIV, and dendritic cells, which transport HIV to lymph nodes where it

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can infect $CD4^+$ T-lymphocytes. Herpes suppressive drug therapy of individuals doubly infected with HSV-2 and HIV reduces viral loads of HIV [4, 5]. However, herpes drug treatment of HSV-2 seropositive individuals did not reduce the risk for HIV infection [6, 7]. Therefore, novel strategies, such as a genital herpes vaccine, are needed to reduce HSV-2 infection and thereby reduce the risk of HIV infection. For these reasons, an effective genital herpes vaccine would be a major advance in global public health.

2 History of HSV Vaccines

Traditionally, viral vaccines were either live, attenuated viruses, or inactivated viruses. Newer forms of inactivated viral vaccines consist of viral protein subunits. There are several examples of successful vaccines of each type. Live, attenuated vaccines have included the smallpox vaccine, the yellow fever vaccine, the Sabin polio vaccine, and the measles vaccine. Killed viral vaccines have included the Salk polio vaccine. Viral protein vaccines have included hepatitis B vaccine and human papilloma virus vaccine.

Several of the early herpes vaccines were tested in trials that were not placebo-controlled, so their efficacy could not be determined (reviewed in [8]). An attenuated HSV recombinant virus, R7020, constructed by Bernard Roizman's laboratory was safe in phase I trials but was not immunogenic [9]. A replication-competent HSV-2 strain constructed in Aurelian's laboratory [10, 11] was tested in a phase I therapeutic clinical trial in Mexico and reported to reduce recurrences [12]. The safety profile of this replication-competent HSV-2 strain has not been described, but it is capable of establishing latent infection albeit at a reduced level [11].

Biovex Inc. has a "novel replication-competent HSV-2 virus," based on inactivation of viral immune response genes, which it is testing as a prophylactic genital herpes vaccine (http://www.biovex.com/immunovex.html), but the precise genotype and properties of this virus have not been reported in the scientific literature. Their ImmunoVEX^{HSV-2} vaccine product was approved for a phase I clinical trial in the United Kingdom.

Recently, Friedman's group has used an HSV-1 glycoprotein E (gE)-null mutant virus to immunize mice, and they observed protection against flank challenge infection by HSV-1 [13]. This approach is discussed in another chapter of this book.

Thus far, it has been difficult to attenuate HSV to make it safe enough to give as a prophylactic vaccine. Therefore, subunit vaccines have been tested as herpes vaccines. The Chiron HSV-2 glycoprotein B and D (gB-2 and gD-2) subunit vaccine in MF-59 muramyl dipeptide adjuvant provided no clinical protection in prophylactic [14] and therapeutic trials [15, 16]. The GlaxoSmithKline gD-2 subunit vaccine in alum and monophosphoryl lipid A (Herpevac vaccine) showed no efficacy in men or in women who were HSV-1 seropositive but showed partial

protection against HSV-2 infection in women who were seronegative for HSV-1 and HSV-2.

Thus, subunit vaccines have not provided broad protection against HSV-2 infection. As a result, novel vaccine strategies including DNA vaccines, peptide vaccines, replication-defective mutant viral strains, and single-cycle mutant viral strains have been tested as potential HSV-2 vaccines (reviewed in [18]). In this chapter, we will consider the replication-impaired viruses as herpes vaccines and vaccine vectors.

3 Replication-Impaired HSV Mutants

Two types of replication-impaired HSV mutant viral strains have been tested as HSV vaccines, replication-defective mutant strains, and single-cycle mutant strains. HSV-1 replication-defective mutant viruses were among the first replication-impaired viruses used as vaccines [19]. An HSV-2 replication-defective mutant induced protection against genital HSV-2 infection in guinea pigs [20]. These replication-defective mutants can infect cells and express immediate-early and early viral gene products and even many late gene products but contain defects in viral DNA replication, so the replication cycle is absolutely blocked. Viral late gene expression is observed with mutant viruses defective for ICP8 even though there is no viral DNA replication, likely because ICP8 and/or a complex of viral DNA replication proteins exerts an inhibitory effect on viral late gene expression in the absence of viral DNA synthesis [21].

To generate a safe, potential vaccine strain for clinical use, we deleted two essential HSV-2 genes, U_L5 and U_L29, from the HSV-2 186 strain virus to generate the *dl*5-29 vaccine candidate virus [22, 23]. The U_L5 gene encodes one of the subunits of the viral helicase–primase complex, while U_L29 encodes ICP8, the viral single-stranded DNA-binding protein. Both are essential for viral DNA synthesis and viral growth. Two deletions separated by a large distance on the viral genome, both unable to recombine with the genes in the complementing cell line, were engineered into the vaccine strain to reduce the likelihood that the vaccine virus could recombine with HSV in the immunized individual to generate a replication-competent virus. Recombination of the HSV-2 mutant virus with wild HSV-2 would generate a replication-competent HSV-2 strain, likely no different from the virus with which the individual was already infected. Therefore, only recombination of the HSV-2 mutant with a wild HSV-1 would generate a new infection with a new replication-competent HSV-2-like virus. The *dl*5-29 mutant virus also has a latency defect in that the small amount of viral DNA that reaches sensory ganglia are not maintained stably [22].

Immunization of mice with *dl*5-29 virus protects them against genital challenge with virulent HSV-2 in that virus shedding from the genital tract was reduced, lesions were reduced, and lethal encephalitis was prevented [22]. Studies in guinea pigs have compared *dl*5-29 immunization with gD-2 protein and plasmid pgD-2

immunization. Prophylactic immunization of guinea pigs with *dl*5-29 virus reduced virus shedding and disease during primary infection and latent viral load similarly to immunization with gD2 in complete Freund's adjuvant and to a greater extent than plasmid immunization [24]. Furthermore, therapeutic immunization of guinea pigs with *dl*5-29 virus was effective in reducing recurrent infection [24]. Surprisingly, immunization with *dl*5-29 induced higher neutralizing antibody titers than immunization with gD-2, a major target for neutralizing antibodies. Immunization with *dl*5-29 virus induced anamnestic T cell responses that were recruited rapidly to sites of viral infection. These studies demonstrated that *dl*5-29 virus induces strong humoral and cellular responses and that it is superior to previously tested vaccine candidates.

The *dl*5-29 virus was licensed by Acambis (now Sanofi Pasteur Biologicals) and is undergoing preclinical studies as a vaccine candidate known as ACAM-529.

4 Durability

One concern about a replication-defective HSV strain that does not establish latent infection is that the immune responses may not be durable. We have observed that the protective immunity induced by HSV-1 *d*301 U_L29 gene mutant persisted for at least 7 months [25]. Similarly, immunization with HSV-2 *dl*5-29 virus induced protective immunity that persisted for at least 7 months in mice [26]. Therefore, immunity induced by replication-defective mutant viruses lasts for a significant portion of a Balb/c mouse's lifetime [27]. Durable immune responses in the mouse may involve (1) persistence of viral genomes that can express viral antigens in various cells and/or (2) trapping of antigen with complement on complement receptors on bone marrow-derived cells in local lymph nodes [28]. In terms of the mechanisms of protection, Morrison [29] has found that $CD4^+$ T cells are required but $CD8^+$ T cells are not essential for protective immunity induced by replication-defective HSV-2 viruses. Therefore, *dl*5-29 induces durable T-cell responses in the murine system.

5 Safety

A prophylactic genital herpes vaccine would ideally be used to immunize children before they became sexually active; therefore, safety is of the utmost importance for this application. Replication-deficient strains are ideal for this because the virus cannot spread significantly beyond the injection site. In mice injected intracerebrally with virus, *dl*5-29 was at least 250,000-fold less virulent than wildtype HSV-2, and *dl*5-29 did not cause any disease in the immunodeficient SCID mice [30]. Therefore, *dl*5-29 has a very desirable safety profile from these preclinical studies.

6 Pre-Existing Immunity

Because the GSK gD2 vaccine in alum and MF59 was not effective in HSV-1 seropositive women, it was conceivable that other vaccines might not be effective in HSV-1 seropositive individuals. To investigate this question, Hoshino et al. [31] tested *dl*5-29 and gD-2 in guinea pigs that were HSV-1-seronegative or -seropositive. In HSV 1-seronegative animals, *dl*5–29 induced the highest titers of neutralizing antibody, and after vaginal challenge with wild-type virus, *dl*5–29 resulted in lower rates of vaginal shedding, lower levels of latent viral DNA in sensory ganglia, and less acute and recurrent genital herpes, compared with the gD2 vaccines. In HSV-1-seropositive animals, both vaccines induced similar titers of neutralizing antibodies and showed similar levels of protection against acute and recurrent genital herpes after vaginal challenge with wild-type virus, but *dl*5–29 reduced vaginal shedding after challenge more than did the gD2 vaccine. Therefore, *dl*5-29 appeared to be efficacious in HSV-1-seropositive guinea pigs.

7 Route of Immunization

The level and type of immunity induced by replication-defective virus immunization depends on the route of delivery. Subcutaneous immunization with HSV-1 induced protective immunity against HSV-1 ocular challenge in mice [25]. Similarly, subcutaneous immunization with HSV-2 5BlacZ U_L29 mutant virus induced protective immunity against HSV-2 genital challenge in mice [32]. Immunization of mice by the intranasal route with HSV-2 5BlacZ also induced protective immunity, but the best protection was observed with combined intranasal and subcutaneous immunization [32]. Intravaginal immunization of guinea pigs with *dl*5-29 was only partially protective against later vaginal challenge [24].

8 Cross Protection Against HSV-1

There is growing epidemiological evidence suggesting an increase in the incidence of genital herpes caused by HSV-1 [33, 34]; therefore, a herpes vaccine should ideally protect against both HSV-1 and HSV-2. Van Lint et al. [35] tested the ability of HSV-2 *dl*5-29 to protect against ocular infection with HSV-1 and observed that *dl*5-29 immunization induced protective immunity that reduced viral shedding from the cornea, ocular disease and reduced latent infection by the challenge virus. Further studies are needed to determine if *dl*5-29 can protect against genital challenge by HSV-1, but these results support the idea that HSV-2 *dl*5-29 might be broadly effective against HSV-1 infection.

9 Single-Cycle Mutant Viral Vaccine

HSV-1 and HSV-2 glycoprotein H mutant viruses were tested as a single-cycle mutant virus vaccine. HSV gH mutant viruses complete the replication cycle in normal cells and form progeny virus particles, but because they lack gH, the progeny virus particles are noninfectious or at least have reduced infectivity [36]. The gH mutant viruses are grown in a complementing cell line that expresses gH, and immunization with these pseudotyped viruses gives immune protection against HSV-1 challenge in the ear pinna [37] and genital HSV-2 challenge in mice and guinea pigs [38]. These viruses may not be completely replication-defective in vivo because latent infection by an HSV-1 gH mutant virus, as detected by latency-associated transcript expression in sensory neurons, was observed [39]. These viruses were named defective infectious single-cycle (DISC) viruses by Cantab Pharmaceuticals. An HSV-2 gH mutant virus was tested in clinical trials by Cantab and was safe but showed no clinical or virological benefit in a therapeutic phase II trial against genital HSV-2 disease [40]. On the basis of available information, the development of the DISC vaccines by Xenova, which acquired Cantab, has been stopped.

10 Future Improvements

10.1 Immune Evasion

HSV encodes a number of gene products that act to reduce the host immune response. These include: (1) ICP47, which binds to TAP and prevents peptide transport into the lumen of the ER for loading on MHC class I molecules [41]; (2) virion host shutoff (vhs), a virion tegument protein that, upon entry into the cytoplasm, becomes a ribonuclease that digests host mRNA and causes shutoff of host protein synthesis [42, 43]; (3) ICP34.5, which activates a phosphatase to reverse phosphorylation of eIF2α by PKR [44]; (4) ICP0, which blocks IRF-3 [45] and Toll-like receptor 2 signaling [68]; and (5) U_S3, which blocks interferon γ responses [46].

Inactivation of the viral genes that inhibit host immune responses could lead to better immune responses to a vaccine strain if the mutation does not reduce viral gene expression. As described above, BioVex Inc. has engineered an HSV-2 strain with mutations in a number of viral immune evasion genes, but the complete description of the viral strain is not available. HSV ICP47 is an obvious gene to target to increase immunogenicity; however, ICP47 affects TAP function only in higher mammals, and there is not a good nonhuman primate model for HSV because rhesus monkey cells are poor host cells for HSV [47] and other nonhuman primates are less available.

The effect of vhs inactivation has been tested in several HSV strains. Inactivation of the *vhs*/U_L41 gene has been shown to increase the immunogenicity of

replication-competent [48] and replication-defective HSV-1 viruses [49]. Inactivation of the *vhs* gene in *dl*5-29 virus generated the *dl*5-29-41L virus, which was more immunogenic and induced greater protection than *dl*5-29 virus in some but not all situations [26, 30]. Replacement of the HSV-2 *vhs* gene with the HSV-1 *vhs* gene, which encodes a less active form of vhs, to give the *dl*5-29-41.1 virus, gave a virus that replicated better than *dl*5-29, expressed slightly lower levels of viral proteins, and induced immune responses and protective immunity that approximated those induced by *dl*5-29 [50]. These results argue that the HSV-1 vhs protein enhances growth of HSV-2 but that the RNase activity may have to be inactivated to give optimal immunogenicity. In total, these studies show the potential of increasing the immunogenicity and/or replicative ability of *dl*5-29 by the incorporation of additional genetic changes in the viral genome.

11 Coexpression of Immune Stimulatory Molecules

An additional potential approach for enhancement of immune responses induced by the replication-defective mutant strains is coexpression of immune stimulatory molecules by the virus. To test this hypothesis, Vagvala et al. [51] constructed a U_L29^- mutant that expressed the B7-2 costimulatory molecule and found that B7-2 expressed by the recombinant virus enhanced its immunogenicity and protective immunity. Therefore, this general approach represents a very promising way to improve the replication-defective virus vaccine candidates.

12 Genetic Diversity of HSV-2 Strains

One of the remaining questions about HSV-2 is the extent to which genetic diversity exists in strains around the world. Certain geographical areas, such as Sub-Saharan Africa, show very high HSV-2 seroprevalence, greater than 40% among antenatal attendees in one clinic in Africa, [52] and as high as 60–95% among female sex workers in Sub-Saharan Africa [53, 54]. Therefore, it is important that a potential genital herpes strain be efficacious against HSV-2 strains found in Sub-Saharan Africa. While little is known about the genetic diversity of HSV-2, one paper has reported that phylogenetic analysis of three genes, U_S4 (encoding glycoprotein G or gG), U_S7 (gI), and U_S8 (gE), of the genomes of 47 HSV-2 isolates from Tanzania, Norway and Sweden show at least two genogroups with genogroup A and B being represented in the Tanzanian isolates and group B in the Scandanavian isolates [55]. Because there is more genetic diversity in the Tanzanian HSV-2 isolates, we reasoned that the Sub-Saharan isolates might also diverge antigenically. We tested the ability of HSV-2 *dl*5-29 mutant virus, which is based on a US isolate, to induce protective immunity against the US isolate, HSV-2 G, or a South African isolate SD-90 (Dudek and Knipe, personal communication). We observed

that *dl*5-29 did induce protective immunity against both viruses but that higher doses of *dl*5-29 virus were needed to protect against SD-90 infection as compared to that needed for protection against G virus infection. Similarly, we observed that a United States U_L29^- virus protected better against three US HSV-2 challenge strains than against three South African HSV-2 challenge strains, and vice versa, a South African U_L29^- virus protected better against South African HSV-2 challenge strains than against the three US HSV-2 challenge strains. Further studies are needed, but these results suggest that optimal protection in South Africa may require a replication-defective virus based on the genomic backbone of an HSV-2 isolate from that region.

13 HSV as a Vaccine Vector

HSV replication-defective mutant virus strains can also serve as recombinant vaccine vectors. There are several advantages to HSV as a vaccine vector. First, HSV infects a wide variety of human cell types, likely because it has multiple virion glycoproteins that facilitate entry through any of several receptor molecules [1]. Second, the HSV genome can be expanded by at least 15 kbp [56], and with deletion of nonessential viral genes, the size of the inserted sequences could be increased further. Third, HSV activates innate responses so an adjuvant is not needed. Fourth, HSV induces Th1 helper T cell responses against antigens expressed by the vector [57]. Fifth, as discussed above, replication-defective HSV strains can be used to immunize systemically or mucosally.

Replication-defective and replication-competent HSV-1 strains expressing simian immunodeficiency virus (SIV) envelope (env) and nef were constructed by insertion of the SIV gene expression cassette into the viral thymidine kinase gene [58]. The HSV recombinants induced antienvelope antibody responses that persisted at relatively stable levels for months after the last administration. Two of seven rhesus monkeys vaccinated with recombinant HSV were solidly protected, and another showed a sustained reduction in viral load following rectal challenge with pathogenic SIVmac239 at 22 weeks following the last vaccine administration. These results provided proof-of-concept for the use of HSV recombinants as AIDS vaccine vectors.

To improve the HSV replication-defective vector, we evaluated the properties of a second-generation HSV vaccine vector, an HSV-1 multiple immediate-early (IE) gene deletion mutant virus, *d*106, which contains deletions in the *ICP4*, *ICP27*, *ICP22*, and *ICP47* genes [59]. Because several of the HSV IE genes have been implicated in immune evasion, we hypothesized that inactivation of the genes encoding these proteins would result in enhanced immunogenicity. The *d*106 virus expresses few HSV gene products and shows minimal cytopathic effect in cultured cells. When we inoculated *d*106 virus into mice, we observed that viral DNA accumulated at high levels in draining lymph nodes, consistent with an ability to transduce dendritic cells and activate their maturation and movement to lymph

nodes. A *d*106 recombinant expressing *E. coli* β-galactosidase induced durable β-gal-specific IgG and $CD8^+$ T cell responses in naive and HSV-immune mice. Finally, *d*106-based recombinants were constructed that express simian immunodeficiency virus (SIV) gag, env, or a rev–tat–nef fusion protein for several days in cultured cells. Thus, *d*106 shows many of the properties desirable in a vaccine vector: limited expression of HSV gene products and cytopathogenicity, high level expression of transgenes, ability to induce durable immune responses, and an ability to transduce dendritic cells and induce their maturation and migration to lymph nodes.

The *d*106 vectors expressing SIV proteins were used to immunize rhesus macaques [60]. Three macaques were inoculated with recombinant HSV vectors expressing Gag, Env, and a Tat–Rev–Nef fusion protein of simian immunodeficiency virus (SIV). Three other macaques were primed with recombinant DNA vectors expressing Gag, Env, and a Pol–Tat–Nef–Vif fusion protein prior to boosting with the HSV vectors. Robust anti-Gag and anti-Env cellular responses were detected in all six macaques. Following intravenous challenge with wildtype, cloned SIVmac239, peak and 12-week plasma viremia levels were significantly lower in vaccinated compared to control macaques. Plasma SIV RNA in vaccinated macaques was inversely correlated with anti-Rev ELISPOT responses on the day of challenge (P value < 0.05), anti-Tat ELISPOT responses at 2 weeks post challenge (P value < 0.05) and peak neutralizing antibody titers prechallenge (P value 0.06). Therefore, the *d*106 vectors were capable of inducing efficacious humoral and cellular immune responses specific for SIV proteins.

HSV-1 *d*106 recombinants expressing HIV clade A env, clade C env, HIV clade B gag, and HIV have been constructed ([61]; Sen et al. personal communication). The recombinant vector expressing clade A env is genetically stable for up to ten passages in cell culture, gives burst sizes of 200–250 PFU/cell, and shows a specific infectivity of less than 100 particles/PFU (Sen and Knipe, personal communication). Therefore, the *d*106 vectors are genetically stable, grow well for production purposes, and show high infectivity. The *d*106 vectors show low infectivity and/or expression of the transgene in rhesus fibroblasts relative to human cells; therefore, the tests of the HSV-1 vectors in rhesus macaques may underestimate the immunogenicity of these viral vectors. Clinical tests of these recombinant vectors in humans are highly justified based on these preclinical results.

14 HSV Amplicons as Vaccine Vectors

HSV amplicons have also been tested as vaccine vectors. HSV amplicons are replication-defective viruses that are deleted for all genomic sequences except for an origin of DNA replication and DNA packaging sequences [62]. The viral proteins needed for replication of the genome and for assembly of the virion are provided by a helper virus [62] or by a set of five cosmids that contain an entire

genome of HSV-1 deleted for DNA packaging signals [63]. An HSV-1 amplicon expressing HIV-1 gp120 induced durable cellular and humoral responses in mice [64]. An HSV-1 amplicon that expressed HIV-1 env elicited polyfunctional T cell responses specific for env and strongly boosted responses to an adenovirus vector primed mice [65]. However, production of these vectors has been a challenge, and expression of the transgene seems to be limited, possibly due to the absence of expression of the HSV IE ICP0 protein, which prevents host chromatin silencing of the viral genome [66, 67]. Immunogenicity and protection studies in nonhuman primates have not been reported for the amplicon vectors.

15 Perspectives

Replication-defective viruses have been very promising as genital herpes vaccines and vaccine vectors in animal model systems. Their real potential needs to be tested in clinical trials in that the animal model systems may overestimate or underestimate the immunogenicity of these vaccine candidates. Testing of these vaccine candidates in humans is a high priority, which would allow an assessment of their efficacy in humans followed by the construction and testing of further generations of recombinant viruses that are designed with the types of improvements described in this article.

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Nucleic Acid-Based Infectious and Pseudo-Infectious Flavivirus Vaccines

Justin A. Roby, Roy A. Hall, and Alexander A. Khromykh

Abstract The genus *Flavivirus* contains a number of important pathogens of humans including yellow fever virus (YFV), dengue virus (DENV), Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), and West Nile virus (WNV). Despite causing significant morbidity and mortality worldwide, commercially available vaccines only exist for YFV (live-attenuated), TBEV, and JEV (inactivated). Flavivirus vaccine research has been driven by the need for cheap, safe, thermally stable, and efficacious preparations amenable to use in developing nations. The creation of infectious cDNA clones of various flaviviruses has led to the development of genetically engineered, nucleic acid-delivered, attenuated live vaccine candidates. These provide effective immunity from a single immunisation, however share the same safety concerns as traditional live-attenuated vaccines. The generation of large internal deletions in the capsid gene of flavivirus genomes creates a vaccine that secretes large amounts of immunogenic prM/E particles from self-replicating RNA but does not form a spreading infection. Packaging of these capsid-deleted RNAs into virus-like particles (VLPs) using a cell line that produces capsid gene from another expression vector creates a pseudo-infectious vaccine that elicits a highly efficient immune response from a single dose and is safer than infectious virus. However, production of these VLPs is cumbersome and the resulting product is heat labile. Providing the capsid gene in trans from another promoter but within the same plasmid DNA as the capsid-deleted viral genome creates a DNA vaccine capable of producing VLPs *in vivo*. Uptake of this plasmid DNA results in the generation of self-replicating, capsid-deleted RNA and the capsid protein in the same cell, leading to production of secreted single-round infectious particles (SRIPs). These SRIPs then deliver capsid-deleted RNA to adjacent cells where it replicates to produce more prM/E particles. As functional

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capsid cannot be produced in SRIP-infected cells, further spread does not occur. SRIP-producing DNA was shown to be highly effective in mice and horses and provides an easier to manufacture and thermally stable alternative to other vaccine candidates currently being developed.

1 Introduction

The genus *Flavivirus* is composed of a number of arthropod-borne disease agents within the Flaviviridae family of positive-strand RNA viruses [1]. The viral genome encodes a single polyprotein that undergoes post-translational cleavage to form three structural proteins; the capsid (C), precursor membrane/membrane protein (prM/M), and envelope protein (E), as well as seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [1].

The most notable human pathogens among the flaviviruses are the mosquito-borne yellow fever virus (YFV), Japanese encephalitis virus (JEV), the four serotypes of dengue virus (DENV), and West Nile virus (WNV), as well as tick-borne encephalitis virus (TBEV) [1, 2]. All of these viruses cause significant morbidity and mortality within their areas of endemicity. Substantial increases in the freedom of trade and travel in the post-World War II era have given these viruses an unprecedented potential to expand their natural geographical range. Perhaps the most demonstrative example of this is the introduction of WNV into New York City in 1999 and the subsequent rapid invasion of the virus into most areas of the United States, parts of Canada, Central America, the West Indies, and substantial areas of South America [3–7].

There are no specific therapies for the diseases of humans or livestock caused by these flaviviruses, thus the development and application of safe and efficacious vaccines is of paramount importance for the management of outbreaks. To date the only commercially licensed vaccines against flavivirus diseases in humans are the live-attenuated 17D YFV vaccine developed in 1936 [8–10], formalin-inactivated whole-virus vaccines against TBEV [11, 12] and JEV (both mouse brain- and cell culture-derived) [13–16], and a live-attenuated SA14-14-2 strain JEV vaccine produced in primary hamster kidney cells in China [17, 18]. These live-attenuated and inactivated whole-virus vaccines have been highly successful in providing effective prophylaxis for communities at risk. However, there are specific challenges that must be overcome in the light of stringent demands on the safety, efficacy, and manufacturing cost of such approaches [19].

Despite their success, recent concerns have been raised in the medical community in regards to adverse reactions observed after administering the currently licensed 17D YFV and mouse brain-derived JEV vaccines. Severe and often fatal yellow fever vaccine-associated neurotropic disease and yellow fever vaccine-associated viscerotropic disease have been estimated to affect 1.3–16 individuals per million vaccinated and 2.13–2.5 individuals per million vaccinated, respectively [8, 20, 21]. The inactivated, mouse brain-derived JEV vaccine has also been

associated with allergic reactions and neurological adverse events (estimated to affect 0.1–1% and 1–2 patients per million vaccinated, respectively) [8, 22–24]. Although these instances of adverse reactions are relatively low, vaccine technology has drastically improved since the development of the currently used vaccines. Modern incarnations of flavivirus vaccines should thus strive to abrogate these negative outcomes.

When administered in compliance with the recommended dosage regimen, contemporary flavivirus vaccine preparations are characterised as having high levels of seroconversion and rates of protection in vaccinees [10]. Any innovations in vaccine research need to demonstrate comparable efficacy. Live-attenuated vaccines hold an advantage in this regard as inactivated and subunit vaccines require booster immunisations to generate sufficient immune response to be protective.

Flavivirus-mediated diseases primarily affect developing nations with comparatively poor healthcare systems and limited budgets in regards to generating and maintaining vaccine stockpiles [2]. Passages of live-attenuated virus in chicken eggs are relatively expensive compared to cell culture, and both live and inactivated viruses require low temperature storage to maintain vaccine integrity. Constructing a cheap and stable alternative to the current vaccine strategies remains an important research goal.

Recently, a range of novel flavivirus vaccine candidates exploiting nucleic acid technologies that overcome the obstacles of cost and safety (Table 1) have been developed. The establishment of infectious cDNA clones has allowed the delivery of live-attenuated virus to the vaccinee as highly stable nucleic acids that are relatively cheap to produce *en masse*. Further manipulation of this technology has engendered the concept of capsid-deletion mutants, the genomes of which are able to replicate in cells; however, virus dissemination is disabled. Such mutants provide an extra degree of safety to vaccine recipients. Capsid-deleted vaccines may be delivered as RNA, DNA, or virus-like particles (VLPs). The latest development of this concept involves the delivery of DNA encoding both the capsid-deleted flavivirus genome and the intact capsid gene from different promoters but on the same plasmid DNA. Replicating capsid-deleted genomes are thus packaged into VLPs in initially transfected cells and undergo one cycle of infection before losing their ability to spread. These single-round infectious particles (SRIPs) produced *in vivo* (otherwise functionally and structurally analogous to VLPs produced *in vitro*) provide a bridge between competing strategies combining the safety, thermal stability, and ease of manufacture of capsid-deleted DNAs with the efficacy close to that of live-attenuated viruses.

2 Infectious cDNA Clones

The generation of infectious cDNA clones of RNA viruses has allowed direct manipulation of their genomes revealing information about their replication and gene expression [36]. In the context of vaccines, cDNA clones have allowed the

Table 1 Replicating nucleic acid-based candidate flavivirus vaccines under development

Class	Vaccine	Flavivirus targeted	Animal model	Delivery (mode/route)	Minimal protective dose	Challenge (route/dose)	References
Infectious cDNA clones	pTNd/cΔ10847	TBEV	Mouse	RNA/GG[a]	Single, 0.6 ng	ND[b]/100 LD_{50}[c]	[25]
	pKUN1	WNV	Mouse	DNA/IM[d]	Single, 0.1 μg	IP[e]/20 IU[f]	[26, 27]
				DNA/GG[a]	Single, 1 μg	IC[g]/20 IU[f]	
					Single, 0.02 μg	IP[e]/100 IU[f]	
	pCMVWN	WNV	Mouse	DNA/IM[d]	Single, 0.01 μg	IM[d]/10 LD_{50}[c]	[28]
Capsid-deleted genomes	C(Δ28–89)-S	TBEV	Mouse	RNA/GG[a]	Two, 1 μg each	IP[e]/>1,000 LD_{50}[c]	[29, 30]
	pCMVWN(ΔC)	WNV	Mouse	DNA/IM[d]	Two, 0.1 μg each	IM[d]/10 LD_{50}[c]	[28]
VLP-delivered capsid-deleted genomes	RepliVAX WN	WNV	Mouse, hamster	VLP/IP[e] or SC[h]	Single, 4×10^4 VLPs	IP[e]/10 LD_{50}[c] (mouse) or 10^f FFU[i] (hamster)	[31–33]
	RepliVAX JE	JEV	Mouse	VLP/IP[e]	Single, 4×10^4 VLPs	IP[e]/5×10^6 FFU[i]	[34]
	RepliVAX D2	DENV-2	Immunocompromised mouse	VLP/IP[e]	Single, 5×10^5 VLPs	IP[e]/1,000 FFU[i]	[35]
DNA-delivered SRIPs vaccine	pKUNdC/C	WNV	Mouse	DNA/GG[a]	Single, 0.02 μg	IP[e]/100 IU[f]	[27]

[a]GG = gene gun; [b]ND = not described; [c]LD_{50} = 50% lethal dose; [d]IM = intra-muscular; [e]IP = intra-peritoneal; [f]IU = infectious units; [g]IC = intra-cranial; [h]SC = subcutaneous; [i]FFU = focus forming units

investigation into defined targets of virus attenuation and provide a novel means of delivery of live-attenuated viruses. In contrast to the traditional, empirical means of live-virus attenuation (i.e. serial passage of the virus to select for growth in chicken eggs, cell culture, or laboratory animals), cDNA clones allow the introduction of targeted mutations at specific sites and a subsequent determination of their phenotypic importance in attenuation [37].

Thus far infectious clones have been constructed for most of the major pathogenic flaviviruses: DENV-1 [38, 39], DENV-2 [40–44], DENV-3 [45, 46], DENV-4 [47], JEV [48, 49], WNV [50], Kunjin [51], TBEV [52, 53], Langat [54], Murray Valley encephalitis virus [55], wild-type YFV [56], and 17D YFV [57]. Mutagenesis and manipulation of these cDNA clones has led to the development of several new vaccine candidates.

Replacing the prM and E genes of a cDNA clone with those of a heterologous flavivirus allows the generation of chimeric vaccines. This technique has been applied to the attenuated 17D YFV vaccine backbone to generate the very promising ChimeriVax series of flavivirus vaccines that have been reviewed extensively elsewhere [58, 59]. This concept has also been applied to a backbone of attenuated DENV-4 [45, 60–65] and of DENV-2 PDK-53 [61, 66, 67].

In addition to using the infectious clones as a platform to generate attenuated viruses, the nucleic acid itself may be utilised for the delivery of the vaccine, creating a more stable preparation that is cheaper to manufacture (Table 1). Mandl et al. [25] were the first group to explore this approach with flaviviruses, using in vitro transcribed RNA corresponding to the genome of the Neudoerfl strain of TBEV with a 470 nucleotide deletion in the 3′ untranslated region (Fig. 1a). RNA was coated onto gold micro-particles for delivery via gene gun with as little as 0.6 ng conferring protective immunity in outbred Swiss-albino mice [25]. The authors showed that coating onto gold micro-carriers improved the stability of RNA when stored at 4°C. However, if a vaccine based upon infectious RNA-coated gold micro-particles is to be used in clinics, further comprehensive studies on its cost-effectiveness, long-term durability, and efficacy in humans are required.

The addition of a eukaryotic promoter upstream of the 5′ untranslated region of an infectious clone allows the plasmid itself to be delivered as a DNA-based vaccine (Fig. 1b), an approach that has been previously utilised for developing alphavirus replicon-based vaccines [69]. DNA delivery has an advantage over RNA vaccines as it is more stable and easier to manufacture. The first demonstrated use of this approach for developing a flavivirus vaccine employed a cytomegalovirus (CMV) immediate–early promoter upstream of an infectious cDNA of an attenuated Kunjin subtype of WNV, creating the plasmid pKUN1 (Table 1) [70]. Later challenge studies indicated that intra-muscular injection of 0.1 μg of the pKUN1 DNA vaccine generated sufficient immune response to protect BALB/c mice from 20 infectious units (IU) of the virulent NY99 strain of WNV, whereas 1 μg of DNA was required for protection when the same challenge dose was administered intra-cerebrally [26]. A later investigation demonstrated that gene gun-mediated

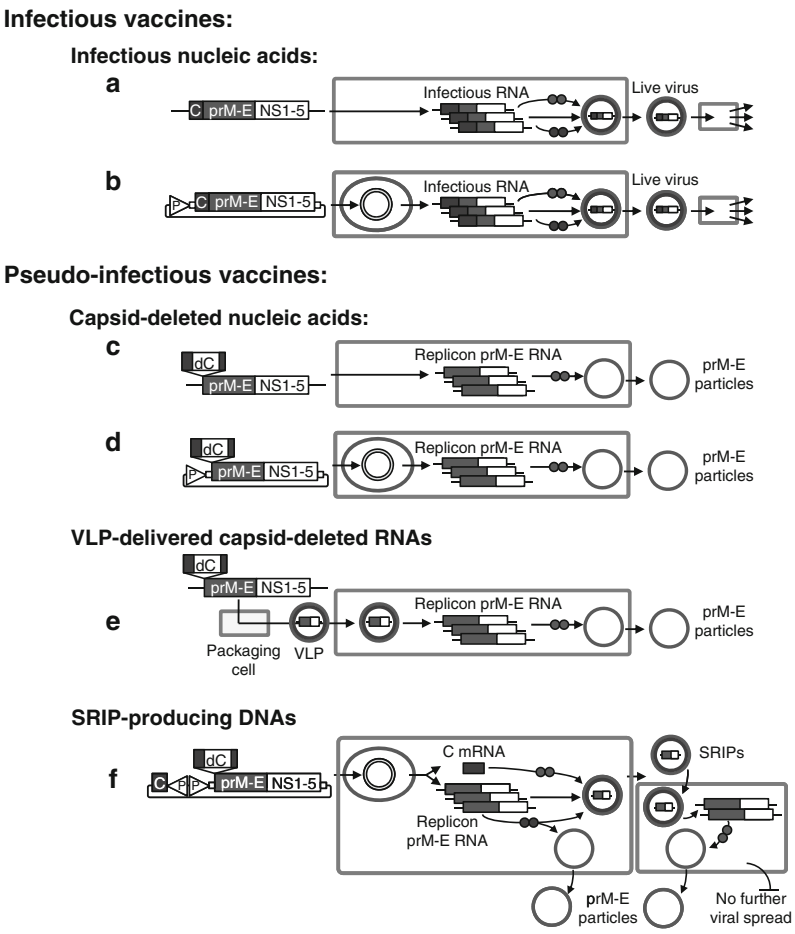

Fig. 1 Schematic representation of various strategies employed for construction, delivery, and mode of action for nucleic acid-based flavivirus vaccines. Infectious vaccines are based on infectious cDNA clones that may be delivered either as *in vitro*-transcribed RNA (**a**) or as plasmid DNA in which the genomic cDNA is placed under the control of a eukaryote promoter (**b**). This approach leads to the generation of live virus *in vivo*. Pseudo-infectious vaccines are based on capsid-deleted flavivirus genomes that may also be delivered either as RNA (**c**) or as DNA (**d**). Capsid-deleted RNA can also be packaged into virus-like particles (VLPs) using a packaging cells expressing capsid protein (**e**). Alternatively, both capsid-deleted RNA and an mRNA for the intact capsid gene can be produced from the same plasmid DNA but under the control of different eukaryotic promoters thus allowing generation of single round infectious particles (SRIPs) *in vivo* (**f**). As functional capsid gene is not encoded within the capsid-deleted RNAs, no further production and spread of infectious virus can occur *in vivo* in any of the pseudo-infectious vaccines based on capsid-deleted genomes. Figure adapted from Khromykh, Chang, and Hall [68]

immunisation of BALB/c mice with as little as 0.02 μg of pKUN1 provided complete protection against intra-peritoneal challenge with 100 IU of virulent NY99 strain of WNV [27].

The cDNA of an attenuated lineage 2 WNV strain 956D117B3 has also been used in the construction of a DNA-delivered live vaccine candidate, pCMVWN (Table 1; Fig. 1b) [28]. A single intra-muscular injection of as little as 0.01 μg of the pCMVWN construct was able to protect NIH Swiss outbred mice from intra-muscular challenge with 10 LD_{50} of WNV NY99 (Table 1). This appears to be a very encouraging preliminary result, and further comprehensive efficacy trials in small and large animal models should reveal full potential of this vaccine candidate.

3 Capsid-Deleted Genomes

Immunisation utilising nucleic acids that encode live-attenuated flaviviruses certainly resolves problems associated with the stability and expense of vaccine preparations. However, this method does little to address the rare instances of vaccine-associated diseases observed with the currently licensed live-attenuated vaccines [20, 21]. An elegant solution to this problem is to limit the infectivity of a vaccine strain by disabling its ability to package replicating RNA into virus particles, an approach that was previously used for developing vaccines against herpesviruses [71]. The principal strategy used to achieve this goal with flaviviruses has been the introduction of internal deletions within the capsid gene (Table 1; Figs. 1c and d and 2) [19]. This concept was first explored using TBEV as a novel means of live-virus attenuation [73], but was soon expanded leading to the application of a non-infectious replicon vaccine [29].

Experimentation with TBEV internal capsid deletions revealed that removal of 62 amino acids (residues 28–89), corresponding to the second, third, and fourth and part of the first alpha helices (Fig. 2), generated a replication-competent, packaging-deficient genome [29]. Smaller internal deletions either did not abolish packaging (1–16 amino acids primarily between alpha helix 1 and 2; Fig. 2) [73] or resulted in spontaneous mutations within the capsid gene that restored the packaging phenotype (19–30 amino acids roughly corresponding to alpha helix 2; Fig. 2) [74]. A later investigation with WNV also demonstrated that removal of all of the second and third alpha helices could in some instances lead to a viable packaging phenotype (Fig. 2) [75]. Early investigations indicated that transfected packaging-deficient replicon RNAs still allowed the secretion of prM/E sub-viral particles (SVPs) (the primary mediators of anti-TBEV humoral immunity in this vaccine approach) (Fig. 1c), but only at relatively low levels [29]. Using rationale devised in previous investigations [76, 77], supplementary point mutations were introduced into the signal sequence upstream of the prM gene to increase the efficiency of signalase cleavage. This "idealised" capsid-deletion mutant C(Δ28–89)-S was thus able to liberate a significantly greater proportion of SVPs following transfection [29]. Gene gun-mediated immunisation of BALB/c mice with approximately 1 μg of C(Δ28–89)-S RNA followed by a booster with the same dose at 4 weeks was sufficient to provide protection against intra-peritoneal challenge with more than 1,000 LD_{50} of the virulent TBEV strain Hypr [29]. Subsequent research by

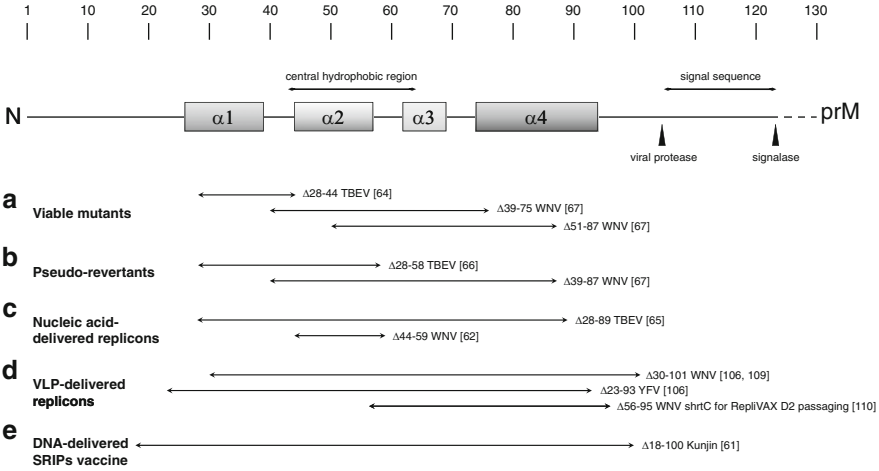

Fig. 2 Schematic representation of the flavivirus capsid protein outlining capsid-deletion mutants utilised in the construction of vaccine candidates. The crystal structure of capsid protein of Kunjin virus was used as a model for the location of the α-helices, hydrophobic domains, and protease cleavage sites in the diagram [72]. (**a**) Internal deletions that do not completely abrogate packaging, leading to generation of a viable viruses. Δ28–44 in TBEV capsid and Δ39–75 and Δ51–87 in WNV capsid were the largest deletions allowing recovery of viable viruses. (**b**) Deletions that initially abrogated recovery of infectious viruses but resulted in compensatory mutations elsewhere in the capsid gene which restored the ability to produce infectious viruses. Δ28–58 in TBEV capsid and Δ39–87 in WNV capsid represent a series of deletions that have this effect. (**c**–**e**) Capsid deletions that completely disabled the ability to produce infectious viruses. Outlined deletions are further separated by the strategy used for delivery of capsid deleted genomes, i.e. naked nucleic acid- (**c**), VLP- (**d**), and SRIPs-producing DNAs (**e**)

the same group demonstrated that the aforementioned consecutive gene gun immunisations of mice were able to induce humoral and cellular (Th1 and CD8+ T cell) immune responses equivalent to those produced by live vaccines and that even a single 1 μg C(Δ28–89)-S RNA dose could induce a long-lasting (1 year) neutralising antibody response [30].

The capsid-deletion approach has also been applied to WNV DNA-delivered replicon vaccines (Table 1; Fig. 1d) [28]. Residues 44–59 of the attenuated lineage 2 WNV infectious clone pCMVWN were deleted to generate the replicon mutant pCMVWN(ΔC) (Fig. 2c). Although incorporating only a relatively small deletion of 16 amino acids, the packaging-deficient phenotype appears to have been stable with no infectious virions present in murine sera after 2 weeks of monitoring following immunisation with up to 10 μg of pCMVWN(ΔC) DNA, and no virus was detected in transfected cell culture during the entire period of observation (48 h) [28]. Serum conversion was demonstrated to be three- to sixfold lower for capsid-deleted pCMVWN(ΔC) compared to infectious pCMVWN DNA following a single intra-muscular injection of mice with comparable amounts of DNA. An equivalent immune response could be achieved via booster immunisation with

pCMVWN(ΔC) DNA [28]. Prime and boost immunisations with as little as 0.1 μg of pCMVWN(ΔC) DNA provided adequate immune response to completely protect mice from intra-muscular challenge with 10 LD_{50} of WNV NY99 [28]. Despite this impressive protective efficacy and a demonstrated stability of the packaging-deficient phenotype, concerns still exist in regards to the long-term durability of the pCMVWN(ΔC) construct. As described earlier, studies using TBEV mutants have shown that capsid-deletions of less than 30 amino acids may result in spontaneous reversion to replicon-packaging competency [74]. A more rigorous investigation into the construct's stability and safety is thus warranted.

4 VLPs for Delivery of Capsid-Deleted RNAs

Immunisation with capsid-deleted nucleic acids is a promising line of research, though this approach is not without shortcomings. Single immunisation with these vaccines generates an insufficient immune response to provide protection against virulent challenge [27], thus necessitating the practice of secondary immunisation. Booster shots are necessary as nucleic acids traditionally have very low transfection efficiency *in vivo* [78, 79]. Temporal separation of two small doses of nucleic acid-based vaccines allows the establishment of a low-level memory immune response prior to subsequent activation and proliferation (the clonal selection hypothesis) [80], leading to an exponential increase in humoral immunity. Simply increasing the initial dose of capsid-deleted DNA or RNA would not reduce the cost and is unlikely to generate an immune response equivalent to that achieved via booster immunisation. One laudable alternative to delivering naked nucleic acid is to deliver the packaging-deficient replicon RNA via VLPs (Table 1; Fig. 1e), a technique that has been successfully applied to alphaviruses, lentiviruses, and poliovirus [81–83]. Flavivirus structural genes that have been deleted in replicons can be provided *in trans* from a different expression vector [84].

Method for the generation of flavivirus VLPs was first developed using Kunjin virus replicons with deletions in the structural genes corresponding to the removal of all of prM and E, and all but the first 20 codons of C (C20DXrep) [84, 85]. C20DXrep RNA was packaged into secreted VLPs by the Kunjin structural proteins produced from a Semliki Forrest virus (SFV) replicon, designated SFV-prME-C107 [84]. Sequential electroporation of 2×10^6 BHK21 cells with 10–20 μg of Kunjin replicon RNA followed by SFV-prME-C107 replicon RNA generated a maximum titre of approximately 1.3×10^5 VLPs/ml of culture fluid, a relatively low titre compared to the wild-type virus (~10^7 infectious virions/ml) [84]. To improve the efficiency of packaging, a stable BHK21 cell line, tetKUNCprME, was later established incorporating the structural gene cassette under the control of a tetracycline-inducible promoter, which facilitated packaging of subgenomic Kunjin replicon RNA with much greater efficiency [86]. In this system, electroporation of 3×10^6 cells with approximately 20 μg of *in vitro*-transcribed Kunjin replicon RNA generated up to 1.6×10^9 VLPs/ml of culture fluid over a 4 day period [86].

The tetKUNCprME cell line also demonstrated an ability to successfully package replicon RNA from other flaviviruses, though at reduced efficiencies (WNV and DENV-2 replicons were encapsidated at approximately 70% and 1% of the efficiency of the Kunjin replicon, respectively) [86].

Although none of these Kunjin-based VLPs were tested as flavivirus vaccines, the establishment of *trans*-encapsidated replicon technology encouraged investigations by other groups. To date, packaging of flavivirus subgenomic replicons by structural genes provided *in trans* has been applied to TBEV [87, 88], WNV [89, 90], YFV [91, 92], JEV [93], and DENV-1 and -2 [94, 95]. Each of these flavivirus encapsidation systems uses an approach resembling that described for the Kunjin replicons. For the majority, one or more of the structural genes sustained large in-frame deletions within the flavivirus replicon, which were in turn complemented by the expression of these structural genes *in trans*, either within a stable cell line [90, 94–96] or via another expression vector (either replicon RNA or eukaryotic expression plasmid) [88, 94, 97, 98]. Other techniques have employed cell lines stably housing flavivirus replicons which are packaged upon transfection with *trans*-complementing constructs expressing structural genes [89, 91, 94] and a dual replicon system with complimentary constructs alternately replacing C with the green fluorescent protein gene, and prM/E with the gene for the red fluorescent protein Cherry [92]. VLP packaging efficiency was quite high for TBEV, WNV, and YFV, with approximately 10^8–10^9 VLPs/ml being produced for each system [89, 92, 96], while DENV and JEV VLP production was low in comparison with only 1.5×10^5 and 4.3×10^5 VLPs/ml produced, respectively [93, 95].

Each of the *trans*-packaged VLPs thus far described have not been applied directly to studies concerning vaccines against flaviviruses. Rather their construction has led to their utilisation as a platform for the transient introduction of foreign genetic information into eukaryotic cells both *in vivo* and *in vitro*. Reporter genes such as β-galactosidase, green fluorescent proteins, and luciferases may be introduced allowing the development of diagnostic and screening tools as well as analysing various steps in virus life cycle such as investigations into RNA replication, visualisation of localised areas of translation of viral proteins within cells, mechanisms of interferon inhibition, sites of initial infection in feeding mosquitoes, virus interactions with host cells, generation of antibody neutralisation tests, and screening for anti-viral drugs [89, 93, 94, 99–109]. Incorporation of genes corresponding to pathogen or tumour antigens and subsequent immunisation with packaged VLPs has been used to elicit strong cell-mediated immunity against specific targets [31–33, 86, 99, 110, 111].

The *trans*-packaged flavivirus VLP configurations described above have primarily employed replicons with in-frame deletions in all of the structural genes. Research into TBEV capsid-deleted genomes [19, 29] provoked interest in the production of replicons that retain the prM/E sequences, requiring complementation of capsid only. Such an approach is advantageous as it allows the production of secreted prM/E particles (SVPs) as well as all the non-structural proteins in cells infected with the VLPs, thus boosting both humoral (anti-E antibody) and cellular

(T-cell responses to non-structural proteins) immunity (Fig. 1e). Hence, this is a system much more amenable to application as flavivirus vaccines.

Initial investigations concerned the production and characterisation of replication deficient VLPs of WNV and YFV [34]. Deletions of codons 30–101 of WNV capsid and 23–93 of YFV capsid were sufficient for stable abrogation of encapsidation without interfering with genome replication (Fig. 2d). Generation of stable BHK21 cell lines harbouring recombinant Venezuelan equine encephalitis virus replicons encoding either the YFV C or WNV C/prM/E genes linked to a puromycin acetyltransferase (Pac) gene (VEErep/C2opt/Pac) allowed packaging of capsid-deleted replicons into VLPs, with titres reaching approximately 10^8 VLPs/ml [34]. Passage of VLPs in packaging cells allowed further amplification of titres. Single intra-peritoneal immunisation of outbred Swiss Webster mice with as little as 3×10^4 WNV VLPs afforded complete protection against challenge with 100 LD_{50} of the virulent NY99 strain of WNV [34].

Later development of the WNV VLP vaccine (designated RepliVAX WN) improved the stability and safety of the packaging cell lines by introducing a ubiquitin gene into the Venezuelan equine encephalitis virus replicon and by removing the prM/E genes from the construct (hence decreasing likelihood of recombination with the capsid-deleted replicon) [35, 112]. Serial passage of RepliVAX WN in packaging cell lines was unable to generate recombination leading to recovery of the infective phenotype, but did select for mutations in the proteolytic cleavage sites flanking the prM signal sequence (Fig. 2) [35]. Mutations of K to M in the viral protease cleavage site upstream of the prM signal sequence and of S to C at the signalase cleavage site upstream of prM both led to an increase in the amount of packaged VLPs; however, only the later mutation was engineered into the candidate RepliVAX WN vaccine [35]. Single immunisation of Swiss Webster mice or Syrian hamsters with as little as 4×10^4 VLPs, either intra-peritoneally or subcutaneously, engendered sufficient immune response to completely protect them from intra-peritoneal challenge with either 10 LD_{50} (mice) or 10^6 focus forming units (FFU) (hamsters) of NY99 strain WNV [35, 112]. When challenged 6 months after this single immunisation, hamsters were still completely protected from challenge [112].

RepliVAX technology has also been used for the generation of JEV vaccines. RepliVAX JE was created by replacing the prM and E genes of the RepliVAX WN replicon with those of JEV strain Nakayama [113]. RepliVAX JE initially presented with low VLP yields; however, blind passage in packaging cell lines was able to select for a mutation in the viral protease cleavage site upstream of the prM signal sequence (K to N) [113]. This mutation was incorporated into the original RepliVAX JE sequence, leading to the generation of titres exceeding 10^7 VLPs/ml [113]. Single intra-peritoneal immunisation of Swiss Webster mice with as little as 4×10^4 VLPs afforded complete protection against intra-peritoneal challenge with 5×10^6 FFU of virulent JEV strain Beijing P3 [113].

DENV-2 vaccines have likewise been generated using the RepliVAX system. RepliVAX D2 was constructed via replacement of the prM and E genes of the RepliVAX WN replicon with those of DENV-2 New Guinea C strain [114].

RepliVAX D2 was initially unable to produce encapsidated VLPs upon expression in packaging cell lines [114]. To overcome this obstacle a new replicon was constructed containing a smaller deletion within C for use in passaging to select for compensatory mutations (Fig. 2d). To summarise, several mutations were found, but only two of them located in the M sequence (G–R and E–V) and one in the E sequence (T–K) were required for RepliVAX D2 RNA to be packaged with reasonable improvements in efficiency [114]. Yield was further increased to titres of approximately 10^6 VLPs/ml by replacing *trans*-complemented WNV C with *trans*-complemented DENV-2 C via generation of a new cell line stably expressing the recombinant replicon VEErep/Pac-Ubi-D2C [114]. Single intra-peritoneal immunisation of severely immunocompromised AG129 mice with 5×10^5 RepliVAX D2 VLPs protected them from intra-peritoneal challenge with wild-type DENV-2 New Guinea C strain, with only 20% of animals showing illness and no recorded deaths [114].

The RepliVAX strategy for flavivirus vaccine production appears very promising. Each construct has demonstrated an impressive capacity to provide protective immunity in animal models after only a single immunisation; stable cell lines allow the passage and production of large titres of VLPs; and the durability of the *trans*-complementation system has been established with a proven lack of recombination leading to recovery of the infectious virus. However, this approach lacks some of the advantages of DNA-based self-replicating vaccines. Production and purification of VLPs is somewhat cumbersome and relatively expensive, and the stability of the VLPs at higher ambient temperatures, while not intensely studied, are unlikely to be as robust as that of DNA.

5 DNA-Based Vaccine Producing SRIPs In Vivo

One of the latest innovations in flavivirus vaccine research combines the advantages of RepliVAX-like technology (efficient delivery and durable immune response) with those of DNA vaccines (easy manufacture and robust genetic and thermal stability). SRIP-producing DNA vaccine technology (Table 1) consists of a capsid-deleted flavivirus cDNA under the control of one eukaryotic promoter combined with cDNA for the complete capsid gene transcribed from another eukaryotic promoter in the same plasmid (Fig. 1f). [27]. The vaccine does not require any additional manipulations, i.e. production of VLPs in a packaging system/cell line, as the capsid-deleted RNA is both produced and packaged into SRIPs *in vivo* (Fig. 1f). As the capsid gene provided in trans is not encoded in the RNA packaged into SRIPs, further spread of infection is not possible (Fig. 1f). Both DNA-transfected and SRIP-infected cells contain replicating capsid-deleted RNA resulting in increased production of immunogenic prM/E particles and CTL-inducing non-structural proteins which in turn leads to an enhanced immune response (Fig. 1f) [68].

A SRIP-producing DNA vaccine targeting WNV (pKUNdC/C) has recently been investigated [27]. The vaccine is based on infectious DNA of Kunjin virus, pKUN1, with the codons corresponding to the amino acid residues 18–100 of the capsid gene deleted (Fig. 2e). The full-length capsid gene is encoded in reverse orientation under the control of a second CMV promoter (Fig. 3a) [27]. Characterisation of pKUNdC/C in vitro demonstrated a production of SRIPs reaching titres of approximately 10^5 particles per 1 μg of transfected DNA by day 2 post-transfection, a yield that was maintained for a 5-day period [27, 68]. Capsid expression has been demonstrated to be limited to initially transfected Vero cells, whereas envelope protein expression was observed both in capsid-expressing transfected cells and in adjacent cells that had been infected with SRIPs released from transfected cells (Fig. 3b) [27]. Importantly, passage of the secreted SRIPs in Vero cells did not show any signs of infectious virus (Fig. 3c), thus, implying an inability of the capsid mRNA to recombine with the genomic replicon RNA [27]. The limited spread of infection was confirmed in an *ex vivo* experiment utilising skin from cattle ears that had been bombarded with gold micro-carriers coated with pKUNdC/C DNA (Fig. 3d). The expression of E was detected in both cells initially bombarded with DNA (containing gold particles) and adjacent cells not containing gold particles for pKUNdC/C and pKUN1 DNAs but not for pKUNdC DNA (Fig. 3d), clearly demonstrating the release and spread of SRIPs.

The pKUNdC/C construct has performed very well in a small animal model. Single immunisation with as little as 0.02 μg of gene gun-delivered DNA provided complete protection to BALB/c mice when challenged intra-peritoneally with 100 IU of virulent WNV NY99 [27]. The construct has also shown promise as a vaccine for horses that are highly susceptible to WNV infection. Two gene gun immunisations with 4 μg of pKUNdC/C were sufficient to elicit detectable neutralising antibody response in all horses against both Kunjin and WNV NY99, with a third immunisation greatly increasing the neutralising antibody titres [27].

SRIP-producing DNA technology thus far appears to be the most comprehensive approach for designing modern pseudo-infectious flavivirus vaccines. The ease of production coupled with the robust genetic and thermal stability make these vaccines highly amenable to use worldwide, including developing nations where specialised resources may be in short supply. The proven efficacy of the pKUNdC/C construct in mice and the immunogenic nature of this vaccine in horses [27] warrant further investigation and should be followed up with a challenge study in larger animals. Considering the relative ease of construction and the availability of capsid-deleted flavivirus clones, adaption of this technique to the development of vaccines against other flaviviruses appears to be straightforward. Given the proven competence of chimeric flavivirus constructs (i.e. ChimeriVax and RepliVAX), changing the Kunjin virus replicon backbone may not even be necessary, as merely swapping the prM and E genes with those of heterologous flaviviruses could in theory generate effective vaccines against different flaviviruses. Although the SRIPs-producing DNA-based approach is relatively recent, all indications point to this strategy being highly attractive as the next-generation of replicating vaccines. As such, continuing developments in this technology should be pursued.

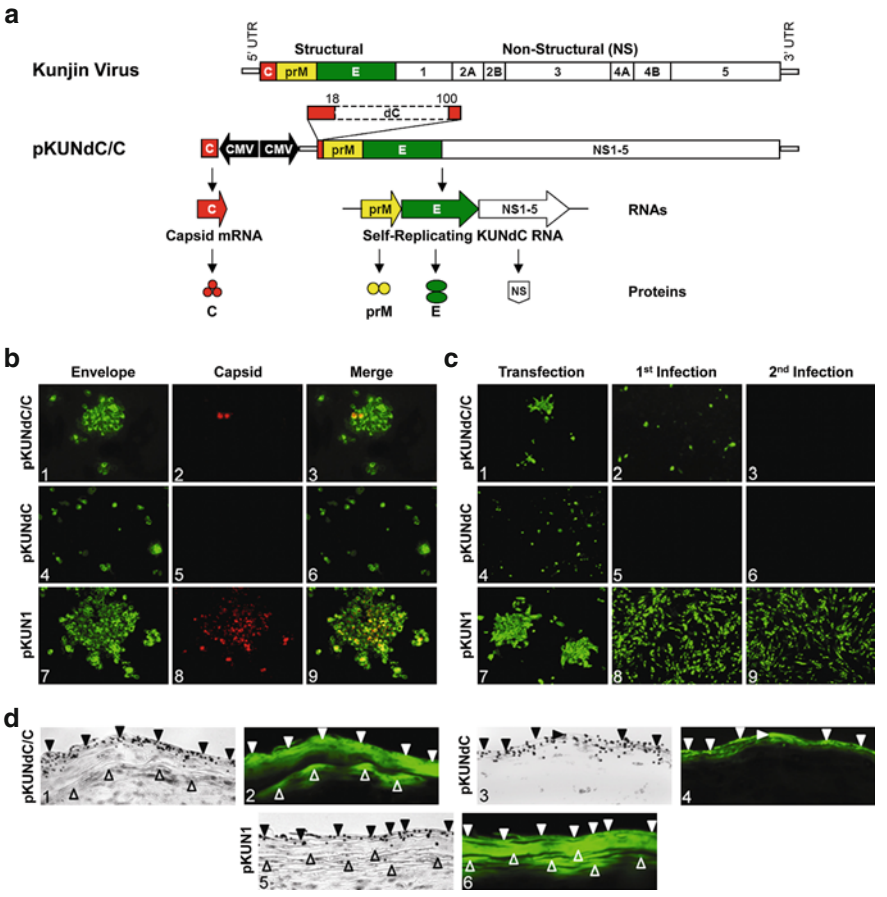

Fig. 3 *In vitro* and *ex vivo* demonstration of the ability of pKUNdC/C DNA to produce SRIPs. (**a**) Schematic diagram of pKUNdC/C DNA. cDNA for capsid-deleted Kunjin virus genome is placed under control of CMV promoter, while cDNA for the intact capsid gene is placed in the reverse orientation under the control of a second copy of CMV promoter. Upon transfection into cell, pKUNdC/C is transcribed to produce an mRNA encoding the capsid gene and a capsid-deleted RNA encoding all the remaining Kunjin virus genes. Subsequent translation of these provides all of the necessary proteins for RNA replication and its packaging into SRIPs. (**b**) Dual immunoflourescence analysis of DNA-transfected Vero cells with anti-E (*green*) (panels 1, 4, 7) and anti-Capsid (*red*) (panels 2, 5, 8) antibodies show capsid production in each of the cells transfected or infected with pKUN1 (panel 9), in none of the cells transfected with pKUNdC (panel 6), and only in those cells transfected with pKUNdC/C but not in the adjacent cells infected with the released SRIPs (panel 3). (**c**) Immunoflourescence analysis with anti-E antibodies shows small foci of positive cells after transfection with pKUNdC/C DNA (panel 1), large foci after transfection with infectious pKUN1 DNA (panel 7), and only individual positive cells after transfection with pKUNdC DNA (panel 4). Three days post-transfection, culture fluids were collected and used to infect naive Vero cells (first infection, panels 2, 5, and 8, respectively). Three days after infection, the culture fluid was again collected and used to infect naive Vero cells (second infection, panels 3, 6, and 9). The pKUNdC DNA did not produce any infective particles, the infectious pKUN1 DNA produced infectious spreading virus as expected, and pKUNdC/C DNA produced infectious

6 Summary

Flaviviruses have been recognised as important agents of human disease for over a century [1]; however, vaccines are currently licensed for only a handful of flavivirus species (YFV, TBEV, and JEV). Several deficiencies have also been identified with existing vaccines. They are in general relatively expensive to produce, difficult to purify, and unstable at ambient temperatures. Considering the global flavivirus disease burden is predominantly afflicting developing nations [2], such complexity and expense limit the efficacy of vaccine utilisation. Safety concerns have also been raised over the currently licensed vaccine preparations. Although they are highly effective at preventing lethal infections when correctly administered, several adverse reactions have been observed in patients following immunisation with commercial flavivirus vaccines [8, 20–24]. Thus, flavivirus research continues to be driven by the need for cheap, stable, safe, and efficacious vaccines.

The generation of infectious cDNA clones of various flavivirus species has allowed their specific, directed attenuation and subsequent use as nucleic acid-delivered, live vaccines. Such vaccines have proven highly effective in animal models; however, they neglect to address concerns over the safety of live-attenuated vaccines in regards to observed adverse reactions. Generation of large in-frame capsid deletions in these cDNA clones has led to the development of vaccines that are safer but at the same time maintain many of the positive immunological features of live vaccines. Capsid-deleted clones are unable to package replicating genomic RNA into virus particles, thus cannot generate a spreading infection in the host. Immunogenicity of the vaccine preparation suffers however, as capsid-deleted replicon vaccines must be administered at least twice in order to generate protective immunity. Single-dose efficacy has been achieved via the *trans*-packaging of capsid-deleted replicons into VLPs and delivery of these into the host. VLP-based vaccines, however, suffer from being relatively expensive to produce, difficult to purify, and they have low thermal stability. SRIP-producing DNA vaccines – one of the latest innovations – provide the complementary capsid gene in trans within the same plasmid as the capsid-deleted genome allowing delivery of the vaccine as naked DNA. Thus, they overcome difficulties with preparation, purification, costs, and stability of vaccine. SRIP-producing DNA vaccine has been demonstrated to not produce any infectious virus and to be highly efficient at generating protective immune response. The low cost, simple manufacture, safety, efficacy, and

Fig. 3 (continued) particles capable of only one round of infection. (**d**) Sections of cattle ear epidermal cells bombarded with DNA-coated gold particles and analysed by light microscopy (panels 1, 3, and 5) and by immunofluorescence with anti-E antibodies (panels 2, 4, and 6). Cells containing gold particles (initially transfected cells) are indicated by *solid arrows*. Cells that express E but do not contain gold particles are indicated by *open arrowheads*. The presence of E-positive but gold particle-negative cells indicates infection with virus particles (pKUN1, panels 5 and 6) or SRIPs (pKUNdC/C, panels 1 and 2). The capsid-deleted DNA pKUNdC does not engender a spreading infection (panels 3 and 4). Figure modified from Chang et al. [27]

high genetic and thermal stability of SRIP-producing DNA vaccines make them an attractive candidate for future flavivirus vaccine development.

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Application of Cleavage Activation Mutants of Influenza Virus as Live Vaccines

Juergen Stech and Hans-Dieter Klenk

Abstract Influenza viruses are major human and animal pathogens. In man, they are responsible for annual epidemics and less frequent but more severe pandemics. Avian influenza viruses cause devastating outbreaks in poultry, and influenza virus infections in pigs and horses also lead to high economic losses. Vaccination is an effective instrument to control the disease burden of human and, to some extent, also of animal influenza. Inactivated human vaccines have been used for more than 60 years. Furthermore, cold-adapted, live attenuated vaccines have been licensed in some countries. Attenuated viruses with reduced pathogenicity can also be obtained when the cleavage site of the hemagglutinin is mutated. Such protease activation mutants have not only been generated for the production of inactivated vaccines against highly pathogenic avian influenza viruses, but they have also the potential to be used as live vaccines. Two types of protease activation mutants have been investigated for use as live vaccines. In the first group, the polybasic cleavage site of the hemagglutinin, a prime determinant of pathogenicity, was cut short to a single arginine. These viruses require additional mutations in other genes for full attenuation. In the second group, polybasic or monobasic cleavage sites are replaced by an elastase cleavage site. These viruses are fully attenuated, yet have retained their immunogenicity.

1 Cleavage Activation of the Influenza Virus Hemagglutinin

Influenza viruses belong to the family of Orthomyxoviridae. Among them are the three genera: influenza A virus, influenza B virus, and influenza C virus. Type A is a large group of viruses comprising 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes. The genome of influenza A viruses consists of eight single-stranded RNA molecules of negative polarity embedded in an enveloped virion. The envelope

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contains HA and NA spikes. HA initiates infection by mediating binding to *N*-acetyl-neuraminic acid-containing receptors and membrane fusion. Fusion activity and therefore virus infectivity depends on proteolytic cleavage of HA. X-ray crystallographic studies have shown that the cleavage site is located in a circular loop projecting away from the surface of the precursor HA into the solvent (Fig. 1). The activating enzymes are provided by the host. Avian influenza viruses show large variations in cleavability, and these differences are prime determinants of pathogenicity. In the following we will give a brief outline of this concept. For more detailed information, the reader is referred to previous reviews by us [2] and others [3].

Mammalian influenza A viruses and low pathogenic avian influenza viruses (LPAIV) have HAs with a single arginine at the cleavage site. They are activated by proteases synthesized by epithelial cells that are present only in respiratory or intestinal tissues. Infection is therefore restricted to these organs (Fig. 1). A number of trypsin-like proteases, such as plasmin, tryptase Clara and factor X, activate HA with a monobasic cleavage site in vitro (for references see [2]), but they are unlikely to activate these viruses in their natural setting. However, recently two serine proteases (TMPRSS2 and HAT) from the human airway epithelium have been found to activate human influenza A viruses as well as LPAIVs [4, 5]. Bacterial proteases may also activate HAs of restricted cleavability and promote the development of pneumonia in mice after combined viral-bacterial infection [6].

Highly pathogenic avian influenza viruses (HPAIV) are activated by a different cleavage mechanism. Their HAs are activated at multibasic cleavage sites by furin, a member of the proprotein convertase family of eukaryotic subtilisin-like serine

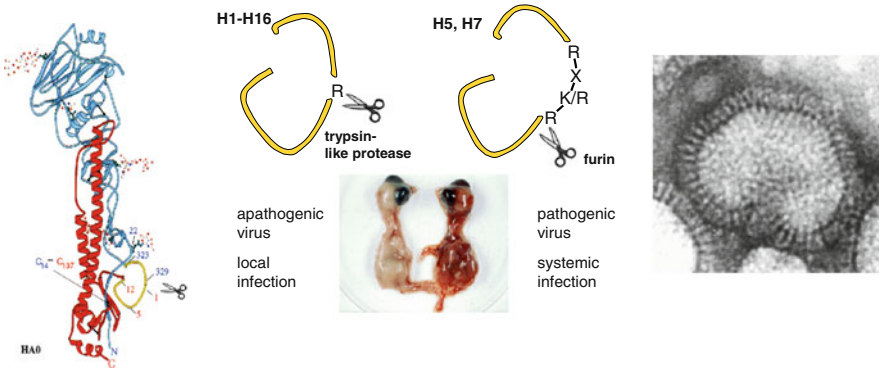

Fig. 1 The cleavage site of HA determines the pathogenicity of avian influenza viruses. HA is cleaved into subunits HA1 (*blue*) and HA2 (*red*). The cleavage site is located in a loop (*yellow*) projecting from the surface of the molecule [1]. LPAI viruses (subtypes H1-H16) have a single arginine at the cleavage site that is recognized by trypsin-like proteases that are present only in specific tissues, such as intestinal epithelia. These viruses cause therefore local infection. HPAI viruses (subtypes H5 and H7) are activated at a multibasic cleavage site by the ubiquitous protease furin. Therefore, these viruses spread throughout the organism. An electron micrograph of a virus particle with HA and NA spikes protruding from the surface is also shown

endoproteases [7]. The ubiquity of this enzyme accounts for the systemic infection typical for these viruses (Fig. 1). Furin is a factor of the constitutive secretory pathway in almost all cells and accumulates in the trans-Golgi network, which is also the cellular compartment where this HA type is cleaved (for references see [2]). Other proprotein convertases, which resemble furin in structure and substrate specificity, are PACE4, PC5/6, and LPC/PC7. Like furin, PC5/6 activates HAs with multibasic cleavage sites, whereas PACE and LPC/PC7 do not [8, 9]. The HAs of most HPAI viruses have the consensus sequences R-X-K/R-R [10] or R-X-X-R [11] at the cleavage site, motifs that are both recognized by furin. The only exception to these rules is the HA of A/chicken/Pennsylvania/83 (H5N2) which contains the unusual tetrapeptide K-K-K-R [12]. Another important determinant is a carbohydrate side chain close to the cleavage site that interferes with protease accessibility. Loss of this carbohydrate resulted in enhanced HA cleavability and pathogenicity [13]. However, masking of the cleavage site by this oligosaccharide was overcome when the number of basic amino acids was increased [12, 14]. It was also shown that HA can acquire high cleavability only if the stretch of basic residues was introduced by insertion, but not by amino acid exchanges in the carboxy-terminus of HA1 [15].

Increased pathogenicity as a consequence of insertions at the cleavage site has first been observed in laboratory studies involving sequential cell culture passages of strains A/seal/Massachusetts/1/80 (H7N7) [16] and A/turkey/Oregon/71 (H7N3). In the latter case, the acquisition of the furin recognition motif resulted from the recombination of the HA gene with 28S ribosomal RNA [17, 18]. The HA gene may recombine not only with cellular RNA but also with other viral gene segments, as has been observed when new HPAIVs emerging in the field have been analyzed. Thus, comparison of A/chicken/Chile/02 (H7N3) isolates revealed that the HA genes of the highly pathogenic strains had an insertion of 30 nucleotides at the cleavage site that was presumably derived from the nucleoprotein gene of the unrelated A/gull/Maryland/704/77 (H13N6) virus [19]. Recombination between HA and matrix protein genes of the same virus generated the highly pathogenic A/chicken/BC/04 (H7N3) viruses [20]. Polymerase slippage has been suggested as an alternative strategy by which a multibasic cleavage site is generated [21, 22]. However, there are other examples where the mechanism of insertion is not clear [23].

It is also not clear yet why the acquisition of a multibasic cleavage site and therefore the generation of HPAI viruses occurs in nature only with subtypes H5 and H7. Interestingly, however, high cleavability was also observed with a subtype H3 HA after in vitro insertion of a multibasic cleavage site and removal of an adjacent oligosaccharide by recombinant DNA technology [15] and with recombinant avian H3 viruses with an engineered multibasic cleavage site [24]. Thus it appears that confinement of HPAIVs to subtypes H5 and H7 cannot be attributed to structural restrictions of the HA protein, but that the responsible mechanisms are at the level of RNA replication [2].

In contrast to natural evolution where HPAIVs generally appear to be derived from LPAIVs, recombinant viruses with reduced pathogenicity can be generated by in vitro mutation at the cleavage site. Because this attenuation technique does not

affect virus yield, it has been employed for the production of inactivated pandemic vaccines. Furthermore, cleavage activation mutants of influenza viruses have the potential to be used as live vaccines.

2 Attenuation by Exchange of a Polybasic for a Monobasic Cleavage Site

For production of inactivated pandemic vaccines, recombinant H5N1 strains have been generated containing HA and NA from a human H5N1 virus and the remainder of their genes from PR8. In these viruses the polybasic cleavage site of HA was replaced by a single arginine to allow safe manufacturing [25–30] (Fig. 2).

To obtain pandemic H5N1 live vaccines, introduction of a monobasic HA cleavage site had to be combined with additional attenuation mechanisms. Thus, 6:2 reassortants have been generated containing a modified HA gene and the NA gene of H5N1 viruses and the other gene segments of a cold adapted influenza virus. These reassortant viruses showed low pathogenicity for chickens and were attenuated in mice and ferrets, while growing to high titres in eggs. Inoculation of ferrets and mice protected against lethality from homologous and heterologous challenge after one dose and conferred protection against viral replication after two doses [31, 32]. In another approach a H5N1 recombinant was generated in which a modified HA cleavage site was combined with an 11-amino acid deletion at the cytoplasmic tail of M2. This virus replicated efficiently in vitro in M2-expressing cells and was attenuated in mice. It provided protection from lethal challenge with homologous

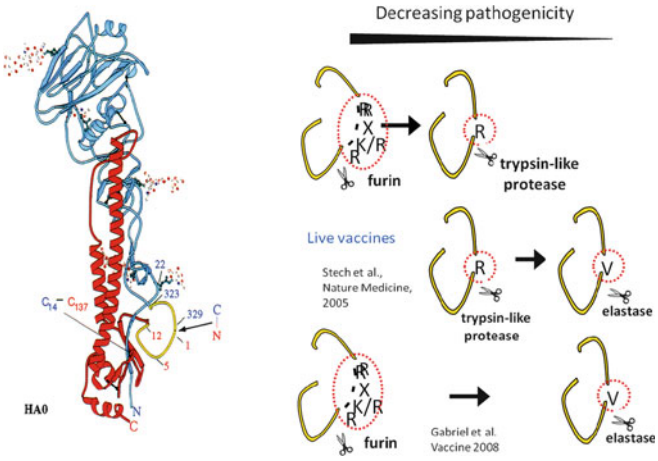

Fig. 2 Generation of vaccine strains by genetic manipulation of the HA cleavage site. Attenuation is accomplished by replacement of (1) a polybasic with a monobasic cleavage site, (2) a monobasic with an elastase cleavage site, and (3) a polybasic with an elastase cleavage site

and heterologous H5N1 viruses in mice [33]. Finally, protease activation mutants of H5N1 virus have been used in which increased interferon sensitivity resulting from a truncated NS1 protein contributed to attenuation [34b].

3 Attenuation by Introduction of an Elastase Cleavage Site

Protease activation mutants of LAIV requiring elastase or another atypical protease instead of trypsin for HA cleavage have been obtained previously by classical virological techniques. These mutants proved to be attenuated and immunogenic [34a]. With the advent of recombinant DNA technology, it is now possible to specifically design such viruses by site directed mutagenesis. Moreover, it has been possible to generate live attenuated vaccines against HPAIVs by this approach.

Using reverse genetics, the HA cleavage site of the strain A/WSN/33 (H1N1) was changed from a monobasic motif to an elastase motif by exchanging the arginine at the cleavage site to valine (Fig. 2), resulting in strict dependence of the generated mutant (WSN-E) on porcine pancreatic elastase. WSN-E replicated in cell culture with elastase equally well as a wild-type (WSNwt) in the presence of trypsin. However, replication of WSN-E in vivo was restricted due to poor access to elastases. Accordingly, in contrast to the lethal wild-type, WSN-E is fully attenuated in mice, confined to the lung and undergoes abbreviated replication, just reaching levels close to the inoculum dose. One intranasal immunization with 10^5 or 10^6 pfu protects mice against lethal challenge with WSNwt four weeks later. Although some animals from the group vaccinated with 10^5 pfu WSN-E developed temporary weight loss and milder disease symptoms, they eventually recovered. All animals vaccinated with the highest dose of 10^6 WSN-E survived the challenge without displaying any weight loss or other visible symptoms of illness. Correspondingly, challenge virus could be detected in lung homogenate from the animals immunized with 10^5 pfu, whereas no virus could be recovered from animals with the highest immunization dose of 10^6 pfu. One intranasal inoculation of WSN-E induced a substantial, dose-dependent local and systemic immune response despite low virus titres in the lung. A dose of 10^5 or 10^6 pfu led to remarkable hemagglutination inhibition titers, serum IgG and mucosal IgA titers. They were lower than those induced by 10^3 pfu of WSNwt because its longer replication enables antigenic stimulation. However, challenged animals showed almost comparable systemic and mucosal antibody titers if immunized with 10^6 pfu WSN-E. This indicates that a notable immunological memory had already been induced in these animals [35].

To check for the emergence of revertants of WSN-E, sequential lung passages in mice were carried out. After the first passage in mouse lung, the entire amount of WSN-E was in the range of 10^5–10^6 pfu. Such virus populations are too small for generation of double-point revertants having an equilibrium frequency of approximately 10^{-5} to 10^{-8}. Therefore, the elastase-dependent WSN-E was passaged on MDCK cells in the presence of trypsin, beginning with different inocula of 10^8, 10^7,

or 10^6 pfu each in 10 parallel cell cultures. From all 10^8 pfu inocula and from six out of ten 10^7 pfu inocula, a trypsin-dependent virus with lysine at its cleavage site was found. No revertants from the lowest inoculum of 10^6 pfu could be obtained. Therefore, the reversion frequency within the WSN-E stock is approximately 10^{-7}. This low reversion rate and the small viral loads of WSN-E in the mouse lung explain the absence of revertants during mouse passages. Another reason for the genetic stability of WSN-E in vivo is the restriction to one replication cycle due to the absence of the appropriate protease [35].

A frequent objection against the use of influenza virus live vaccines is the possibility of reversion to pathogenicity. Because of the double point mutation (from any arginine to the valine codon) within the cleavage site, two nucleotides together must be replaced for back-mutation; suppressor mutants outside of the cleavage region seem to be very unlikely. This explains the low reversion frequency in cell culture. In hen eggs, a factor X-like protease is present, which should cause a considerably higher proportion of revertants. Therefore, eggs might not be suitable for vaccine production. However, in cell culture the substitution of trypsin by elastase for propagation of WSN-E leads both to positive selection for elastase-dependent virus and to negative selection against revertants. A reversion frequency of approximately 10^{-7}, as found for WSN-E, underlines that a live vaccine virus should carry several independent attenuating mutations; the modified cleavage site would be useful as one attenuating component of such a vaccine.

The suitability of elastase cleavage site mutants for use as influenza live vaccines was further investigated with the mouse-adapted highly pathogenic H7N7 strain SC35M$_H$ [16, 37]. This virus has been obtained by repeated passages of the isolate A/seal/Massachusetts/1/1980 (H7N7) in chicken embryo fibroblasts and afterwards in mouse lungs [16, 36]. The elastase cleavage site mutant SC35M$_H$-E, which was generated by replacing the polybasic cleavage site of HA by a single valine, grew in cell-culture in the presence of elastase as well as the wild-type (Fig. 2). In mice, SC35M$_H$-E was attenuated. Animals inoculated intranasally with doses of 10^3 to 10^6 pfu survived. In contrast, the parent virus had an LD_{50} of $10^{1.4}$ pfu. SC35M$_H$-E could be detected on day 1 at titers close to the inoculum dose. Moreover, the organ tropism of SC35M$_H$-E was severely restricted compared to that of the wild-type, which replicates to high titers in lung, brain, and heart from day 1 to 7 post inoculation. For the immunization studies, two 6:2 reassortants were also generated: WSN-H7N7-E is composed of the internal protein genes from A/WSN/33 (H1N1) and the surface protein genes from SC35M$_H$-E; and reciprocally, SC35M-H1N1-E carries the internal protein genes from SC35M and the surface protein genes from WSN-E (H1N1). Intranasal inoculation with SC35M$_H$-E, SC35-E, WSN-H7N7-E or SC35M-H1N1-E induced high titers of serum IgG and mucosal IgA antibodies detectable 4 weeks later. Four weeks after intranasal immunization, mice that had received a dose of 10^6 pfu of any of the viruses survived the challenge with 100 LD_{50} of SC35M$_H$. The lower immunization doses of 10^3, 10^4 or 10^5 pfu also ensured survival of all animals with the homosubtypic SC35-E, SC35M$_H$-E and WSN-H7N7-E (except that three of four mice inoculated with either SC35-E 10^4 pfu or SC35M$_H$-E 10^3 pfu survived). In contrast, immunization with the

heterosubtypic SC35M_H-H1N1-E led to lower rate of survival (two of four mice). This lower efficacy corresponds to the absence of antibodies that neutralize H7 virus in the latter group and suggests viral clearance due to cell-mediated immunity. Taken together, this pilot study demonstrates that HPAIVs are highly attenuated when the polybasic cleavage site is replaced by an elastase cleavage site and that these mutants can be used as live vaccines [37].

Recently, elastase-dependent live attenuated viruses have been tested in vaccination trials in pigs, a relevant host for influenza A viruses. Two elastase-dependent mutants were generated from strain A/swine/Saskatchewan/18789/02 (H1N1): R345V and R345A. These viruses were administered intratracheally and were shown to be attenuated in pigs [38]. Both mutants induced IgG and IgA in serum and bronchoalveolar lavage fluid, high serum hemagglutination inhibition titers, enhanced levels of lymphocyte proliferation and higher numbers of interferon-gamma secreting cells at the infection site. After a second immunization, the animals developed cross-reactive antibodies and cell-mediated immune responses at higher levels. Accordingly, pigs were vaccinated twice with R345V at a dose of 4×10^6 pfu and challenged with either the homologous wild-type, the homosubtypic H1N1 strain A/swine/Indiana/1726/88 or the heterosubtypic H3N2 strain A/swine/Texas/4199-2/9/98. No vaccinated animals developed signs of illness after the challenge. Unvaccinated animals infected with either H1N1 virus developed fever and mild respiratory symptoms; infection with the less virulent H3N2 virus did not lead to any apparent clinical signs even in the mock-immunized group. Pigs challenged with the homologous H1N1 viruses displayed no detectable viral replication in lungs. The animals challenged with the heterologous H3N2 virus were protected partially: four of seven vaccinated pigs developed significantly milder macroscopic and microscopic lung lesions accompanied by significantly lower viral replication compared to the mock-immunized animals. These data suggest that the very brief replication of the elastase-dependent mutants in vivo requires two vaccinations to generate an adequate immune response and that two vaccinations could mimic natural immunity after infection with wild-type. Taken together, these results demonstrate that elastase cleavage site mutants are candidate vaccines for pigs. More practical routes of application, such as intranasal immunization, remain to be developed [39]

4 Conclusions

Proteolytic activation of the hemagglutinin is essential for multicycle replication of influenza A and B viruses. The required monobasic and multibasic cleavage sites are attractive targets for virus attenuation. Conversion of the multibasic cleavage sites of HPAIVs to a monobasic motif leads to an avirulent phenotype. However, it should be taken into account that any H5 or H7 strain with a monobasic cleavage site found in poultry surveillance is still considered a potential HPAIV precursor. It is therefore common practice to cull the whole flock. Our proof-of-principle

studies in mice demonstrated that elastase cleavage site mutants are attenuated and immunogenic. Further immunization studies in pigs revealed the suitability of this approach for relevant hosts; vaccinated animals were protected against homosubtypic challenge and partly protected against heterosubtypic challenge. For safety in the field, an influenza live vaccine probably must be attenuated by several mechanisms, among which introduction of an elastase cleavage site appears to be particularly attractive. Moreover, such a modified cleavage site prevents reassortment of the hemagglutinin gene of the vaccine strain into circulating viruses.

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Alphavirus Particle-Based Vaccine Vectors

Scott J. Balsitis, Clayton W. Beard, and Peter W. Mason

Abstract Most of the vaccines in use today are live attenuated, killed, or protein subunit vaccines. Although these vaccines have saved countless lives, there is still a need to develop safer and more efficacious ones. These improved new generation vaccines will enable us to protect more people from a greater number of different infectious disease threats. One such new vaccine technology is derived from the alphaviruses, which are single-strand, positive-sense RNA viruses in the family *Togaviridae*. By removing the genes that encode for the viral coat proteins, and replacing them with an antigen-encoding gene, these viruses become a replicon that can replicate its genome but cannot propagate new virus particles. The resources that the virus once expended to make new progeny are now diverted to making vaccine antigen within the host. As a result of this molecular alteration, the alphavirus particle-based vectors serve as an attractive technology for the development of new and improved vaccines.

1 Overview

At a very basic level, the goal of vaccination is to trick the body into believing that it is under attack from a pathogen. If this deception is successful, the immune system will mount a robust response to the perceived threat with the end result being the generation of a long-lived and protective immune state. One of the most effective ways to carry out this ruse has been through the use of live-attenuated vaccines (LAV) because they actually do infect the host, but with lowered pathogenicity. The fact that LAVs are live agents that can potentially regain the ability to cause disease (or are pathogenic in a subset of the population) results in concerns about safety. A safer alternative is to use vaccines that deliver a killed pathogen, or pieces of a pathogen as subunits. These vaccines are quite safe but tend to stimulate weak

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immune responses. The alphavirus-based vaccine vectors described in this chapter are able to fully convince the immune system that the host is infected with a pathogen such that a full-blown immune response is launched. These vectors simultaneously produce large amounts of any specified protein antigen that is then fed into the strong immune response that they have stimulated. The end result is a LAV-type immune response, but with the safety profile of a killed or subunit vaccine.

2 Alphavirus Biology

2.1 Classification, Transmission and Epidemiology

Alphaviruses are positive-sense RNA viruses that form the largest genus in the Family *Togaviridae*. The *Alphavirus* genus has approximately 30 members, all of which are known to be arthropod-borne [1]. A number of alphaviruses are important human pathogens, including Venezuelan equine encephalitis, eastern equine encephalitis, and western equine encephalitis (VEEV, EEEV, WEEV). These three agents are maintained in nature in highly restricted mosquito-warm blooded vertebrate infection cycles, in which their vertebrate hosts undergo acute self-limiting infections that produce viremias sufficient to reinfect the appropriate mosquito vectors. Infections of vertebrates, including man and domestic animals, can result in severe morbidity and mortality, producing a range of illnesses of varying severity [1]. The encephalitic alphaviruses can cause lethal infections, with case fatality rates in human beings ranging from 30 to 50% (EEEV and WEEV) to less than 5% (VEEV). VEEV, WEEV, and EEEV are restricted to the New World, while a different group of alphaviruses, whose pathogenesis is limited to arthralgias and rashes is largely (but not exclusively) found in the Old World [1]. These Old World alphaviruses include the prototype virus of the family, Sindbis virus (SINV), which is the best-studied member of the alphavirus genus, as well as Semiliki Forest virus (SFV), Ross River virus (RRV), and Chikungunya virus (CHIKV) [1]. Among these, SINV and SFV are not considered to be important human pathogens, whereas RRV and CHIKV are known to cause debilitating, but rarely fatal, arthralgias. Despite the global distributions of alphaviruses, infections are low in most human populations [2].

2.2 Alphavirus Molecular Biology

The alphavirus particle is a 70 nm diameter icosahedron that contains a single-strand, positive-sense RNA genome that is capped and polyadenylated. The genome is approximately 11.5 kb in length and is complexed to the capsid protein (C), producing a nucleocapsid core that is enveloped in a cell-derived lipid bilayer

studded with 80 spikes, consisting of three heterodimers of the E1 and E2 glycoproteins. Both glycoproteins have transmembrane domains (TMDs) that anchor the spike to the bilayer, and the internal extension of the E2 protein contacts the nucleocapsid core of the virion, orienting the glycoprotein spikes on the nucleocapsid core [3].

The alphavirus replication cycle begins with cell surface binding. A number of different cellular receptors have been identified for alphaviruses, consistent with the broad in vivo and in vitro host ranges of these viruses. E2 appears to mediate cell binding to cell-surface proteins, and repeated passage of alphaviruses in culture has been shown to select viruses that have an increased affinity for ubiquitous cell surface glycosaminoglycans (GAGs). As expected, viruses selected in this manner display GAG-dependent increases in specific infectivity. Following attachment, alphavirus particles are internalized into an endocytic compartment in a clathrin-dependent manner, where a drop in pH triggers the dissociation of E1 and E2, exposing a fusion peptide on E1 that is responsible for virion fusion to the endosomal membrane, transferring the nucleocapsid core to the cytoplasm, where it dissociates and releases its RNA cargo.

The released genomic RNA is translationally competent, and produces two polyproteins, which encode nonstructural (ns) proteins nsP1-nsP2-nsP3 and nsP1-snP2-nsP3-nsP4 [3]. The former is more abundant, due to the positioning of an opal stop-codon between nsP3 and nsP4. Both polyproteins are processed by enzymatic activity in nsP2 to yield the four functional components of the viral replication apparatus: nsP1 methyltransferase/guanylyltranferase, nsP2 proteinase/helicase (responsible for processing of both polyproteins), nsP3 phosphoprotein, and the nsP4 RNA-dependent RNA polymerase (RdRp). The translated ns proteins form replication factories on the surface of intracellular membranes that transcribe full-length negative-strand copies from the infecting genome, and this negative-strand copy then serves as a template for two positive-strand RNA molecules: the genomic RNA and a shorter collinear subgenomic RNA that corresponds to the 3′ third of the genomic RNA. This subgenomic RNA, also known as the 26S RNA, encodes the structural proteins of the virus (C and E1/E2). This RNA is transcribed at extremely high levels later in the viral replication cycle, permitting the accumulation of the structural proteins during the later phases of the viral life cycle, facilitating efficient assembly of the progeny virus. The alphavirus structural proteins are synthesized as a polyprotein precursor. The C protease is only active in cis, and its activity per se is not required for its assembly into the virion (this property is critical to certain types of transpackaging systems, see below).

The C coding region is followed in the 26S RNA by sequences encoding a glycoprotein precursor, which is co- and post-translationally processed into the mature viral glycoproteins by cellular proteases [3]. The glycoprotein portion of the 26S coding region encodes four “mature” peptides, in the order E3-E2-6K-E1. The complex cleavage scenario that produces these mature products requires both early (signal peptidase) and late (furin) cleavages to allow for the sequential formation of multiple folding intermediates that are needed to produce the mature virion spikes, consisting of three E2–E1 heterodimers that accumulate on the plasma membrane

of the infected cell. In the final step of virion assembly, the preformed nucleocapsid cores bud through spike-containing regions of the plasma membrane to produce mature virions [3].

2.3 Pathogenesis

Early work on the characterization of a cell culture-adapted, live-attenuated viral vaccine for VEE (TC-83) important provided insights into the mechanism of alphavirus pathogenesis. The strain was shown to lack the neuroinvasiveness of its parental Trinidad donkey (TRD) strain in multiple VEEV-susceptible animals. Interestingly, this attenuated strain was also shown to be much more quickly cleared from the circulatory system than the parental strain [4], and this property was proposed by these same authors to be related to a previous finding, that the attenuated virus bound more tightly to cells in culture than its parent [5]. The parental and attenuated viruses differ at a handful of genetic loci, and subsequent studies identified specific mutations in the 5′UTR and E2 regions as the major sources of the attenuation of TC-83 [6]. The E2 mutation (AA120 from Thr to Arg) [7] results in an increase in positive charge of the attenuated glycoprotein, which increases binding to heparan sulfate (HS), a negatively charged GAG. Similar mutations have been selected by cell passage of a number of alphaviruses [5], as well as other RNA viruses that are subjected to cell culture passaging regimens. In fact, similar E2 mutations have been identified for SINV [8, 9], and short-term passaging of VEEV in the presence of trypsin, designed to produce rapidly penetrating variants of the virus, selected variants with attenuated phenotypes, and a number of HS-binding mutations, including one at precisely the same position found in E2 of TC-83 [7]. Based on the expected increase in affinity for ubiquitous cell surface molecules, all of these mutations would be expected to enhance viral clearance from the circulatory system, that would be expected, in turn, to reduce a virus' ability to cross the blood–brain barrier, correlating with the reduced neuroinvasiveness of many cell culture-selected isolates. The second major attenuating mutation found in TC-83, which was found at nucleotide 3 in the genome [6], allows the mutant virus to produce less subgenomic (26S) RNA in infected cells [10]. This property could contribute to its attenuation, since VEEV virulence is controlled, in part, by the C protein (see below).

Work on adaptation of Old World alphavirus replicons to persist within cells led to the discovery that their nsP2 protein was a major virulence factor. These studies showed that wild-type replicons of SINV or SFV were virulent in cells in culture, but that forcing these replicons to persist in cells produced variants that had acquired mutations in nsP2 that rendered the replicons less cytopathic [11–14]. The selected mutations were shown to have dramatic effects on the persistence phenotype that promotes the ability of the mutant replicons to persist by up to a 100,000-fold relative to the WT replicons [11–13]. Interestingly, work by Frolov

and coworkers to adapt replicons from VEEV to persist in cells found that non-cytopathic VEEV replicons were much easier to obtain, and although the mutations associated with persistence were found in the same region of nsP2, these mutations had a less dramatic effect on this New World replicon than on its Old World counterparts [15]. Additional studies on VEEV led to the conclusion that its ability to inhibit host gene expression and counteract innate antiviral responses were associated with its C protein, rather than nsP2 [16]. Further support for the role of the C protein of New World alphaviruses in inhibition of the host's innate immune response came from work on EEEV [17].

2.4 Alphaviruses as Vectors

Several groups have adapted alphaviruses for use as vaccination vectors [18–20]. In the simplest form, this is accomplished by replacing the structural protein genes of the alphavirus with a heterologous gene of interest (see Fig. 1). The resulting RNA, called a replicon, is capable of directing its own replication and heterologous gene expression when introduced into the cytoplasm of host cells, but is incapable of forming virions or spreading to adjacent cells because it does not encode the alphavirus capsid or glycoprotein genes. If these replicons are introduced into a helper cell, in which the capsid and glycoprotein genes are expressed in trans, output virions are produced which are structurally identical to wild-type alphaviruses, but which encapsidate the replicon RNA in place of a normal alphavirus genome. These virus replicon particles (VRPs) are capable of infecting cells in vitro or in vivo, and expressing the encoded gene of interest, but are single-cycle particles incapable of cell-to-cell spread due to the lack of structural protein genes in the replicon.

Several features of alphaviruses and alphavirus replicons make them attractive candidates for use as vaccine vectors. First, VRPs stimulate strong immune responses against the encoded antigen. The broad tropism of alphaviruses allows VRPs to deliver antigen to a variety of cell types, including antigen-presenting cells [21–23]. Antigen is expressed at very high levels from the replicon subgenomic RNA, comprising up to 25% of total cell protein in cultured cells [19]. The production of antigen in vivo from replicating RNA stimulates innate immune defenses which recognize RNA virus infection and potentiate adaptive immune responses [24], while allowing antigen presentation both intracellularly and extracellularly. This results in a balanced T_H1/T_H2 profile of cellular and humoral immune responses similar to those induced by LAVs (see studies reviewed in [25, 26]).

In addition to inducing strong systemic immune responses, alphavirus VRP vaccination also has further, unexpected benefits. First, subcutaneous or intramuscular immunization with VRPs induces potent mucosal immune responses and efficiently protects against challenge with mucosal pathogens [27–33]. This allows for development of vaccines against mucosally transmitted pathogens without the need to vaccinate at a mucosal site. Second, VRPs have an adjuvant effect on

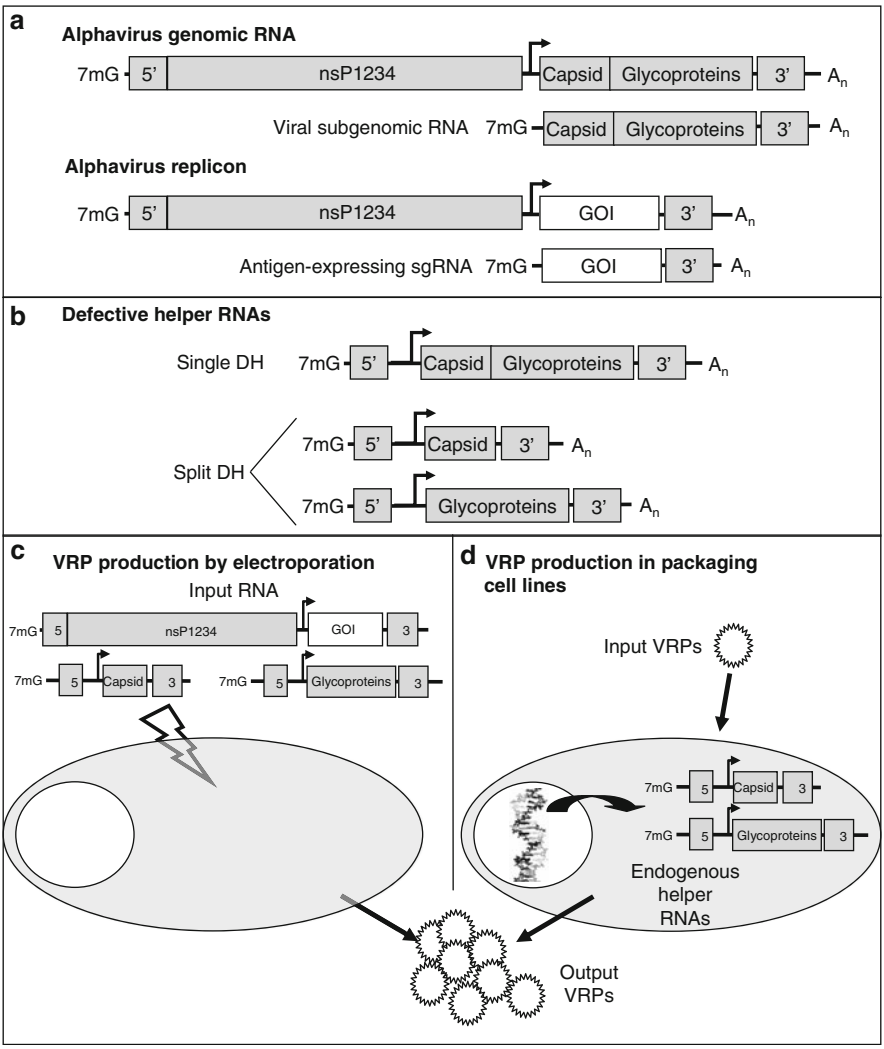

Fig. 1 Alphavirus vector constructs and production methods. (**a**) Schematics of alphavirus genomic and subgenomic RNAs as produced by wild-type viruses, and an alphavirus replicon RNA in which the alphavirus capsid and glycoprotein genes are replaced by a gene of interest (GOI). (**b**) Schematics of the defective helper RNAs most commonly used for VRP production. Single-DH constructs contain all the alphavirus structural genes in one RNA, while split-DH constructs divide these across two RNAs. (**c**) VRP production by electroporation. In this method, the replicon RNA and helper RNAs are introduced to an unmodified susceptible cell, such as BHK or Vero cell. (**d**) VRP production in packaging cell lines (PCLs). In this method, a cell line is modified to express the helper RNAs from DNA cassettes integrated into the host genome. Infection of a PCL with a replicon then triggers helper activation and VRP production

immune responses to purified protein antigens codelivered with VRPs in the inoculum [24, 31, 34], likely as a result of immune stimulation by replicating RNA. This adjuvant effect opens some intriguing possibilities, such as development of new vaccines that utilize "killed" antigens but elicit a "live" vaccine immune response [31], or making combination vaccines in which VRPs stimulate immunity to one antigen, while simultaneously potentiating responses to another, unrelated antigen, provided that the formulation and delivery are suitable for both the VRP and protein components.

Importantly, published studies suggest that the excellent immunogenicity profile of alphavirus VRPs is not compromised by pre-existing immunity to either the vector or the encoded antigen. Unlike poxvirus or adenovirus vectors, the immunogenicity of alphavirus VRPs is not subject to widespread pre-existing antivector immunity in the human population, as seroprevalence of antibodies against alphaviruses in humans is low [35, 36]. Furthermore, even the antivector immunity induced by repeated immunization with VRPs, did not impede subsequent immunization with VRPs encoding a different antigen in mice [20]. Similarly, a boost in immunity to a VRP encoded target antigen was observed in humans even when two prior immunizations had elicited substantial antivector neutralizing titers [37]. Additionally, in contrast to LAV, the immunogenicity of VRP vaccines is not greatly affected by the presence of maternal antibody against the target antigen [38].

Complementing the potent immunogenicity of alphavirus VRPs are their safety features. In contrast to the spreading infection caused by LAVs, the single-cycle replication of VRP infection ensures that vaccination cannot initiate a pathogenic infection, provided that the VRPs are free of any contaminating replication-competent viruses. An additional layer of safety is added by the RNA genome of alphavirus vectors, which only persists for about 7 days postvaccination, does not disseminate to systemic sites, and has no potential to integrate into the host genome [39]. This contrasts with plasmid DNA vaccination, where the vector DNA persists for months postvaccination, both at the injection site and at distal sites, and which could potentially integrate into host DNA [39].

In light of all these advantages of VRP vaccination, it is perhaps not surprising that VRPs effectively elicit protective immunity against an extensive list of bacterial, viral, and protozoan pathogens in a variety of small animal and primate models (reviewed in [25, 26]). While immunizing against one pathogen at a time is the most straightforward approach, multiple VRPs expressing different antigens can also be combined into a single immunization to simultaneously induce protective immunity against multiple pathogens [40]. These studies confirm that VRP vaccination is a versatile approach to inducing protection against a broad array of important human and animal pathogens.

3 Alphavirus Vector Vaccine Production

While alphavirus vectors have advantages over other vaccine vector candidates in several respects, clinical application of alphavirus VRPs has lagged behind other viral vectors, in part due to the challenges of producing VRPs at large scale. Cost-effective, scalable systems for manufacturing alphavirus VRPs have not been fully worked out, although considerable progress has been made. This section will review the issues inherent in producing alphavirus VRPs at industrial scales, and review work performed to date to overcome these limitations.

3.1 Electroporation Systems

Alphavirus VRP production occurs whenever permissive cells contain both an alphavirus replicon RNA and the necessary helper RNAs expressing alphavirus structural proteins. In most published work, VRPs are produced by coelectroporation of these RNAs into a susceptible cell, typically BHK cells, and harvesting output VRPs which are present at high titer by the next day. Such an approach has been used to efficiently produce SINV, SFV, and VEEV VRPs at small scales [18–20]. However, two problems with electroporation systems have thus far limited their utility: (1) the ability of alphavirus replicons to recombine with or copackage with helper RNAs, and (2) the difficulty of performing electroporation at industrial scale and in GMP-quality cell lines. Nevertheless, several products produced from electroporated Vero cells have made it into clinical studies [37, 41].

3.2 VRP By-Products

Recombinant viruses. The earliest attempts to produce alphavirus VRPs used an alphavirus replicon introduced into cells with a single defective helper RNA (encoding the capsid and glycoproteins as a single polyprotein, as it occurs in wild-type alphavirus genomes) [18–20]. Although these initial systems produced high titers of VRPs, this single helper system also produced plaque-forming viruses (also called replication-competent virus, RCV) at a very high frequency, with approximately 1 RCV detected for every 10^4–10^5 VRPs produced [18–20]. These RCV were generated by a recombination event (or RdRp strand switch) joining the helper genome structural protein coding regions to the replicon genome, thus reforming a genome that encoded all of the proteins necessary for producing progeny virions and a spreading infection. This outcome was consistent with studies which found alphavirus replicons to be highly prone to intragenomic RNA–RNA recombination events in electroporation systems [42], provided that the helper RNA has functional 3′ end replication signals [43]. Interestingly, these

and other studies found that alphavirus RNA recombination appears to be predominantly nonhomologous recombination, and thus cannot be prevented simply by eliminating regions of homology shared by the replicon and helper RNAs [20, 42, 43]. Moreover, SFV or VEEV VRP preparations containing RCV contaminants are lethal to intracranially inoculated mice, demonstrating that RCV poses a very real threat to vaccine safety [18, 20].

Segmented genome RCV. Importantly, the frequency of RCV detected in VRP preparations produced by electroporation of replicon and single-defective helper RNAs can be higher than the frequency of recombination events, and if the defective helper RNA contains signals for efficient packaging into virions, RCV titer can be similar to VRP titer [18, 20]. These observations suggest that these plaque forming RCVs are the result of efficient packaging of single helpers and replicons into the same infectious particle. In these scenarios, the alphavirus has, in effect, been converted into a virus with a segmented genome. Indeed, investigators have succeeded in designing two or three genome SINV that are efficiently replicated [44, 45], emphasizing that efficient helper RNA packaging could enable production of unwanted segmented genome RCV in electroporation of replicons and helper genomes.

Helper expression. The problem of segmented-genome RCV can be alleviated to a great degree by eliminating packaging signals from helper RNAs to make helper packaging less efficient [18, 20, 44]. However, even if reduced, expression of helper RNA in VRP-infected cells in vivo would be expected to elicit immune responses to vector structural proteins, which may impact the immunogenicity of subsequent vaccinations with the same vector. Furthermore, efficient helper packaging into VRPs could provide the opportunity for replicon-helper recombination to occur in vivo and produce RCV in the vaccinated host, even if RCV was not present in the initial inoculum.

3.3 Safety Improvements

RCV prevention. Of the three by-products of VRP production listed above, replicon-helper recombination has received the most attention because it appears to have the greatest potential to produce an RCV that compromises vaccine safety, especially for alphaviruses that are highly pathogenic in humans such as VEEV. Consequently, considerable effort has been made to reduce the frequency with which recombination events produce functional infectious virus.

One of the most successful approaches thus far to prevent RCV while retaining high VRP yields has been the “split-defective helper” approach [46]. In this system, the single defective helper RNA used in earlier studies is “split” into two helper RNAs, one encoding the capsid gene, and a second encoding the glycoprotein genes, so that a replicon RNA must make two independent recombination events to form a virus genome containing all of the alphavirus genes. This approach, first published with SINV [46], was also adapted to SFV and VEEV

VRPs [20, 47], and in all cases no RCV was detected in 10^8–5×10^9 VRPs produced using the split-DH system. As an added benefit, the split-DH system probably also reduces the likelihood of forming segmented-genome RCV, as a three-component genome would be less likely to form and efficiently passage than a two-component genome.

In the SFV iteration of this split helper system, a further safety enhancement to the split-DH system was made by ablating the autoprotease activity of the capsid protein so that even if two recombination events rejoined the capsid and glycoprotein genes into a single polyprotein gene, the resulting protein would not function [47]. This innovation would certainly prevent regeneration of a wild-type alphavirus genome, with a single subgenomic promoter driving a structural protein polyprotein, but would not prevent the formation of an RCV replicon encoding the capsid and glycoprotein genes under the control of two separate subgenomic promoters.

Although the split DH system has eliminated detectable RCV in small research-scale VRP lots, mathematical considerations suggest that the split-DH approach may not reduce RCV to levels needed to ensure safe use in humans. Since recombination between a replicon and one helper RNA occurs at a frequency of 10^{-4}–10^{-5} [18, 20, 48], the expected frequency of a dual-recombination event between a replicon and two helper RNAs would be approximately 10^{-8}–10^{-10}. This is consistent with the published literature which did not find RCV in lots of this size when split-DH RNAs were used [20, 46, 47], but suggests that an industrial-scale lot of 10^{16} VRP could be contaminated with 10^6–10^8 RCV. Thus, either additional safety features may still be needed for alphavirus VRPs to reach commercialization, or the RCV formed must be sufficiently attenuated to ensure that it does not pose a human health risk.

One design innovation that may be able to reduce the frequency of replicon-helper recombination events is the removal of the subgenomic promoter from the helper RNAs [49]. Doing this reduces the theoretical number of possible recombination events that would still result in functional structural protein expression from the recombinant replicon, because the helper-replicon recombination must occur in a precise way that places the structural protein under the control of the subgenomic promoter that was present in the replicon, as opposed to the structural protein gene having an attached promoter that can function from a variety of recombination sites. This approach can be used successfully without a reduction in VRP titer [49], but the theoretical benefit has yet to be tested experimentally to show that the rate of RCV is actually reduced in this system.

RCV attenuation. Alphavirus envelope glycoproteins strongly affect both viral tropism and pathogenicity. Consequently, if VRPs are made with glycoproteins from alphaviruses with low pathogenicity in humans, then the health risk posed by RCV is reduced. Two groups have pursued such a strategy to improving VRP safety, in both cases with the use of VEEV replicons.

In the first approach, attenuating mutations can be introduced into the envelope glycoproteins of VRPs. For example, mutations that increase virion affinity for heparan sulfate decrease the neuroinvasiveness of alphaviruses by reducing the

level of viremia that is established during infection [7]. Introducing these mutations into defective helpers would attenuate any RCV produced, but would also alter VRP tropism, and therefore could affect immunogenicity, in vivo. However, VEEV VRPs packaged using envelope glycoproteins with varying heparan sulfate affinity showed only moderate variation in immunogenicity in vivo [50], demonstrating that attenuating mutations can be used in VRPs without dramatically altering immunogenicity. Thus VRP vaccine safety can be improved by packaging replicons in virions derived from attenuated virus strains; however, even attenuated strains of VEEV retain some pathogenicity by peripheral inoculation in animal models [7], so it is unclear whether introduction of attenuating mutations into glycoproteins alone will be sufficient to ensure that RCV by products do not compromise the safety of VRP vaccines.

A much greater degree of RCV attenuation appears to be possible with a second approach based on chimeric VRPs. In this case, VRPs were made in which the replicon is derived from VEEV, but the structural proteins are derived from SINV [51]. To ensure efficient packaging of VEEV replicons into SINV virions, the well-defined SINV packaging signal was introduced into the nsp3 region of the VEEV replicon. The resulting VRPs are similar to VEEV VRPs in production yields from electroporated BHK cells, RNA replication and antigen expression in infected cells, interferon resistance, and immunogenicity in vivo [51].

Thus, VEE/SIN chimeric VRPs retain important features of VEE VRPs, but there are several reasons why any RCV produced from a VEE/SIN VRP system is likely to be much less pathogenic than RCV produced from a VEEV-only system. While VEEV is a virulent human pathogen capable of causing fatal encephalitis, SINV is not neurotropic in humans, causes disease only rarely, and is not life-threatening [1]. Furthermore, several chimeric alphaviruses have been made in which the replicon genome of a low-pathogenicity virus (SINV) is combined with the structural proteins of a more pathogenic virus (VEEV, WEEV, EEEV, or CHIKV). In all cases, the chimeras were attenuated compared to the pathogenic parent viruses [52–56], suggesting that chimerization is inherently attenuating to alphaviruses. This was confirmed in vitro for a chimeric virus constructed between the VEEV nonstructural proteins and SINV structural proteins [57], which resembles the RCVs that could occur during VEE/SIN chimeric VRP production. This chimeric virus is unable to shut down host cell gene expression, is noncytopathic in vitro, is cleared from cultures of interferon-competent cells, and was reported to be nonlethal when inoculated intracranially into mice [57]. This degree of attenuation is likely a result of the fact that the VEEV capsid and glycoprotein genes are both major pathogenicity determinants for VEEV, and VEE/SIN RCV encodes neither of these proteins. Combined, these data suggest that VEE/SIN RCV may be severely attenuated and pose little or no human health risk. Safety could be enhanced even further if attenuating mutations were added to glycoprotein genes of the chimeric VRP, so that multiple layers of protection prevent RCV from being pathogenic in vivo. However, more detailed animal pathogenicity studies are still needed to thoroughly document the attenuation of chimeric RCV.

3.4 Industrial-Scale Production

Electroporation has been the most common methodology for VRP production because at small scale it is simple and affordable, and the only specialized equipment required is a commercially available electroporator. However, it is not clear if electroporation can be performed at industrial scales in a cost-effective manner for all target populations. A typical VRP electroporation protocol requires trypsinization of adherent cells, followed by multiple wash steps and electroporation of cells in individual cuvettes, followed by cell plating in adherent format and harvest the next day. While such a multistep protocol is easy to carry out on a bench top with a limited number of cells, automating electroporation for industrial use is difficult and will require specialized equipment that does not currently exist. While these hurdles may be surmountable, they are likely to add considerably to the cost of developing an alphavirus VRP-based vaccine. Nevertheless, electroporation has been successfully performed under GMP at scales at least sufficient for phase I clinical trials [37].

As an alternative, VRP packaging cell lines (PCLs) could be used for VRP production. One type of PCL that has been developed consists of an alphavirus-permissive cell line with DNA cassettes expressing the defective helper RNAs stably integrated into the host genome. In this PCL, the helper RNAs are constitutively expressed, but the alphavirus structural proteins are not, because the genes are under the control of an alphavirus subgenomic promoter [58]. Upon introduction of an alphavirus replicon into the PCL by transfection (or VRP infection), the replicon-encoded replicase enzymes are produced, and trigger replication of the cell-encoded capsid and glycoprotein genomes and subgenomes, producing the structural proteins needed to package the replicon genome (Fig. 1). Thus, PCLs allow VRPs to act as self-propagating viruses. This technology allows VRPs to be produced in much the same manner, and using the same equipment and methodologies that are in use for the production of traditional or genetically engineered live-attenuated viral vaccines. Despite these advantages, amplifying VRPs through multiple passages on PCLs also may provide multiple opportunities for recombination and RCV formation, although in published work PCLs utilizing the split-DH system did not produce detectable RCV at small-scale [58].

In summary, while considerable progress has been made in alphavirus VRP production, issues of safety and scalability must be more fully addressed before the potential of alphavirus VRPs will be fully realized.

4 Preclinical/Clinical Evaluation

Many alphavirus replicon-based vaccines have been tested at the preclinical level. Replicons have been used to express antigens from infectious disease agents such as influenza, HIV, SHIV, SIV, Louping ill, dengue virus, *Plasmodium falciparum*,

hepatitis C virus, infectious bursal disease virus, human papilloma virus, Lassa virus, Marburg virus, equine arteritis virus, and Ebola virus, as well as tumor antigens. Most of these constructs have performed well in small animal models, and those that have moved further into preclinical development have functioned well in nonhuman primates. A VEEV-based replicon expressing the G protein from Marburg virus was able to completely protect nonhuman primates (cynomolgus macaques) from viremia and disease upon lethal Marburg virus challenge [59]. Both Semliki Forest virus-based [60] and VEEV-based [61] replicon vaccines expressing SIV immunogens have been found to confer partial protection from lentiviral disease as exemplified by lowered peak viral titers or lessened disease signs. A chimeric replicon system consisting of a VEE –based replicon packaged with SINV structural proteins has been used to develop a prototypic next generation measles vaccine, expressing the measles virus hemagglutinin (H) or hemagglutinin and fusion (H+F) proteins. When tested in nonhuman primates, the vaccine was found to induce neutralizing antibody titers and T cell responses that were similar to those induced by the current live attenuated measles vaccine [62]. These animals were protected from measles virus challenge, but did show a reappearance of measles virus RNA in their PBMCs 4 months after challenge. This recrudescence was not seen in macaques that received the measles LAV, and further experiments will be required to fully understand the immunological mechanisms that surround measles virus clearance and to determine if additional antigens are required for more effective vaccination. The chimeric replicon expressing the measles hemagglutinin protein, was compared to a formalin-inactivated alum-precipitated vaccine in mice. The LAV measles vaccine is unable to replicate in mice and was therefore not included. The replicon-based vaccine induced a balanced B and T cell response to the H protein that produced high affinity neutralizing antibody titers, [63] while the formalin-inactivated vaccine elicited no neutralizing titers. This work fully demonstrates the benefits that the alphavirus-based replicon system provides over a formalin-killed vaccine.

Recently, the results of a phase 1 clinical trial of a VEE alphavirus replicon-based vaccine against cytomegalovirus (CMV) has been reported [64]. This vaccine contained two replicon particles, one that expressed CMV gB, and the second that expressed a CMV pp65/IE1 fusion protein. Four groups of eight individuals each received either a lower dose (1×10^7 IU) or higher dose (1×10^8 IU) by subcutaneous or intramuscular injection at 0, 8, and 24 weeks. The vaccine was found to be safe and produced only mild to moderate reactions at the injection site. All individuals generated neutralizing antibody titers against CMV following the initial immunization and these titers increased following each subsequent immunization, despite the development of antivector immune responses. This alphavirus replicon vaccine also induced polyfunctional CD4+ and CD8+ antigen-specific T cell responses to CMV pp65, gB, and IE1 in almost all of the study participants. The ability of these vaccine vectors to safely stimulate both humoral and cellular immune responses in healthy adults provides good support for their continued development for use in human vaccine development.

5 Future Prospects

The future of alphavirus-vectored vaccines is bright. They offer a vehicle that can produce a desired antigen within the vaccinated individual in such a way that the immune system reacts with both cellular and humoral arms. This response results in CD4 and CD8 T cell, systemic antibody, and mucosal antibody responses that can effectively prevent infection by a vast number of pathogens. The large amount of data investigating the molecular mechanisms of alphavirus replication and pathogenesis leaves us with the road maps and tools to fine-tune these vaccine candidates for optimal antigen production and immune responses.

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Recombinant, Chimeric, Live, Attenuated Vaccines Against Flaviviruses and Alphaviruses

Thomas P. Monath

Abstract Many arthropod-borne flaviviruses are important human pathogens responsible for diverse illnesses, including yellow fever (YF), Japanese encephalitis (JE), and TBE and dengue. Live, attenuated vaccines have afforded the most effective and economical means of prevention and control, as illustrated by YF 17D and JE SA14-14-2 vaccines. Recent advances in recombinant DNA technology have made it possible to explore a novel approach for developing live attenuated flavivirus vaccines against other flaviviruses. Full-length cDNA clones allow construction of infectious virus bearing attenuating mutations or deletions incorporated in the viral genome. It is also possible to create chimeric flaviviruses in which the structural protein genes for the target antigens of a flavivirus are replaced by the corresponding genes of another flavivirus. By combining these molecular techniques, the DNA sequences of DEN4 containing a deletion in the 3′NCR, a DEN2 PDK-53 candidate vaccine and YF 17D vaccine have been used as the genetic backbone to construct chimeric flaviviruses with the required attenuation phenotype and expression of the target antigens. Encouraging results from preclinical and clinical studies have shown that several chimeric flavivirus vaccines have the safety profile and satisfactory immunogenicity and protective efficacy to warrant development as products for human use. The chimeric flavivirus strategy has led to the rapid development of novel live, attenuated vaccines against DEN, TBE, JE and WN. This chapter reviews an extensive body of work on the development of these vaccine candidates, one of which is licensed and others are in advanced clinical development.

A similar approach is being used to create vaccines against alphaviruses. Here the experience is less, but some promising data have been developed, particularly using SIN virus as a vector for structural genes of heterologous alphaviruses. The principal issues for this technology will be to achieve convincing nonclinical data on safety, the proper balance of attenuation and immunogenicity, and proof of concept in large animal models and ultimately humans.

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1 Introduction

This chapter focuses principally on live, chimeric vaccines against flaviviruses, since these vaccines are in clinical development or are licensed. Similar technology is being used for development of new vaccines against medically important alphaviruses, and this topic will be discussed more briefly.

It is assumed that the reader has a general knowledge of flavivirus and alphavirus structure and replication, and an understanding of the medical need for new vaccines against flaviviruses and alphaviruses. Chimeric vaccines contain the structural genes of one flavivirus (alphavirus) against which immunity is desired and the genes encoding nonstructural (NS) proteins of another flavivirus (alphavirus), which serve as the vector or "backbone". The NS proteins from the vector provide the replicative machinery of the chimeric vaccine, whereas the immune response to infection with the vaccine virus is directed principally at the envelope (E) protein (flaviviruses) or proteins (E1 and E2, alphaviruses) provided by the gene donor. The E protein(s) contains critical antigens for stimulation of neutralizing antibodies, which are the principal mediator of protective immunity against flaviviruses and alphaviruses. Attenuation of virulence is determined by a combination of factors, including chimerization itself and mutations in structural and/or vector sequences resulting from natural evolution, passage in laboratory hosts or cell culture, or site-directed mutagenesis. Assembly and replication of chimeric virions are efficient due to the consistency of genome organization and function across members of the genus Flavivirus (Alphavirus).

The extraordinary immunity provided by natural infection with flaviviruses (alphaviruses) provides an important benchmark for defining immune correlates of protection during the development of new vaccines. Fortunately, the evolutionary diversity of members of the genus Flavivirus (Alphavirus) defined by neutralization is very different from influenza, HIV, and enteroviruses, where antigenic diversity greatly complicates vaccine development. Each medically important flavivirus represents a single serotype; the same is true of medically important alphaviruses, with the exception of Venezuelan equine encephalitis (VEE), which occurs in multiple subtypes. There are few examples of important human or veterinary flavivirus diseases caused by multiple virus species that cocirculate in sympatric fashion, requiring a multivalent vaccine. Dengue (caused by four distinct viruses) is the only important example. Even where a syndrome (encephalitis) is caused by sympatric flaviviruses [e.g., Japanese encephalitis (JE) and either West Nile (WN) or tick-borne encephalitis (TBE) viruses in parts of Asia, or WN and St. Louis encephalitis (SLE) in the Americas], one disease so overshadows the other that multivalent vaccines have not been a priority for development. The situation is similar for the medically important alphaviruses, with eastern equine encephalitis (EEE), western equine encephalitis (WEE), epizootic VEE subtype I, Getah, Ross River (RRV) and (except for some areas of Africa) chikungunya (CHIK)[1] having

[1]In parts of Africa, a related virus o'nyong nyong has an overlapping range with CHIK.

geographically distinct distributions. In addition to the lack of antigenic diversity and relatively simple epidemiological situation, a tremendous advantage to vaccine development is the quality of the immune response to flaviviruses (alphaviruses) and the availability of well accepted immunological correlates of protection. Infection is always followed by natural immunity, and it is strong and highly durable (essentially life-long). These qualities associated with natural infection have been embodied in artificial immunization with the live, attenuated vaccines, especially yellow fever (YF) 17D vaccine. The basis for the remarkable immunogenicity of YF 17D virus has been the subject of recent enquiry, which showed, as had been long suspected, that infection efficiently activates multiple pathways of the innate immune system, a prerequisite for effective adaptive immunity and long term memory [1–3]. It is not surprising, therefore, that this property of live flavivirus infection would be harnessed by using live vaccines as vectors for foreign genes, with the expectation that the vector would impart a similar quality of immune response on the foreign gene carried by it. Over the last 15 years a wealth of information has accumulated on this strategy, with the result that there are multiple chimeric flavivirus vaccines in clinical development, using several vector species. As with any platform technology, it takes dedicated industrial development, years of evidence based science, and regulatory consensus before applications "take off". One chimeric live vaccine (against WN) is now approved for veterinary use, another (against JE) is in registration in several countries, and, perhaps most importantly, several vaccines against dengue (DEN) are in clinical development.

2 Principles for Use and General Properties of Chimeric Vaccines

This review focuses on live, chimeric vaccines against flavivirus, and, more briefly alphavirus infections. Other chapters address different vaccine technologies, including single cycle replicons and DNA immunization, that could utilize a chimeric approach. Chimeric recombinant E proteins [4] represent another yet approach not covered in this chapter.

Multiple live vector platforms have been developed in addition to flaviviruses and alphaviruses, poxvirus, adenovirus, cytomegalovirus, vesiculovirus, paramyxoviruses (Newcastle's disease virus), and others. A major problem common to these technologies is that pre-existing immunity to the vector or immunity generated to the vector after priming constrains their use. A principal approach to skirting this problem is to remove protective antigenic determinants from or to switch serotypes of the vector. This, however, raises a number of issues, since one serotype of the vector may be significantly less immunogenic than another (a common problem for adenoviruses). Flavivirus and alphavirus vectors have the advantage that the structural genes can be substituted across multiple viruses in the genus and that the

remaining genes (of the vector backbone) do not contribute materially to protective immunity. In this way novel vaccines can be constructed against multiple members of each genus without interference from immunity to the vector.

This chapter will describe the use of flaviviruses (and alphaviruses) for constructing live vaccines against other flaviviruses (alphaviruses). Use of flavivirus (alphavirus) vectors for foreign genes will not be considered, but the reader should be aware that there is interest in this approach. For example, it is possible to insert relatively small foreign gene coding inserts at one or more sites within the envelope (E) gene of the flavivirus vector, so that 180 copies of the gene product would be displayed on each E protein monomer on the virion surface; this approach is restricted to small inserts, the size of a single epitope, that do not perturb virion assembly. In another example, larger genes (up to 1–2 kb) may be positioned in a flavivirus vector downstream of the structural genes. Various insertion points have been successfully employed, including the intergenic regions between the E and NS1 genes [5] or the NS2b and NS3 genes [6, 7] , or the placement of an internal ribosome entry site and foreign sequence between NS5 and the 3′ untranslated region (UTR). Since the structural genes of the vector are preserved, these approaches are complicated by antivector immunity; it may be necessary to use a vector for which there is very low immunity in the intended target population and to construct one or more vectors with different E proteins for use in boosting immunity. In the case of foreign genes inserted in the flavivirus (alphavirus) backbone, it is possible to avoid anti-vector immunity by exchanging structural gene sequences for one of up to 70 flaviviruses (29 alphaviruses) comprising the genus.

The terminology used in this chapter to refer to chimeric constructs is to place the virus donating structural genes and the target for immunization first, and the vector second. Thus a chimeric virus with structural genes of JE and the backbone of YF is referred to as a "JE/YF chimera."

3 Flavivirus Vaccines

3.1 Molecular Construction and Rationale Design

The construction of chimeric Flavivirus vaccines depends on recombinant techniques employing cDNA plasmids derived from flavivirus RNA, site directed mutagenesis to introduce desired deletions and mutations, an understanding of the structure and function of the Flavivirus genome, and knowledge of the molecular basis of virulence and antigenic structure. These aspects are covered in recent reviews [8]. Briefly, the 10.6 kb single strand, positive sense RNA genome is organized with a single long open reading frame with three structural genes encoding the capsid (C), premembrane (prM) and envelope (E) genes at the 5′ end and genes for seven NS proteins at the 3′ end. The viral RNA is translated as a polyprotein and is post-translationally processed into the individual viral proteins

by host and virally encoded enzymes. There are short 5′ and 3′NCRs at the respective termini which play important roles in translation and replication of the viral RNA. Rice et al. [9] first described the generation of full-length infectious RNA transcripts derived from cDNA of yellow fever 17D (YF 17D) virus. These transcripts were prepared using a two-plasmid system by cloning the 5′ and 3′ halves of the genome separately and then ligating them in vitro prior to transcription. Instability of full-length cDNA in *E. coli* proved to be an impediment for several other flaviviruses. The two-plasmid system was also used to obtain the infectious RNA transcripts of JE virus strain JaOArS982 [10]. A stable full-length cDNA copy of wild type DEN4 strain 814669 was first cloned in *E. coli* strain BD1528 for transcription of infectious RNA [11]. Full-length cDNA clones of many other flaviviruses including DEN, WN, and Langat viruses have subsequently been described.

There are several general approaches to the construction of a flavivirus chimeric vaccine. The first and most commonly used approach substitutes the prM-E structural protein genes in the vector for the corresponding genes of the heterologous virus against which immunization is desired. All three structural genes (C-prM-E) can be replaced, but in general these constructs are more attenuated, less likely to replicate efficiently for manufacturing and less immunogenic. Construction of a chimera requires that the structural genes be sourced from another flavivirus, since proper assembly of progeny virions requires a high degree of sequence homology.

The use of cDNA clones for introduction of mutations into the vector backbone or E gene(s) by site-directed mutagenesis allows analysis of their effects on the phenotype of the recovered viruses. Many studies have shown that the virulence phenotype can depend on a single mutation that decreases or increases the efficiency of viral replication in cultured cells or in animals. Genetically defined mutants have been constructed for YF [12], DEN4 [13], DEN2 [14], JEV [15, 16], WN [17] and TBE [18]. Mutations in DEN chimeric viruses have been engineered to improve viability and increase yields in cell cultures [19]. Mutations in the NS genes have been introduced to attenuate DEN4 virus [20]. Deletions engineered into the 3′ and 5′ NCRs of several flaviviruses produced attenuation in cell culture and in animals [21–26]. Mutations in the hinge region spanning domains I and II of the flavivirus E glycoprotein [16, 17, 27] and in the upper–lateral surface of domain III [18] have been shown to reduce virulence; but, in one case a hinge region mutation increased virulence [28]. Ablation of the glycosylation site in E or in NS1 has been shown to markedly attenuate viremia and neuroinvasiveness [29]. Site directed mutagenesis is an important method for rationally attenuating vaccine candidates and for deciphering the biological effects of mutations that occurred during passage in the empirical development of live vaccine strains used as gene donors or backbones in constructing chimeras. Wild type and mutated DEN4, the DEN2 PDK53 candidate vaccine, and YF 17D vaccine strains have been explored as vectors in constructing chimeric vaccines.

Infectious clone technology has many advantages for manufacture of live, attenuated vaccines in cell culture. The manufacturing method begins with full length chimeric RNA transcribed from cDNA, and transfection of the RNA into an

acceptable cell line for manufacture. This approach is recognized by regulatory authorities as reducing the likelihood of contamination with adventitious viruses, and such testing may potentially be avoided. Moreover, the recombinant vaccine virus is clonal and can be precisely defined by sequencing for quality control and assessment of genomic stability. However, since normally flaviviruses and alphaviruses are genetic swarms, and their biological behavior is determined by the population of variants, care must be taken to determine that the clonal population has the desired biological phenotype. A powerful strategy for evaluating clonality and expression of recombinant inserts is limiting dilution cloning and evaluation of 50–100 clones by sequencing.

A critical aspect of the use of any live vaccine, including chimeric viruses, is obtaining the correct balance of attenuation and immunogenicity (Fig. 1). This balance, which also determines the rate of growth and yields in cell cultures used for manufacturing, is determined by both the gene insert and the vector backbone. Some confidence can be derived by use of a vector with known properties of attenuation and immunogenicity, for example an attenuated live vaccine with a long history like YF 17D. As will be described below, both the structural gene insert and the NS backbone sequences influence the biological phenotype of the chimera, so it is sometimes necessary to further attenuate the virus by introducing mutations or deletions in the structural gene insert or backbone. It is well established that the process of chimerization is in itself attenuating and that substitution of structural genes or the nonstructural backbone for sequences from wild-type virulent viruses usually does not usually confer a virulence phenotype exceeding the parental viruses [30–32]. This is not always so. When the backbone virus is attenuated, the insertion of structural genes from a virulent virus can confer a higher degree of virulence (an example being the increased neurovirulence observed when TBE structural genes were inserted in a DEN4 backbone [33]). Therefore each construct must be subjected to empirical study.

Attenuation must be assessed carefully in various model systems before introducing a chimeric virus into humans. These assessments generally include evaluation

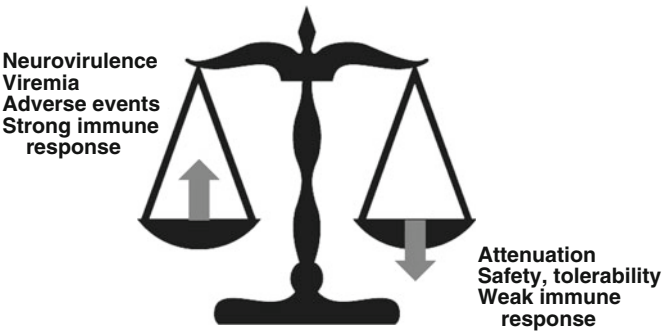


Fig. 1 Phenotype of live vaccines requires proper balance of attenuation and immunogenicity that must be sought through empirical testing

of neuroinvasiveness and neurovirulence in suitable animal models [34], since all flaviviruses have the capacity to infect and cause damage to brain and spinal cord tissue. Viscerotropism or special pathogenic features may be more difficult to assess in animal models, but viremia (mean peak, mean duration, area under the curve), levels of virus replication in tissues of the host, tissue specific enzymes, and levels of proinflammatory cytokines may be useful markers [28, 30]. It may be instructive to employ hosts that are deficient in adaptive or innate immune responses, such as interferon receptor-deficient mice, to reveal residual virulence of a chimeric construct or to demonstrate safety in the presence of immune deficiency. Attenuation of DEN chimeric viruses has been assessed using SCID mice reconstituted with human hepatoma (HuH-7) cells [20, 25, 26] since liver is a target organ for DEN viruses in humans.

A theoretical concern about the use of chimeric viruses for immunization is the reliance on epitopes within the structural (transplanted) proteins to confer "complete" immunity. While neutralizing antibodies to the E glycoprotein are accepted correlates of protective immunity, and are specific for the pathogen represented by the foreign genes, NS proteins that may contribute to immunity are derived from the heterologous vector. Relatively little is known about the contribution of the NS proteins to protective immunity, but there is evidence that both anti-NS1 antibodies [35] and T cell responses [36] play a role in protection and immunological memory. Immunity to shared T cell epitopes in the vector and target virus may account for some cross-protection. As experience has accumulated, these concerns have not proven critical to vaccine development. First, multiple T cell epitopes, including MHC-I restricted epitopes, reside in the flavivirus E protein [37–40]. Second, the contribution of anti-NS immune responses to protective immunity may not be great. In one illustrative example, a WN/DEN2 chimera induced high titers of DEN2 specific NS1 antibodies (and presumably T cell responses to DEN2 NS proteins) but afforded limited protection against DEN2 virus challenge [41]. Moreover, immunity to chimeric live vaccines has been durable (lasting years) and recall occurs on boosting. In the specific case of DEN vaccines, it may in fact be desirable to use a heterologous vector, since T cell responses to DEN (in the absence of neutralizing antibodies) may play a role in the pathogenesis of dengue hemorrhagic fever (DHF) [42].

When developing a new vaccine it is important to consider carefully the indications for use and the target product profile (Table 1). This can be an early guide to the applicability of a chimeric flavivirus vector.

3.2 Chimeric Flaviviruses Using Yellow Fever 17D Vaccine as the Vector

Yellow fever 17D vaccine was developed in 1936 by empirical passage as a highly effective live attenuated vaccine, and has been used in 500–600 million travelers

Table 1 Target product profile for a live, attenuated vaccine

- Disease indication
- Incidence, severity and lethality of the disease to be prevented (important in assessing risk: benefit equation)
- Vaccine formulation [virus(es), stabilizers, adjuvant, excipients]
- Vaccine potency (minimum and maximum), test method, units
- Presentation (liquid, lyophilized, vial size and no. doses/vial, prefilled syringe, etc.)
- Storage temperature (before and after reconstitution)
- Thermostability (before and after reconstitution)
- Toxicity (if any) in nonclinical tests (e.g., lethal for infant mice 5 days of age or less when inoculated IC)
- Age group(s) indication
- Geographic, occupational, vocational indications
- Dose (volume, potency units)
- Route of inoculation
- Safety (incidence of severe and serious adverse events; list specific expected adverse events if known)
- Tolerability (incidence of common side effects, if any; list expected adverse events, if known)
- Viremia level and incidence
- Precautions
- Contraindications
- Optimal immunogenicity (e.g., seroprotection rate, if level of protective antibodies known; or non-inferiority of GMT to a licensed vaccine)
- Minimal acceptable immunogenicity
- Secondary immune response measures (e.g., T cell assays)
- Specificity (immunity vs. all antigenic variants or subtypes, genotypes of virus targeted?)
- Cross-protection against heterologous, related virus species
- Interactions with other vaccines
- Transmissibility by vector mosquitoes (ticks)
- Shedding, secretion, environmental concerns

Each of these factors should be described early in the development process, and refined as work proceeds

and residents of endemic countries in Africa and South America (reviewed in [43].). YF 17D is delivered as a subcutaneous (SC) inoculation, but can also be administered by the intramuscular, intradermal (ID) or epidermal [44, 45], or intranasal [46] routes. Wild-type YF virus has been shown to infect monkeys by the oral route (intragastric inoculation) [47], and there are two recent reports of adverse events caused by inadvertent YF 17D infection in infants breastfeeding on mothers who were recently vaccinated (CDC, unpublished), suggesting that use of 17D vectors for oral or enteric immunization might be possible. Yellow fever 17D is one of the most, if not *the* most effective vaccines, rapidly inducing neutralizing antibodies – the mediator and surrogate of protection – in 90% of vaccinees within 10 days and in $\geq$99% within 30 days after inoculation [43]. Yellow fever 17D also evokes robust cytotoxic T cell and memory T cell responses [48, 49]. The vaccine contains a large excess of virus, about 4.7 $\log_{10}$ plaque-forming units (PFU)/0.5 mL dose, whereas the 50% immunizing dose is only approximately 50 PFU [43]. This remarkable efficacy demonstrated by low dose requirements was also observed for a JE/YF chimeric virus (ChimeriVax™-JE) in humans [50]. The YF 17D

vaccine is indicated for persons 9 months of age or older. Immunity lasts decades and is probably life-long [51]. However, sterilizing immunity is not complete, since booster doses result in an increase in antibody levels, albeit typically blunted [52]. Based on these observations, it is likely that a similar negative boosting effect might occur when a chimeric vaccine with prM-E proteins against a virus for which preformed neutralizing antibodies were present is used for primary immunization or boosting. Yellow fever 17D vaccine is well tolerated, and local and systemic side effects are minimal (reviewed in [43].). The incidence of serious neurotropic and viscerotropic adverse events is 0.8 and 0.4 per 100,000, respectively [53]. The occurrence of these adverse events raises the obvious question of whether similar reactions will be seen with chimeric viruses utilizing the 17D as a vector. However, data to be presented below indicate that YF chimeric vaccines are more attenuated than parental 17D with respect to neurovirulence, are attenuated for growth in human liver cells [54], and are more rapidly cleared from tissues of nonhuman primates [39]. Finally, the YF 17D vaccine virus and chimeric vaccines with the YF 17D backbone are incapable of infecting mosquitoes by the oral route.

Based on the biological characteristics outlined above, YF 17D is considered an ideal vector for heterologous genes encoding protective antigens. Multiple groups are engaged in vaccine development using this strategy, including Sanofi Pasteur (formerly Acambis), Fiocruz, and the Rockefeller University. An underlying hypothesis for use of YF 17D as a live vector is that it will impart to the resulting chimera a phenotype that resembles the parental virus. This is an assumption that needs to be tested on a case by case basis, but it has held up quite well.

What accounts for the remarkable immunogenicity of YF 17D and for the durability of the immune response? It is generally understood that the live virus infection strongly up-regulates innate immunity, including activation of the AIM2/inflammasome pathway and toll-like receptor pathways, which lead to production of IL-1β and interferon-α/β, driving the adaptive immune response [3]. The tropism of flaviviruses for cell receptors is determined by ligands on the E glycoprotein. An important target cell for YF 17D and other flaviviruses are dendritic (DC) cells [7, 54] including Langerhans cells in the skin [55]; the receptor is the lectin DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) and the ligands on flaviviruses are glycosylation sites on the E protein. Thus, YF 17D hijacks DCs for initial replication and movement to regional and systemic lymphoid tissue and functional maturation. In doing so, multiple activators of innate immunity are triggered, including toll-like receptors (TLR) 2, 7 and 8 [1]. TLR ligands enhance effector functions of other innate immune cells and synergize with T cell receptor and B cell signaling to enhance cytotoxic T cell and antibody responses. Querec et al. [2] and Gaucher et al. [3] studied the early innate immune gene activation and cytokine profiles in humans vaccinated with YF 17D and found multiple correlations between specific gene activation signatures and B and T cell responses. Since many if not all flaviviruses utilize DCs in early stages of replication, it is likely that chimeras utilizing YF 17D as the backbone but with heterologous flavivirus prM-E genes will similarly activate innate immune pathways.

Use of an established commercial vaccine as a vector provides a benchmark against which the chimeric virus phenotype can be compared. Thus the nonclinical and clinical behavior of chimeric YF vaccines can be measured against comparator groups that receive the commercial YF 17D vaccine. The safety of YF 17D vaccine and chimeric vaccines derived therefrom may be tested preclinically by intracerebral (IC) inoculation of mice and monkeys, and neurovirulence compared to parental YF 17D in dose response experiments (establishing PFU/LD_{50}) or by semiquantitative scoring of histopathologic lesions [34, 56]. These methods (particularly dose response neurovirulence tests in infant mice) can detect the biological effects of single mutations [34]. A chimeric vaccine that is more neurovirulent than YF 17D in animal models would be expected to be associated with a higher incidence of neurotropic adverse events in humans, as established many years ago by Fox et al. [57] In a recent study, a Langat/DEN4 chimera proved more neurovirulent than YF 17D in monkeys and thus potentially unsuitable for further development [58]. Similarly, viscerotropism of a chimeric virus can be benchmarked against YF 17D by determining viremia profiles or other markers such as liver enzymes or levels of proinflammatory cytokines. See the section on chimeric WN/YF and JE/YF viruses for specific examples.

3.2.1 Chimeric JE/YF Vaccine (ChimeriVax™-JE, IMOJEV™)

The vaccine candidate ChimeriVax™-JE was originally developed by Acambis Inc. and was acquired by and now branded as IMOJEV™ by Sanofi Pasteur. Since published work to date refers to the vaccine as ChimeriVax™-JE, this terminology will be used below.

ChimeriVax™-JE is a live, attenuated, genetically engineered virus, prepared by replacing the prM-E genes of YF 17D vaccine virus with the corresponding genes of JE virus [59]. The E protein contains redundant epitopes specifying neutralizing antibodies and T cell responses [40, 60]. At least four major B cell epitopes have been identified in the E protein of JE virus at amino acid residues 327–333, 337–345, 373–399 and 397–403 [61–63] and important T cell epitopes at amino acid residues 60–68 and 436–445 [64, 65]; collectively, these epitopes as well as others not yet defined confer protective immunity.

The chimeric virus was constructed from a YF 17D infectious clone, substituting the 17D prM-E genes for the corresponding genes of the SA14-14-2 strain, a live, attenuated JE vaccine licensed for use in China, South Korea, India and elsewhere in Asia. The genetic rearrangement was accomplished by standard cloning techniques, employing two bacterial plasmids containing cDNA copies of the prM-E genes of JE SA14-14-2 virus and the remaining genes of YF 17D [59]. In constructing a viable chimera, a critical element was precision of the C-prM junction to ensure that the yellow fever 17D specific signal sequence (KRRSHDV) for the NS2b-2 protease was maintained.

The biological properties of the SA14-14-2 strain are well documented and it is a safe and effective human vaccine [66]. The E protein sequence of SA14-14-2 has

Table 2 Comparison of the amino acid differences in the E protein of chimeric JE/YF, JE SA14-14-2 and wild-type JE viruses SA14 and Nakamaya

Virus	E 107	E 138	E 176	E 177	E 227	E 244	E 264	E 279	E 315	E 439
JE SA14-14-2 PDK[a]	F	K	V	T	S	G	Q	M	V	R
JE SA14-14-2 PHK	F	K	V	A	S	G	H	M	V	R
YF/JE SA14-14-2	**F**	**K**	**V**	*A*	*S*	*G*	*H*	**M**	**V**	**R**
YF/JE Nakayama	L	E	I	T	P	E	Q	K	A	K
JE Nakayama[b]	L	E	I	T	P	E	Q	K	A	K
JE SA14/JAP[c]	L	E	I	T	S	G	Q	K	A	K
JE SA14/CDC[d]	L	E	I	T	S	G	Q	K	A	K
JE SA14/USA[e]	L	E	I	T	S	E	Q	K	A	K

Six residues distinguish the ChimeriVax™-JE virus (JE SA14-14-2/YF) virus from wild-type, virulent strains SA14 and Nakayama (shown in bold). Residues that are shared between ChimeriVax™-JE and SA14 substrains but distinguish ChimeriVax™-JE from JE Nakayama virus are shown in italics

[a]JE SA14-14-2 vaccine strain sequenced after passage in primary hamster kidney used to manufacture the vaccine (Aihara et al. [67]) and after additional passages in primary dog kidney (PDK) cells [68]

[b]Wild-type (prototype) JE virus (virulent)

[c]SA14 strains sequenced by Aihara et al. [67] (virulent)

[d]SA14 virus sequenced by Nitayaphan et al. [68] (virulent); sequence corrected by Ni et al. [69]

[e]SA14 virus sequenced by Ni et al. [70] (virulent)

been sequenced by a number of laboratories and compared to the virulent parental SA14 strain (Table 2). The mutations in the E protein of SA14-14-4 underlie the attenuated phenotype of the vaccine, and it was hypothesized that they would also contribute to the safety profile of the chimera. The E protein sequence of the JE (SA14-14-2)/YF chimeric virus (ChimeriVax™-JE) was identical to the JE SA14-14-2 vaccine strain, sequenced after passage in primary hamster kidney used to manufacture the vaccine at Chendu (China) [67]. Six amino acid differences at positions E107, 138, 176, 279, 315 and 439 distinguish all attenuated SA14-14-2 substrains from both virulent JE viruses, SA14 (the parental strain) and Nakayama (the JE prototype strain). Four additional mutations at E 177, 227, 244, and 264 distinguish the ChimeriVax™-JE virus from the JE prototype strain, Nakayama. Mutations at positions E177 (A) and 264 (H) seem to be host cell dependent because they are found in SA14-14-2 primary hamster kidney (PHK) cell grown virus, (and ChimeriVax™-JE derived from PHK passed virus) but not in primary dog kidney (PDK) cell passaged virus, or parental SA14 virus. Since the PDK vaccine has wild-type amino acids at these positions, but is attenuated, the E177 and E264 mutations in ChimeriVax™-JE are unlikely to play a significant role in attenuation. The putative attenuating amino acid residues map to three subregions of Domains I and II of the flavivirus E protein model. These include the fusion peptide (position 107), the hinge cluster (positions 138, 279) and the exposed surface of Domain I (positions 176).

The complete consensus nucleotide sequence of the ChimeriVax™-JE virus was determined and the vector backbone sequence compared to published sequences for

other YF 17D vaccine strains. Genomic heterogenity between YF 17D substrains has been noted by several authors, reflecting the uncloned nature of these viruses and differences in passage level [71, 72]. A single nucleotide difference at position 4025 (causing a V→M amino acid mutation in NS2a) was found in the YF sequence of ChimeriVax™-JE compared to other vaccine substrains.

Plaque-reduction-neutralization tests ($PRNT_{50}$) were performed on the chimeric viruses containing SA14-14-2 or Nakayama prM-E genes using YF and JE-specific hyperimmune polyclonal ascitic fluids and YF-specific purified IgG monoclonal antibody (2E10). The JE (SA14-14-2)/YF and JE (Nakayama)/YF chimeras and SA14-14-2 virus were neutralized only by JE hyperimmune ascitic fluid, whereas YF 17D was neutralized in a specific fashion by YF hyperimmune ascitic fluid and by 2E10 monoclonal antibody.

To determine whether the SA14-14-2 mutations were important to the attenuation of ChimeriVax™-JE two lines of evidence were followed. In the first, a chimeric virus was constructed in which the prM-E genes were derived from a virulent wild-type JE strain (Nakayama). The second approach evaluated the contribution of each of the SA14-14-2 specific mutations to attenuation. Virulence was assessed in mice inoculated by the IC route. The JE(Nakayama)/YF virus was 100% lethal for adult mice inoculated by the IC route with 4 $\log_{10}$ PFU, whereas ChimeriVax™-JE was fully attenuated (0% mortality). Therefore attenuation was determined by the prM-E genes, and one or more of the SA14-14-2 mutations in ChimeriVax™-JE caused a dramatic attenuation of neurovirulence. There are ten amino acid changes in the E protein of ChimeriVax™-JE when compared to the JE (Nakayama)/YF chimera (and wild-type JE Nakayama virus). Six of these ten amino acids (E107, 138, 176, 279, 315 and 439) are suspected to be critical neurovirulence determinants based on: (1) sequence comparison studies between SA14-14-2 virus substrains, ChimeriVax™-JE and the virulent parent strain SA-14 envelope proteins (Table 3); (2) the observation that these residues map within putative functional domains of the E protein; (3) published studies showing that several attenuated vaccine strains of JE (SA14-14-2, SA14-5-3, and SA14-2-8) differ from virulent parental SA14 virus at amino acids E138, E176, 315 and 439 [69, 73]; and (4) identification of a virulence determinant at E138 by mutagenesis of a full-length JE infectious clone [15].

A nominal requirement for a genetically stable vaccine is the presence of multiple mutations independently conferring the attenuation phenotype to avoid reversion to virulence with a single back mutation. To exclude the possibility of a single reversion event altering the virulence phenotype of ChimeriVax™-JE, a series of chimeras with single and multiple changes in amino acid residues was generated, converting SA14-14-2-specific mutations to the corresponding wild-type (Nakayama) residues [16]. To define the neurovirulence phenotype of the revertant viruses, weanling ICR mice were inoculated IC with 4 $\log_{10}$ PFU of each revertant. Based on these results, revertants were classified as lethal (showing 100% mortality), partially attenuated (<100% mortality), or fully attenuated (0% mortality) (Table 3). The analysis showed that reversion of three or four amino acids was required to restore the mouse neurovirulence typical of the wild-type JE virus. This finding, as well as the stability of the SA14-14-2 specific residues during sequential

Table 3 Neurovirulence of single site and multisite revertants of SA14-14-2 specific mutations in ChimeriVax™-JE to wild-type (Nakayama) amino acid residues, from Arroyo et al. [16]

Virus or revertant[a]	No. of dead mice/total no. of mice (%)[b]	AST (days)
YFV/JEV SA14-14-2	0/8 (0)	
1 (107; F→L)	0/8 (0)	
2 (138; K→E)	0/8 (0)	
3 (227; S→P)	0/8 (0)	
4 (244; G→E)	0/8 (0)	
5 (264; H→Q)	0/8 (0)	
6 (279; M→K)	1/8 (13)	
7 (315; V→A)	0/8 (0)	
8 (439; R→K)	0/8 (0)	
9 (176/177; V/A→I/T)	0/8 (0)	
10 (107, 176/177)	0/8 (0)	
11 (107, 138)[c]	1/8 (13)	9.0
12 (138, 176/177)	1/8 (13)	13.0
13 (107, 138, 176/177)[d]	9/9 (100)	9.4
14 (107, 138, 279)	3/8 (38)	11.3
15 (138, 227, 264, 279)	2/9 (22)	11.5
16 (107, 138, 227, 264, 279)	8/9 (89)	9.4
17 (138, 176/177, 227, 264, 279)	9/9 (100)	9.0
18 (107, 176/177, 227, 264, 279)	0/8 (0)	
19 (107, 138, 176/177, 227, 264, 279)	8/8 (100)	8.1
YFV/JEV Nakayama	8/8 (100)	9.3
YFV/JEV Nakayama	7/8 (88)	11.6

[a]Viruses with neurovirulent phenotypes are indicated by *boldface*. For viruses 1–9, the positions of and reversions at the single sites are indicated. For viruses 10–19, the positions of the multiple reversions are indicated

[b]For revertants 1 to 12 compared with YFV/JEV SA14-14-2, there was no significance. For revertants 14 and 15 compared with YFV/JEV Nakayama, *P* was 0.01 and 0.002, respectively. For revertants 13, 16, and 17 and YFV/JEV Nakayama compared with YFV/JEV SA14-14-2, *P* was 0.0001, 0.0005, 0.0001, and 0.0001, respectively

[c] One of two experiments; the mortality ratio for the second experiment was 0%

[d]One of two experiments; mortality was identical for the second experiment (AST was 9.9 days)

passage (see below) indicated that the risk of reversion to virulence was exceedingly remote.

Since attenuation relied on multiple specific mutations in the E protein, it was critical to assess genetic stability of the ChimeriVax™-JE vaccine, as RNA viruses have a high mutation rate due to lack of proof-reading enzymes. To ascertain genetic stability of the chimeric virus, and to search for "hot spots" in the genome susceptible to mutation, the virus was serially passaged at high multiplicity of infection (MOI) in two substrates considered for manufacturing [diploid fetal rhesus lung (FRhL), Vero] and partial or complete genomic sequencing and mouse neurovirulence studies performed at low and high passage levels [74]. None of the SA14-14-2 specific mutations were affected by up to 18 serial passages in vitro. In each of two different Vero cell passage series and with passage in FRhL, a single E protein mutation appeared, but these

mutations appeared at different sites, indicating that there were no amino acid-specific "hot spots". However the changes all occurred in the in hinge 4 (bounded by amino acids E266 to E284) of the molecular hinge region of the E protein responsible for a pH-dependent conformational change during virus penetration from the endosome into the cytoplasm of the infected cell. The molecular hinge therefore appeared to represent a region of relative instability. No changes occurred in the YF 17D backbone. There were no changes in neurovirulence for mice associated with the hinge region mutations. However a plaque size change (from large to small in Vero cells) was associated with a mutation (T→K) at E281 in the FRhL cell passage series. In vivo stability was also assessed [74]; no changes in brain titer or neurovirulence were found upon six sequential brain passages in mice. Collectively, these studies led to the development of specifications for quality control of ChimeriVax™-JE, namely: (1) each lot would be consensus sequenced across prM-E and all SA14-14-2 specific mutations were to be retained (other mutations were tolerated); (2) the passage level would be maintained by a seed lot system; and (3) a statistically powered test for neurovirulence (in infant mice) would be performed on each lot in mice, and would meet criteria for complete attenuation.

Interestingly, during the early attempts to manufacture ChimeriVax™-JE in FRhL cells, a reversion (M→K) at E279 (one of the SA14-14-2 specific sites) appeared at passage 5 and was associated with a small plaque phenotype. The E279 mutation is located in a beta-sheet in the hinge 4 region of the E protein. The reversion was not seen in the seed viruses. This mutation caused concern because it was close to the mutation at E281 observed in the genetic stability studies in FRhL cells that had also resulted in a small plaque size alteration, indicating genetic instability at that region during FRhL cell passage. Moreover, Arroyo et al. [16] had shown that the single-site E279 revertant was the only one with any evidence of a change in virulence, with 1 of 8 animals succumbing after IC inoculation (Table 3). These observations triggered a change to Vero cells for manufacturing and a further investigation of the role of the E279 site in the attenuation phenotype of ChimeriVax™-JE. The neurovirulence of the E279 revertant was studied in mice and monkeys. Outbred mice 4 days of age were inoculated IC with graded doses of ChimeriVax™-JE $FRhL_3$ (FRHL passage 3, no mutation), ChimeriVax™-JE $FRhL_5$ (E279 M→K), or a JE(SA14-14-2)/YF chimera in which a single mutation E279 (M→K was introduced by site-directed mutagenesis [16]. The LD_{50} values of the two viruses containing the E279 mutation were >10-fold lower than the $FRhL_3$ construct without the mutation, indicating that the E279 M→K mutation increased the neurovirulence of the chimeric virus. The $FRhL_5$ virus was 18.5 times more virulent than $FRhL_3$ ($p < 0.0001$). This sensitive methodology for detecting differences in neurovirulence was subsequently employed more widely in developing chimeric flaviviruses [34].

In the formal monkey neurovirulence test, the $FRhL_5$ virus containing the E279 reversion was significantly more neurovirulent than $FRhL_3$ (no mutation), but less neurovirulent than commercial YF 17D vaccine. Interestingly, there was an inverse relationship between neurovirulence and viscerotropism of the E279 revertant as

reflected by viremia, the $FRhL_5$ revertant virus displaying decreased viscerotropism (statistically lower viremia following IC inoculation of monkeys) compared to $FRhL_3$ virus [28]. Sera from monkeys inoculated with ChimeriVax™-JE $FRhL_3$ and $FRhL_5$ were examined for the presence of plaque size variants. Only large plaques were observed in sera from monkeys inoculated with the $FRhL_3$, whereas the virus in the blood of monkeys inoculated with $FRhL_5$ had the E279 revertant small plaque morphology. These studies reinforced the specification for the vaccine requiring stability of SA14-14-2 mutations and a phenotypic in vivo release test for attenuation.

During the course of further development of the vaccine, it was decided to change the cell substrate from the original Vero cell bank grown in medium containing fetal calf serum to a new cell bank grown in serum-free (SF) medium. In addition, whereas the original chimeric virus used to prepare seed stocks had not been biologically cloned, it was decided to plaque purify the new seed virus. The starting place for developing the new seeds was the RNA transcript used to prepare the original virus. SF Vero cells were transfected, and a series of expansion and plaque purification steps were then undertaken, leading to a newly derived pre-master seed at Passage 10. During this passage series a mutation (R→C) at amino acid position 60 in the M protein occurred and became the dominant genotype. Analysis of virus containing the M60 mutation revealed an increased rate of replication in SF Vero cell culture, with a peak titer approximately 0.5–1 $\log_{10}$ higher when compared to the nonmutant ChimeriVax™-JE. This suggested that the presence of the M60 mutation could potentially improve genetic stability by preventing accumulation of unwanted mutations associated with restricted growth, and could also increase virus yields during manufacturing. The genetic stability of this virus was ascertained by sequencing following three large-scale passages in stationary culture and stirred tank bioreactors[2].

Preclinical studies evaluated the safety profile, immunogenicity and protective activity of ChimeriVax™-JE in different hosts [16, 74]. ChimeriVax™-JE virus was fully attenuated for weaned mice inoculated by the IC route, whereas commercial YF 17D vaccine (commercial YF-VAX®) caused lethal encephalitis with an LD_{50} of 1.67 $\log_{10}$ PFU. Since the chimeric virus causes no illness in mice after IC inoculation, it was of interest to determine whether the virus replicated in brain tissue. Ten groups of 3–4 week old ICR mice were inoculated by the IC route with 3.0 $\log_{10}$ PFU of ChimeriVax™-JE virus. The virus replicated to a peak titer of approximately 6 $\log_{10}$ PFU/g on day 6 after inoculation; titers then decreased over time. Thus the virus is able to replicate to reasonably high titer in mouse brain without causing illness.

[2]The manufacture of ChimeriVax™-JE is performed by infecting Vero cells grown in serum free medium on microcarrier beads in a stirred tank bioreactor. The cell culture fluid containing the virus is harvested and the virus purified by depth filtration, ultrafiltration and diafiltration [early lots also included a nuclease (Benzonase®) digestion step, but it was later determined that acceptable levels of residual host cell DNA were achieved without nuclease digestion.] The final product is lyophilized in a proprietary stabilizer. The vaccine is administered as a 0.5mL SC injection containing approximately 4.7 $\log_{10}$ PFU.

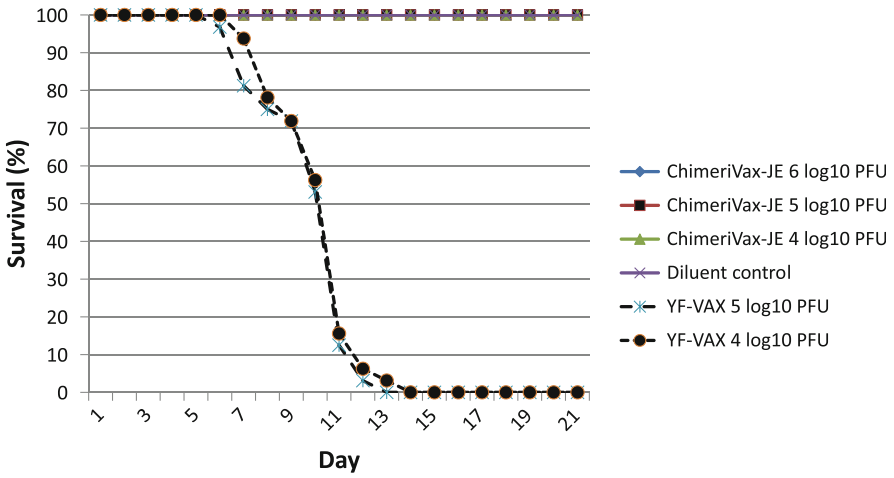


Fig. 2 Results of neurovirulence release test (survival curve) used for a typical lot of ChimeriVax™JE. Infant mice 8 days of age were inoculated IC with ChimeriVax™-JE 4,5, or 6 $\log_{10}$ PFU (32 mice/group); YF-VAX® 4 or 5 log10 PFU (32 mice/group), or diluent (32 mice). Dose groups did not differ and were pooled for analysis. The ChimeriVax™-JE survival curve is significantly different from that of YF-VAX® ($p < 0.0001$, log rank test)

The ChimeriVax™-JE virus was neurovirulent after IC inoculation in mice up to 6 days of age, whereas YF 17D is neurovirulent in weaned and adult mice [74]. Partial resistance to IC infection with ChimeriVax™-JE was observed in mice 7 and 9 days of age. The age at which mice become fully resistant to the chimeric virus occurs between 10 and 28 days of age. Since mice 7–9 days old were partially susceptible to IC inoculation with ChimeriVax™-JE, this host system was used to develop a sensitive test for changes in the attenuation phenotype in vaccine lots. The test was statistically powered to show a difference between a test article and YF-VAX® in mortality ratio and employed three groups of 32 mice precisely 8 days of age inoculated with 4, 5 and 6 $\log_{10}$ PFU by the IC route. A reference control (YF-VAX®) and negative control were included in the test. Results of a typical study are shown in Fig. 2, with all three dose groups of the ChimeriVax™-JE pooled for analysis (all had 0% mortality). The new plaque purified vaccine seeds and drug product developed in serum-free medium and containing the M60 R→C mutation were also assessed for attenuation in this way. The M60 mutation did not change the neurovirulence profile when compared to virus lacking the M60 mutation.

The principal control test for safety of YF 17D is the monkey neurovirulence test. The monkey neurovirulence test was performed according to Good Laboratory Practice (GLP) regulations using the methods described in the World Health Organization (WHO) requirements for testing YF 17D vaccine for preclinical safety [75]. The WHO test was modified to include determinations of clinical laboratory tests and microscopic examination of selected visceral organs in addition to the brain to search for unexpected extraneural organ dysfunction or pathology. The test involves the inoculation of 0.25 mL of the Master Virus Seed into the frontal lobe of a minimum of ten healthy, nonimmune rhesus or cynomolgus monkeys. Another group of ten monkeys is inoculated with the vaccine virus. A reference control preparation

Table 4 Neurovirulence test, cynomolgus monkeys, ChimeriVax™-JE plaque-purified seed viruses made in SF Vero cells vs. YF-VAX®

Parameter	ChimeriVax™-JE		YF-VAX® ($N = 7$)
	Master Virus Seed ($N = 9$)	Production Virus Seed ($N = 10$)	
Proportion with clinical signs	0%	0%	0%
Proportion viremic (%)	78%	90%	100%
Viremia mean peak (PFU/mL) ±SD	351 ± 472	272 ± 263	239 ± 188
No. viremic days (Mean ±SD)	2.89 ± 1.76	2.80 ± 1.40	2.86 ± 0.38
Neuropath score (target areas, mean ±SD)	0.160 ± 0.20[a]	0.223 ± 0.35	0.436 ± 0.19
Neuropath score (discriminator areas, mean ±SD)	0.155 ± 0.17[a]	0.106 ± 0.14[a]	0.610 ± 0.42
Neuropath score (combined, mean ±SD)	0.159 ± 0.16[a]	0.167 ± 0.23[a]	0.526 ± 0.19

Test articles (5 $\log_{10}$ PFU in 0.25 mL) inoculated IC on Day 1, observed for 30 days, then euthanized and necropsied

[a]Significantly different from YF-VAX® group

(commercial YF-VAX®) is inoculated into a third group of ten monkeys, and the test article is compared to the control, with respect to specific outcome measures including clinical observations, viremia and scoring of lesions in defined areas of the brain and spinal cord 30 days after inoculation. ChimeriVax™-JE (Master Virus Seed and vaccine lot) were significantly less neurovirulent and produced significantly lower viremia than YF 17D [76]. The neurovirulence test was repeated on the plaque purified seed viruses prepared in serum-free Vero cells containing the M60 mutation (Table 4).

An additional safety feature was the finding that ChimeriVax™-JE vaccine was incapable of infecting *Culex* and *Aedes* mosquitoes by oral feeding and had markedly restricted replication after intrathoracic inoculation [77]. Other work in Australian and Asian JE virus vectors (*Culex annulirostris, Culex gelidus,* and *Aedes vigilax*) also showed that these mosquitoes failed to become infected feeding on 6.1 $\log_{10}$ PFU/mL of ChimeriVax™-JE vaccine, which is >10,000 times greater than the peak viremia in seen in vaccinees [78].

In a pilot study, the susceptibility of rhesus monkeys to challenge by the intranasal (IN) and IC routes with wild-type JE virus was explored. Three young adult monkeys were challenged by the IC route and five monkeys were challenged IN with a high dose of JE IC-37 virus (0.25 mL containing 5.4 $\log_{10}$ PFU). None of the monkeys challenged by the IN route developed signs of illness, whereas all three monkeys inoculated by the IC route developed encephalitis. The time to onset of specific neurologic signs of encephalitis was 8–13 days. All monkeys inoculated by the IN and IC routes developed viremia, but the virus titers in serum were variable. Based on these results, it was concluded that the IC challenge could be used to model severe JE in the monkey, and predicted that the long incubation period between inoculation and illness would provide sufficient time for immunity induced by prechallenge vaccination to abrogate infection in the central nervous system.

Studies in mice [74] and monkeys [76, 79] showed that a single dose of ChimeriVax™-JE was highly immunogenic and protected the animals against lethal IC challenge with wild-type JE virus. Monkeys were inoculated by the SC route with graded doses of ChimeriVax™-JE virus (Table 5). All monkeys

Table 5 Representative studies in non-human primates of the viremia and antibody response following subcutaneous inoculation of graded doses of ChimeriVax™-JE and protective efficacy following intracranial challenge with wild-type JE virus

Parameter	Study 1 (rhesus macaques) [76]		Study 2 (rhesus macaque) [79]		Study 3 (Cynomolgus macaque) (not published)		
	Uncloned ChimeriVax™-JE vero passage 2	Not vaccinated	Original uncloned ChimeriVax™-JE FRhL passage 5 (no mutations)	Not vaccinated	Original uncloned ChimeriVax™-JE vero passage 5 GMP vaccine lot	Plaque-purified ChimeriVax™-JE (M60 R–>C mutant) vero passage 13 GMP vaccine lot	Diluent
Number of animals	6	4	12	2	5	4	4
Vaccine dose, route	4.3, 5.3 $\log_{10}$ PFU, SC ($N = 3$/group)	N/A	2, 3, 4, 5 $\log_{10}$ PFU, SC ($N = 3$/group)	N/A	4.0 $\log_{10}$ PFU, SC	4.0 $\log_{10}$ PFU, SC	N/A, SC
Viremia (% viremic)	100%	0	100%	0%	100%	100%	0%
Viremia duration (mean days)	4.0	0	1.7–2.1	0	3.4	3.75	0
Viremia (mean peak $\log_{10}$ PFU/mL	1.2–1.8	0	1.8–2.3	0	2.4	2.2	0
$PRNT_{50}$ Day 30/31 GMT[a]	1,016	<10	320–761	0	1,689	761	<10
Seroconversion %	100%	0%	100%	0%	100%	100%	0%
Pre-challenge $PRNT_{50}$ GMT[a]	449[b]	<10[b]	640–1,280	<10			
Challenge virus[c] dose, route	5.4 $\log_{10}$ PFU, IC	5.4 $\log_{10}$ PFU, IC	5.4 $\log_{10}$ PFU, IC	5.4 $\log_{10}$ PFU, IC			
Challenge % ill	33% (17% severe)	100%	0%	100%			
Challenge % dead	17%	100%	0%	100%	Not challenged		
Challenge % viremic (mean peak $\log_{10}$ PFU/mL)	0%	100% (2.8)	0%	100% (1.5)			
$PRNT_{50}$ Day 30 Post challenge GMT	4,222[b]	<10[b] (dead)	6,089–10,240	<20 (dead)			

[a]To homologous virus (ChimeriVax™-JE) unless otherwise specified
[b]To Nakayama strain
[c]Challenge virus JE (IC-37)

developed low-level, transient viremias similar to those induced by YF 17D. Neutralizing antibodies appeared between days 6 and 10, and by day 30, responses were similar across dose groups, with $PRNT_{50}$ titers between 320 and 2,560. Immunized monkeys and sham-immunized controls were challenged by the IC route with 5.4 $\log_{10}$ PFU of wild-type JE virus. None of the immunized monkeys developed viremia, few developed signs of illness, and survivors had mild residual brain lesions 30 days after challenge, while sham-inoculated controls all developed viremia, lethal encephalitis, and severe histopathologic lesions. Challenge by the IC route is a severe test of immunity, since natural infection occurs by intradermal inoculation during mosquito feeding and the virus must undergo extraneural replication and cross the blood brain barrier.

To evaluate the role of antibodies in protection, hyperimmune ascitic fluids were prepared in mice to either ChimeriVax™-JE or a commercial inactivated JE vaccine (JE-VAX®). The antisera neutralized a battery of wild-type JE virus strains in an equivalent fashion. C57 Bl6 mice were passively immunized and then challenged IC with a variety of different wild-type JE strains representing different genotypes [80]. Antisera against ChimeriVax™-JE and JE-VAX® passively protected strongly against challenge with genotypes II and III JE strains, and less well against genotypes I and IV. However, overall the antiserum against ChimeriVax™-JE appeared slightly superior. The study confirmed that protection was mediated by neutralizing antibody.

Clinical trials with ChimeriVax™-JE are summarized in Table 6. The initial, randomized, double blind, outpatient Phase I study of ChimeriVax™-JE was conducted in 36 healthy young volunteers to assess safety, tolerability and immunogenicity in comparison to YF 17D [81]. This study also addressed the theoretical concern that prior YF immunization could blunt the efficacy of ChimeriVax™-JE vaccine by antivector immunity (anti-NS1 antibody or cell-mediated responses against nonstructural YF proteins). Mild, transient injection site reactions and flu-like symptoms were noted in all treatment groups, with no significant difference between the groups. Nearly all subjects inoculated with ChimeriVax™-JE at both dose levels developed a transient, low-level viremia similar in magnitude and duration to that following YF-VAX®. There was a suggestion of slightly higher viremias in subjects with preexisting YF immunity. Neutralizing antibody seroconversion rates to JE were 100% in the high and low dose groups in both naïve and YF-immune subjects. Geometric mean neutralizing antibody responses were higher in the ChimeriVax™-JE high dose groups (naïve subjects $PRNT_{50}$ 254; YF immune subjects $PRNT_{50}$ 327) than in the low dose groups (naïve subjects $PRNT_{50}$ 128; YF immune subjects $PRNT_{50}$ 270). Prior yellow fever vaccination increased the neutralizing antibody response to the chimeric vaccine, probably due to memory responses to shared antigenic determinants in the E glycoprotein or carrier priming mediated by preexisting immunity to nonstructural proteins.

In a follow-up study, ten adults who had been inoculated 9 months earlier with ChimeriVax™-JE vaccine and ten control subjects were challenged with noninfectious JE virus antigen [inactivated mouse-brain JE vaccine (JE-VAX®)],

Table 6 Summary of clinical studies performed with ChimeriVax™-JE

Phase	Location	Age (years)	Treatment (no. subjects)	Dose (log_{10} PFU)	Adverse events		Viremia			Neutralizing antbodies to JE	
					Serious, related	Nonserious	Proportion viremic (%)	Duration (range, days)	Peak, mean log_{10} PFU/mL	GMT ($PRNT_{50}$[a]) Day 28–31	Seroconversion (%)[a] Day 28–31
1	US	18–49	CV-JE[b] ($N = 12$)	5.0	0	AEs were qualitatively	83%	0–3	1.1–1.6[c]	254–327[c]	100%
			CV-JE ($N = 12$)	4.0	0	and quantitatively	92%	0–5	1.4–1.5[c]	128–270[c]	100%
			YF ($N = 12$)	5.0	0	similar across the three treatment groups	100%[d]	2–3[d]	1.6[d]	15−13[c]	42%
1	US	Follow-on study in subjects who were immunized in the study above to determine memory response to "challenge" with inactivated JE antigen (see text)									
2	US	18–59	CV-JE ($N = 10$)	5.8	0	AEs were qualitatively	50%	0–4	0.8	262	100%
			CV-JE ($N = 44$)	4.8	0	and quantitatively	67%[e]	0–5	1.1	299	100%
			CV-JE ($N = 11$)	3.8	0	similar across all	82%	0–3	1.2	210	100%
			CV-JE ($N = 11$)	2.8	0	treatment groups	100%	1–6	1.6	103	91%
			CV-JE ($N = 11$)	1.8	0		82%	0–5	1.3	285	100%
			YF ($N = 11$)	5.0	0		64%	0–3	1.3	<10	9%
			Placebo ($N = 11$)		0		0%	0	0	<10	0%
2	Australia	18–55	CV-JE ($N = 201$)	3.8	1[f]	AEs were qualitatively and quantitatively similar across the treatment groups	Not determined; a sample for viremia taken 14 days after vaccination was negative			258–389[g]	98.5%
			Placebo ($N = 199$)		0					<10	0%
2	Australia	18–55	CV-JE and YF ($N = 108$)	3.8		See text and Table 7					
2	Australia	18–49	CV-JE ($N = 32$)	5.0	0	AEs were qualitatively	28%	0–11[h]	0.5	2,060	93.5%
			CV-JE ($N = 32$)	4.0	0	and quantitatively	53%	0–7[h]	0.8	2,152	93.8%
			CV-JE ($N = 32$)	3.0	0	similar across the	44%	0–3	0.6	1,809	100%
			Placebo ($N = 32$)		0	treatment groups	0%	0	0	<10	0%
3	Australia & US	≥18	CV-JE [$N = 410$ (safety); 346 (efficacy)]	~4.0[i]	0	Lower incidence of AEs in CV-JE group than				1,392[j]	99.1%[j]
			JE-VAX® [$N = 410$	Licensed product	0	JE-VAX® group ($p = 0.031$)				37.4	74.8%

			(safety); 365 (efficacy)]			
3	Australia & US	≥18	CV-JE ($N = 1{,}600$)	4.0	1[k]	Similar incidence of AEs Day 0–30 between active and placebo treatment groups ($p = 0.203$; higher incidence in CV-JE group Day 0–7 and 0–14 ($p = 0.038$). Headache, myalgia, inj site pain, generally mild
			Placebo ($N = 404$)		0	

[a]Titers are to homologous strain (also see text); $PRNT_{50}$ = 50% plaque reduction neutralization test
[b]ChimeriVax™-JE; YF = YF 17D (YF-VAX® or Stamaril®)
[c]YF nonimmune – YF immune
[d]YF-nonimmune subjects
[e]There were 33 subjects in this group tested for viremia
[f]"Acute viral illness" onset 8 days post vaccination with diarrhea, fever, dehydration, hospitalized; recovered w/out sequelae
[g]The study was a cross-over design in which half of the subjects received CV-JE first and 30 days later received placebo, and the other half received placebo, then CV-JE. The range of GMTs represents the 30 day post CV-JE titers for each cross-over subgroup. The seroconversion arte is combined
[h]Viremias were intermittent, with 0–4 days positive out of 14 days tested; the latest day positive was taken as the longest duration in the range shown
[i]Three consistency lots were tested in 136 subjects each
[j]The titer and seroconversion are to homologous JE strains (ChimeriVax™-JE for the CV-JE treatment group and Nakayama for the JE-VAX® treatment group. The statistical noninferiority and clinical consistency (between lots) endpoint were met
[k]Pyrexia, hospitalized, recovered w/out sequelae

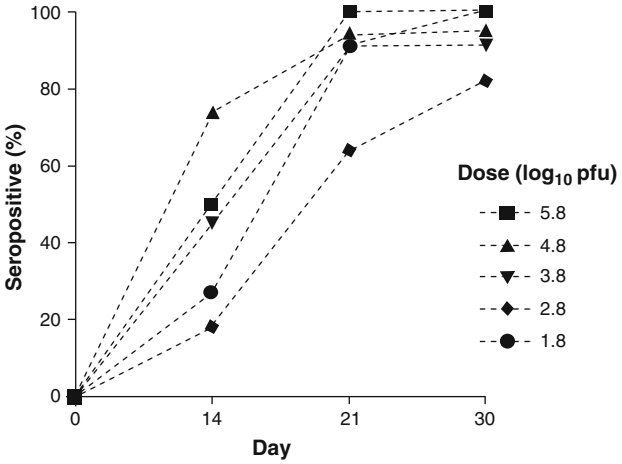


Fig. 3 Kinetics of the neutralizing antibody response to a single SC inoculation of graded doses of ChimeriVax™-JE (From Monath et al. [50], with permission)

representing a surrogate for exposure to live virus transmitted by mosquitoes under conditions of natural exposure [50]. Subjects who had previously received ChimeriVax™-JE responded with a rapid rise in neutralizing antibodies (Fig. 3). Mean antibody levels on Day 7 were approximately 20-fold higher and on Day 14 100-fold higher than pre-inoculation levels. In contrast, JE-VAX® control subjects responded more slowly and had low antibody levels. Differences in mean antibody levels between the treatment groups were highly significant on all study days. These results demonstrate that strong immunological memory is induced by the ChimeriVax™-JE vaccine. Vaccinated individuals would be expected to have a rapid anamnestic response if exposed to wild-type JE virus.

A randomized, double blind, placebo-controlled, out-patient Phase 2 study was conducted in the US in 99 healthy subjects, 18–59 years, to further evaluate the safety and immunogenicity of ChimeriVax™-JE vaccine (Table 6). Subjects received one or two inoculations (30 days apart) across a wide dose range (1.8–5.8 $\log_{10}$ PFU) [82]. Treatment groups included volunteers who received YF 17D 30 days before or after inoculation of ChimeriVax™-JE to investigate vaccine interactions. The vaccine at all dose levels was well tolerated, and there were no differences in the incidence of adverse events across active vaccine and placebo treatment groups after the first or second dose. ChimeriVax™-JE vaccine was associated with a brief viremia of low magnitude. Viremias were somewhat higher in the low dose than high dose treatment groups. This inverse relationship between dose and response has also been noted in the case of YF 17D vaccine [43]. The 90% effective dose was estimated to be 17 PFU, which is not dissimilar to the ED_{90} of YF 17D (50 PFU) [43]. Eighty-two (94.3%) of 87 subjects who received a single inoculation of ChimeriVax™-JE at all dose levels (Groups 1–6, 8 and 9) seroconverted to JE by neutralization test within 30 days. Seroconversion rates varied between 82 and

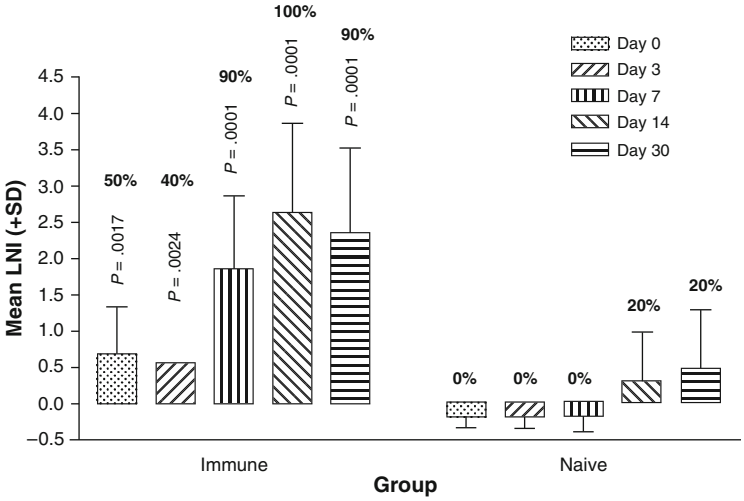


Fig. 4 Seroconversion rate (% above bars) and mean neutralizing antibody levels [$\log_{10}$ neutralization index (LNI, *histogram*] for subjects in the Phase 1 trial (Table 6) who (*left*) had been immunized with ChimeriVax™-JE 9 months earlier or (*right*) naïve subjects by day after "challenge" with inactivated JE vaccine (JE-VAX®). P c values (t tests) compare immune and naïve treatment groups. From Monath et al. [50], with permission

100% across the range of doses from 1.8 to 5.8 $\log_{10}$ PFU without relationship to dose. No statistical differences in mean antibody titers were found across dose groups. Antibody titers increased rapidly over 2–3 weeks after primary inoculation (Fig. 4), appeared to peak around Day 30, did not increase after boosting, and in fact tended to decrease slightly by Day 60. Follow up studies have now documented persistence of antibody for >3 years following a single dose. Neutralization tests were performed against ChimeriVax™-JE and three wild-type JE virus strains (Beijing-1, Nakayama and 902/97); seroconversion rates were high to all strains across all dose groups, but GMTs were higher to the homologous (ChimeriVax™-JE) virus. The antibody response to ChimeriVax™-JE was not influenced by vaccination against YF performed 30 days previously; however there was a suggestion that prior inoculation of ChimeriVax™-JE diminished the serological response to YF 17D. 64% of ChimeriVax-JE-immune subjects seroconverted to YF, compared to 91% of ChimeriVax™-JE-naïve subjects; the difference was, however, not statistically significant. The mean antibody titer to YF 30 days after inoculation was lower in ChimeriVax-JE-immune subjects than in ChimeriVax-JE-naïve subjects, but again the difference was not statistically significant. It was concluded from these results that ChimeriVax™-JE has a safety profile and viremia pattern similar to those of YF 17D vaccine. ChimeriVax™-JE rapidly elicited high titers of neutralizing antibodies after a single inoculation at very low doses, an advantage over existing inactivated vaccines that require multiple inoculations.

Another randomized double-blind Phase 2 study conducted in 2004 at a site in Australia further evaluated the interaction of ChimeriVax™-JE and YF 17D

vaccine (Stamaril®). The study enrolled 108 subjects 18–55 years of age. Thirty-six subjects received ChimeriVax™-JE followed 30 days later by Stamaril®; 36 subjects received Stamaril® followed by ChimeriVax™-JE; and 36 subjects received the two vaccines simultaneously in different arms. Subjects who were Flavivirus naïve at baseline (JE, YF, MVE, KUN, ALF) were analyzed (Table 7). YF 17D elicited higher levels of neutralizing antibodies against YF than ChimeriVax™-JE against JE. Subjects who received ChimeriVax™-JE before Stamaril® had higher antibody responses than those who received Stamaril® in advance of or concurrently with ChimeriVax™-JE. However, the two vaccines evoked strong responses in nearly all subjects regardless of the schedule of immunization.

Another Phase 2 study in 201 military personnel in Australia included a double-blind stage during which subjects received two vaccinations, one SC administration of 3.8 $\log_{10}$ PFU of ChimeriVax™-JE and one 0.5 mL SC dose of placebo (diluent), 28 days apart in a cross-over, parallel group design. The seroconversion rate on Day 30 to homologous virus was 98.5% and the GMT was 258–389. The seroconversion rate and GMT to wild-type JE virus strains representing different genotypes of JE virus were determined (Table 8). The seroconversion rate to genotype IV virus was lower than to the other genotypes. Genotype IV is the most evolutionarily divergent subgroup of JE viruses and is also the least likely to be associated with human disease. The GMTs were lower to genotypes II and IV than to genotype I and

Table 7 Seroconversion rate and geometric mean antibody titer (GMT) by 50% plaque reduction neutralization test to JE and YF 30 days after administration of two sequential vaccinations or coadministration with ChimeriVax™-JE or YF 17D (Stamaril®)

Statistic	ChimeriVax™-JE then Stamaril®	Stamaril® then ChimeriVax™-JE	Coadministration
JE seroconversion 30 days after both vaccinations	17/17 (100%)	21/23 (91%)	22/23 (96%)
JE GMT 30 days after both vaccinations	688	426	344
YF seroconversion 30 days after both vaccinations	17/17 (100%)	23/23 (100%)	23/23 (100%)
YF GMT 30 days after both vaccinations	3,289	2,175	2,094

Table 8 Seroconversion rates and neutralizing antibody geometric mean titer (GMT) to wild-type JE strains representing different genotypes 28 days after vaccination with ChimeriVax™-JE

	ChimeriVax-JE	Genotype I Korea TVP-8236	Genotype II Thailand B1034/8	Genotype III Beijing	Genotype IV Indonesia JKT 9092
Seroconversion rate[a]	194/197 98.5%	194/197 98.5%	181/197 91.9%	194/197 98.5%	175/197 88.8%
GMT group A[b]	388.6	209.2	65.4	211.7	55.7
GMT group B[b]	258.4	236.9	72.7	228.0	64.3

[a]Seroconversion is presented for the combined groups A and B

[b]Group A and B represent subjects in the different arm of this cross over study, subjects in group A received ChimeriVax™-JE then placebo, subjects in group B received placebo then ChimeriVax™-JE

III. These differences are not believed to be clinically significant since neutralizing antibody titers $\geq$10 are considered to be protective [82].

A randomized placebo controlled Phase 2 study was designed to test safety and immunogenicity of the plaque-purified, lyophilized formulation of ChimeriVax™-JE. Groups of 32 healthy subjects received graded doses (3.0, 4.0, 5.0 $\log_{10}$ PFU) of ChimeriVax™-JE or placebo. The safety profile was good, with no differences in adverse events across dose groups or placebo. Viremias were very low in all dose groups. Based on historical data the viremia was lower than that observed following inoculation of YF 17D. Seroconversion rates in the ChimeriVax™-JE 3.0 $\log_{10}$ PFU, 4.0 $\log_{10}$ PFU and 5.0 $\log_{10}$ PFU groups were 100%, 93.8%, and 93.5%, respectively. The homologous GMT in the ChimeriVax™-JE 3.0 $\log_{10}$ PFU, 4.0 $\log_{10}$ PFU and 5.0 $\log_{10}$ PFU treatment groups were 1,809, 2,152 and 2,060, respectively. There were no statistical differences in seroconversion or GMT across dose groups, confirming earlier dose response data. Tests were conducted with wild-type JE strains; as in the previous study (Table 8), responses to genotypes II and IV were lower than to genotypes I and III.

Two Phase 3 (pivotal) trials were conducted by Acambis in 2006. The first study was a multicenter, randomized, double-blind, study of the comparative immunogenicity, safety and tolerability ChimeriVax™-JE vs. inactivated mouse brain JE vaccine (JE-VAX®) and was conducted in Australia and the US. The trial enrolled 410 subjects who received placebo on Days 0 and 7 and ChimeriVax™-JE on Day 30 and an equal number of subjects who received JE-VAX® on Days 0, 7 and 30. The primary endpoint was a test for non-inferiority of ChimeriVax™-JE to JE-VAX®, with the test intended to rule out a 5% difference in JE neutralizing antibody seroconversion rates to each respective homologous virus (ChimeriVax™-JE or JE Nakayama) 30 days after immunization. Secondary endpoints examined GMT at 30 days and early response (Day 14 seroconversion and GMT). The trial met all of its endpoints; the seroconversion rate and GMT were statistically higher in the ChimeriVax™-JE group after a single dose than following three doses of JE-VAX® (Table 6). The GMT following ChimeriVax™-JE was 37 times higher than after JE-VAX®. In addition, the study showed that the antibody response was more rapid than that following JE-VAX®, with 93% seropositive after 14 days.

In parallel, a randomized double blind multicenter Phase 3 study was conducted in 2006 in 2000 subjects >18 years of age in Australia and the US. The objectives of the study were to assess safety in 1,600 subjects receiving ChimeriVax™-JE and 400 subjects who received placebo (0.9% saline). The study showed that the new vaccine was safe and well tolerated.

In 2006, an open label Phase 3 trial of ChimeriVax™-JE was initiated in India, enrolling children aged 9 months to 5 years. A smaller Phase 2 trial addressed interactions of ChimeriVax™-JE and measles vaccine in infants.

Following the successful Phase 3 trials, a partnership with Sanofi Pasteur was formed for commercialization of the vaccine. In 2008, Sanofi Pasteur acquired Acambis. The ChimeriVax™-JE vaccine, now called IMOJEV™, is in registration in Australia, Thailand and elsewhere.

ChimeriVax™-JE vaccine was shown to cross protect against Murray Valley encephalitis [83] virus (a member of the JE antigenic complex) in mice. If confirmed, this strategy could be a useful intervention in the event of outbreaks of MVE, which occur at infrequent intervals in Australia, but further development would be needed. It would be of interest to test subjects who receive IMOJEV™ for cross-neutralizing MVE antibodies.

3.2.2 Chimeric Flavivirus Vaccines Against Dengue

Because of the worldwide importance of dengue fever and severe dengue [previously called dengue hemorrhagic fever (DHF)], there has been a sustained interest in the development of vaccines. Before the development of infectious clone technology allowing rational vaccine design, efforts focused on empirical derivation of live, attenuated DEN vaccines by serial passage; these efforts have largely been abandoned due to difficulties in getting the correct balance of attenuation and immunogenicity (Fig. 1). Major issues that complicate the development of DEN vaccines include: (1) the role of enhancing antibodies and T cells in sensitizing the host to severe dengue on exposure to a heterologous dengue virus to which solid immunity is not induced; (2) the requirement therefore to evoke (preferably simultaneously) durable protective immune responses against all four dengue serotypes; (3) interference between the four DEN serotypes when combining them in a live vaccine formulation; (4) difficulty in establishing immunological surrogates of protection due to the inability to distinguish between homotypic neutralizing antibody and cross-reactive heterotypic nonprotective antibody.

With the advent of infectious clone technology, several groups have developed DEN vaccine candidates based on chimeric flavivirus constructs [84, 85]. Three different strategies are being deployed currently. The farthest along in development is the DEN/YF chimeric (ChimeriVax™) DEN vaccine first constructed by Tom Chambers at St Louis University, subsequently developed by Farshad Guirakhoo, Konstantin Pugachev, and Thomas Monath at Acambis [86, 87], and acquired (in 2008) by Sanofi Pasteur (Acambis and Sanofi Pasteur had collaborated on this project beginning in 1999). Ricardo Galler and colleagues (BioManguinhos/FioCruz) in Brazil are independently developing DEN/YF chimeric vaccines [88]. At NIAID, Michael Bray and Ching-Juh Lai pioneered the construction and testing of intertypic dengue chimeras [89, 90], and a full vaccine development program ultimately evolved under the leadership of Steven Whitehead and Brian Murphy [85, 91]. At CDC (Ft. Collins) Richard Kinney and Claire Huang developed an intertypic dengue vaccine platform [92] that was licensed to and is under development by Inviragen Inc.

A fundamental question in selection of an appropriate vector backbone is whether it is preferable to utilize a heterologous backbone virus (e.g., YF 17D) or a homotypic vector backbone (DEN) that may contribute to anti-DEN immunity via T cells and anti-NS1 antibody. The central objective in vaccine immunity is to stimulate strong neutralizing antibody responses directed against the E glycoprotein. That will

be determined principally by the ability of the vector backbone to replicate in the host, generating sufficient antigenic mass, and its ability to activate innate immune signaling pathways. In mouse models, the DEN NS proteins have not contributed materially to protection [41]. From a safety perspective, in order to prevent sensitization to severe DEN in the event antibody levels fall, it may be preferable not to have cytotoxic T cells directed against DEN NS proteins (particularly memory T cells directed against cross-reactive epitopes, that may have lower avidity for the infecting virus resulting in altered proinflammatory T-cell functional responses), especially in the absence of strong neutralizing antibodies [42]; therefore, in the case of a DEN vaccine, a heterologous (non-dengue) backbone might have some advantages.

3.2.3 Chimeric DEN/YF Vaccines (ChimeriVax™-DEN and Others)

Several groups have independently reported the successful construction of chimeric DEN/YF viruses [86, 88, 93, 94]. Guirakhoo et al. [86, 95] used donor prM-E genes from low passage, recent isolates from Asia for construction of all four YF/DEN chimeras. Various other donor genes have been used, e.g., DEN2 prototype New Guinea C virus, DEN2 Americas I genotype (PR159) or DEN1 from Venezuela VeMir95 [88, 93, 94, 96]. In contrast to the JE/YF chimera, wild-type, unmodified donor genes were used for construction of a vaccine candidate without modification (mutagenesis), and reliance was placed on the chimerization process itself and the vector NS genes to confer appropriate attenuation. The rationale for this approach was the successful empirical development at Mahidol University of a live DEN2 vaccine by 53 serial passages in primary dog kidney cells (PDK53), which was shown to be attenuated in clinical studies and to contain no mutations in the E gene [97]. Therefore it was presumed that presentation of the wild-type dengue 2 prM-E sequence in the background of another vector (YF 17D) with attenuating mutations in the backbone would yield an acceptable phenotype. The first biological assessments of various chimeric DEN2/YF viruses showed them to be less neurovirulent than YF 17D after IC inoculation of mice while eliciting antibodies to DEN2. In contrast, a DEN4/YF chimeric virus constructed by Chambers et al. [94] utilized a mouse-neuroadapted and neurovirulent DEN4 gene donor strain and the resulting chimera was neurovirulent, showing [as for JE (Nakayama)/YF] that the structural gene insert influences the virulence phenotype of chimeras constructed on a highly attenuated backbone. The fact that wild-type DEN viruses are naturally attenuated for neurotropism suggested that it would be possible to use unmodified donor prM-E genes for constructing chimeras, but each serotype and construct would have to be tested empirically.

Guirakhoo et al. [86] showed that the DEN2/YF chimeric vaccine inoculated SC over a wide dose range, caused low-titer viremia in rhesus monkeys compared to wild-type virus, elicited strong neutralizing antibody responses, and protected monkeys against wild-type DEN2 challenge. Based on these promising results, manufacture and testing of clinical grade vaccine was undertaken, with the first

IND for a monovalent DEN2/YF chimera (ChimeriVax™-DEN2) filed to the Food & Drug Administration (FDA) in early 2002. The method of manufacture was similar to that described above for ChimeriVax™-JE. Seed virus was tested in infant mice and by the monkey neurovirulence test and the chimeric virus shown to be significantly less neurovirulent than parental YF 17D. An interesting aspect of the preclinical assessments in monkeys was the observation that viremia patterns following chimeric DEN vaccination differed from those seen after YF 17D in that virus could be detected (at very low levels or intermittently) for a more prolonged period, sometimes into the second week.

An important milestone was the clinical proof-of-concept study [98]. Parenthetically, there was considerable internal debate within the development team about the practical value of conducting clinical studies of each monovalent YF/DEN serotype independently. It was concluded that human testing of the tetravalent YF/DEN vaccine would provide more conclusive data on the interaction of viruses in the formulation than further studies in nonhuman primates or clinical tests of monovalent components.

The monovalent ChimeriVax™-DEN2 trial was conducted in healthy subjects, 18–49 years of age, at a single center in the US (Table 9) [98]. The study randomized 42 eligible healthy adult subjects without prior immunity to YF to receive a single SC vaccination of either ChimeriVax™-DEN2 (5 $\log_{10}$ or 3 $\log_{10}$ PFU dose) or YF 17D (YF-VAX®). In addition, 14 subjects previously vaccinated to YF received high dose (5 $\log_{10}$) ChimeriVax™-DEN2 as an open-label vaccine. The vaccines were well tolerated, with no differences in adverse event profiles between ChimeriVax™-DEN2 and YF-VAX®. More YF-naïve subjects vaccinated with ChimeriVax™-DEN2 compared to YF-VAX® developed viremia: 8 (57%) in the ChimeriVax™-DEN2 5.0 $\log_{10}$ group; 9 (64%) in the ChimeriVax™-DEN2 3.0 $\log_{10}$ group compared with 2 (14%) in the YF-VAX® group. The proportion of subjects who developed viremia following vaccination with ChimeriVax™-DEN2 5.0 $\log_{10}$ PFU was slightly greater in YF-immune than in YF naïve subjects (79% v 57%). The mean peak viremia level following ChimeriVax™-DEN2 5.0 $\log_{10}$ PFU

Table 9 Phase 1 clinical trial of monovalent ChimeriVax™-DEN2; viremia and antibody responses. (Data from Guirakhoo et al. [98])

Group	No. subjects	YF immune	Vaccine	Dose $\log_{10}$ PFU in 0.5 mL	Viremia (mean)			PRNT50[a]	
					% Viremic	Duration (days)	Peak (PFU/mL)	Seroconversion (%)	GMT
Double-blind, randomized									
1	14	No	ChimeriVax™-DEN2	5.0	57	1.4	12.1	100%	921
2	14	No	ChimeriVax™-DEN2	3.0	64	1.2	11.4	100%	570
3	14	No	YF-VAX®	≥5.04	14	0.4	20.0	0%	<10
Open label									
4	14	Yes	ChimeriVax™-DEN2	5.0	79	1.9	29.3	100%	975

[a]antibodies to the homologous ChimeriVax™-DEN2 virus

was greater in YF immune subjects than that in YF naïve subjects. While none of these values were statistically significant with this small sample, the finding may be evidence of immune enhancement of ChimeriVax™ replication in persons with pre-existing YF immunity, an observation similar to that shown for ChimeriVax™-JE in some studies. ChimeriVax™-DEN2 was highly immunogenic; 100% of subjects seroconverted; geometric mean neutralizing antibodies were statistically higher in the higher dose group. The sera were also tested against three different wild type DEN2 strains as well as heterotypic dengue viruses (types 1, 3 and 4). Nearly all subjects (93–100%) vaccinated with ChimeriVax™-DEN2 seroconverted to the wild-type DEN2 viruses but GMTs were lower than to the homologous strain; minimal, low titer responses were seen to heterotypic dengue serotypes.

Interestingly, prior YF immunity had a dramatic effect on stimulating broad heterotypic responses to DEN 1, 3 and 4 following immunization with ChimeriVax™-DEN2. The results suggested that in areas where YF immunity is prevalent, e.g., South America, DEN immunization might be enhanced. This result was anticipated by earlier studies of empirically developed live dengue vaccines in YF immune vs. nonimmune subjects [99]. T cell responses were measured by IFNγ production by PBMC cultured for 7 days in the presence of inactivated ChimeriVax™-DEN2 virus. All four vaccine groups showed a significant increase in IFNγ production at day 31 relative to day 1. A slightly lower IFNγ response was seen in YF-immunized subjects, but it was statistically similar to the response in either dose group of ChimeriVax™-DEN2 immunized subjects. This suggested that nonstructural proteins present in the YF backbone of the ChimeriVax™-DEN2 vaccine made a significant contribution to the T cell response. The IFNγ response to ChimeriVax™-DEN2 vaccination was not diminished by prior immunity to YF.

Importantly, neutralizing antibody titers to DEN2 in this trial were substantially higher than observed in subsequent trials of the tetravalent vaccine (see below), illustrating the effect of interaction of multiple dengue strains in a combined (mixed) vaccine.

The first recombinant tetravalent DEN vaccine was developed by Acambis [95]. ChimeriVax™-DEN1–4 viruses were constructed by inserting prME genes of wild-type DEN viruses into core and nonstructural genes of YF 17D virus. The origin of the donor wild-type strains used to construct these viruses is shown in Fig. 5. Different methods for genetic constructions were employed, including the standard two-plasmid system, and in vitro ligation of an overlap extension PCR amplicon (DEN1/YF) or multiple DNA fragments (DEN3/YF). The initial viruses derived by transfection of recombinant RNA were not plaque purified and accumulated a number of mutations after passages in Vero cells.

Biological characterization of the chimeric candidate viruses was performed in mice and monkeys [95]. The candidates exhibited a high level of attenuation in mice. A focus of the preclinical evaluation was whether or not there was interference between the four subtypes in the tetravalent vaccine. Monkeys inoculated with the tetravalent mixture developed satisfactory immune responses to all four serotypes, although the response to DEN2 appeared to predominate [95]. Signs of

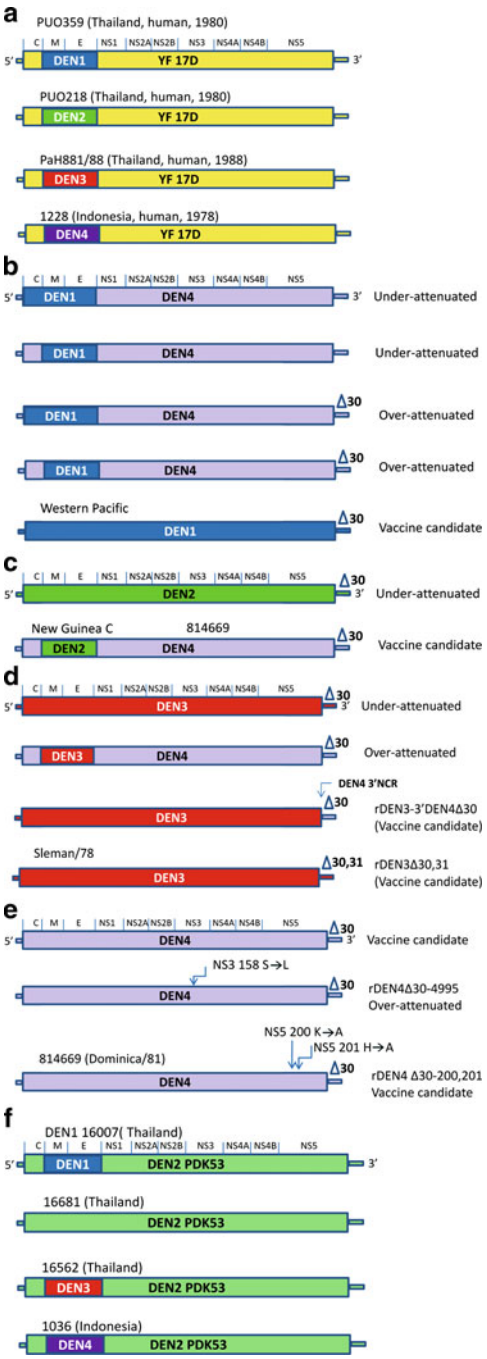


Fig. 5 The three major approaches to construction of rationally designed live, attenuated DEN vaccines. (**a**). Chimeric vaccines in which the prM-E genes of YF17D are replaced by the

interference were relatively subtle; the incidence and duration of viremia to DEN 1 and 3 were less than in monovalent controls [86, 95].

To improve the genetic stability, the viruses were rederived and plaque purified in an attempt to make pre-master seed (PMS) viruses with wild-type consensus sequences and minimize mutations arising on passage [100]. A principal goal was to derive DEN 1, 3 and 4 viruses that had wild-type sequences, since it was noted that the original constructs contained a small number of mutations in the prME genes [100], whereas the DEN2 chimera did not have mutations. It was possible that this accounted for the predominant immunogenicity of the DEN2/YF chimera in monkeys [86, 95]. Plaque-purified pre-master seeds (PMS) at passage 7 (P7) were produced in Vero cells, and passed three times under cGMP to produce vaccine lots at P10.

Preclinical studies of ChimeriVax™-DEN1-4 viruses demonstrated that the vaccine candidates had the following product profile:

- Produced high yields in Vero cell culture for vaccine production
- Underwent minimal genetic changes during passage up to P20 in Vero cells
- Were not neurovirulent in 3–4 week old mice inoculated by the IC route
- Were less neurovirulent than YF-VAX® in infant mouse and monkey models
- Did not become more neurovirulent upon extensive in vitro passaging (measured by a sensitive infant mouse neurovirulence test)
- Had restricted replication in mosquito vectors, similar to the YF 17D virus, and significantly lower than their parental wild-type DEN viruses
- Did not interfere with each other in terms of replication in host (mouse, monkey [101] and mosquito models)
- Gave a balanced response (low viremia and high neutralizing) when administered at an equal mixture (e.g., 3,3,3,3 or 5,5,5,5 $\log_{10}$ PFU formulation of the chimeras for DEN 1, 2, 3 and 4 serotypes) and
- Protected monkeys against a severe heterologous challenge after a single monovalent or tetravalent dose [100]

Genetic stability was assessed by full genomic sequencing at various passage levels, including pre-master seed (P7), Master Seed (P8), Working Seed (P9), vaccine (P10) and P20. The biologically cloned viruses had no mutations from the parental wild-type sequence at P7. With passage, a small number of mutations were observed, but these were fewer than seen in the original uncloned preparations and no mutations arose in prME of DEN3 at P10 (vaccine lot) or in DEN4 at P20 (genetic stability passage) (Fig. 6). Mutations in the YF backbone (NS4B) chimeras were likely adaptations to growth in SF Vero cells and had been observed previously when passing uncloned chimeric DEN2. To ensure that the accumulated

Fig. 5 (Continued) corresponding genes from wild-type DEN 1–4 strains (Acambis/Sanofi Pasteur); (**b–e**). Various approaches used at NIAID to develop mutagenized and chimeric DEN vaccines against DEN1 (*Panel B*), DEN (*Panel C*), DEN3 (*Panel D*) and DEN4 (*Panel E*); (**f**). Chimeric vaccines in which the prM-E genes of DEN2 PDK53 vaccine are replaced by the corresponding genes from wild-type DEN 1,3 and 4 strains (CDC/Inviragen)

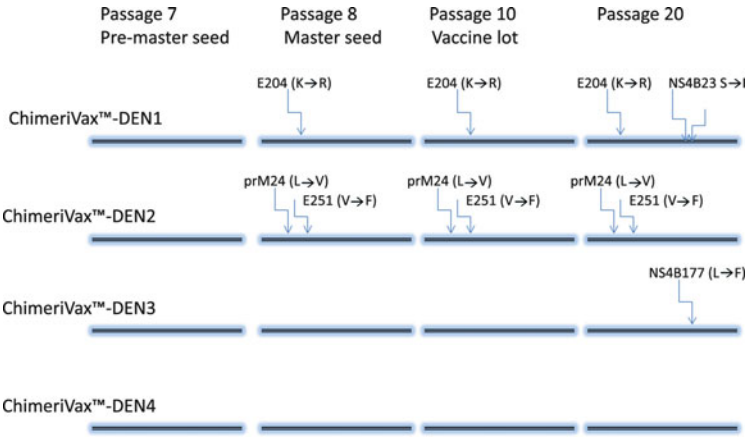


Fig. 6 Mutations in plaque-purified, reconstructed ChimeriVax™-DEN monovalent seeds and vaccine lot during GMP production in serum-free Vero cells

mutations during cell culture passages had not increased the neurovirulence phenotypes of these viruses, 4-day-old suckling mice were inoculated IC with various doses of P7 (Pre-Master Seed) and P20 viruses. Neurovirulence of the chimeras was not increased from P7 to P20; in fact, some chimeras lost their neurovirulence upon in vitro passages. In addition to the in vitro stability studies, virus recovered from the sera of monkeys were sequenced and if mutations were found, tested in the 4-day mouse test. Point mutations were found in DEN1 and DEN3 chimeras but neurovirulence for 4-day-old mice remained less than that of YF 17D.

The E protein mutation (K→R) at residue 204 that appeared between P7 and P8 in ChimeriVax™-DEN1 was the subject of investigation to determine the effects on biological phenotype [102]. Although mutations were found in the other chimeras (Fig. 6), none affected neurovirulence. However, the E204 (K→R) mutation in ChimeriVax™-DEN1 increased the plaque size of the virus and reduced neurovirulence in the 4-day mouse test. At P7 (no mutations), the LD_{50} for 4-day-old mice was <2.0 $\log_{10}$ PFU, whereas for the virus containing the mutation the LD_{50} was >5.1 $\log_{10}$ PFU. Monkeys inoculated SC with P7 (no mutations) developed higher and more prolonged viremia than monkeys inoculated with virus containing E204 (K→R), indicating a linkage between attenuated neurovirulence and viscerotropism for monkeys. Fortunately, this mutation and attending attenuation had minimal effect on immunogenicity of the E204 mutant virus. As predicted from the SC inoculation experiment, monkeys inoculated IC with P10 vaccine had lower viremias (mean peak titer 48 PFU/mL than monkeys inoculated with P7 (no mutation) virus (722 PFU/mL) ($p = 0.0432$ ANOVA). All monkeys in both groups developed DEN 1-specific neutralizing antibodies. On Day 31, antibody titers ranged from 640 to 10,240 and from 2,560 to >20,480 in the ChimeriVax™-DEN1 P7 and P10 treated groups, respectively, indicating that attenuation due to E204 (K→R) did not impair immune responses.

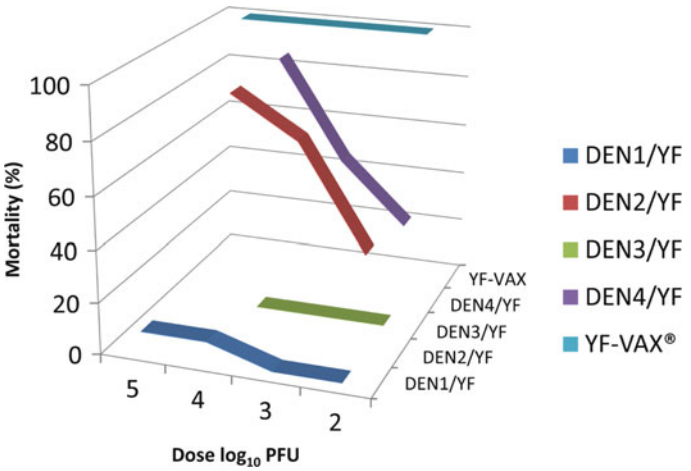


Fig. 7 Mortality ratios by dose, 4 day-old infant mice inoculated IC with plaque-purified P10 ChimeriVax™-DEN vaccines and YF-VAX®. All chimeric candidates were significantly attenuated for neurovirulence compared to YF-VAX®

Safety of each final monovalent component and the tetravalent formulation was assessed by studies of neurovirulence in mice using a sensitive 4-day-old suckling mouse test and shown to be improved compared to YF 17D (Fig. 7). No interference for replication in mouse brain tissue between serotypes was found when the mixture and individual components were inoculated IC [100]. A GLP monkey neurovirulence test was performed on the tetravalent vaccine. As predicted by mouse studies, the histopathological lesion scores were significantly lower than seen in monkeys inoculated with YF 17D.

The ability of the chimeric dengue vaccine viruses and tetravalent formulation (P10 vaccine level) to infect *Ae. aegypti* mosquitoes by intrathoracic (IT) inoculation or oral feeding was evaluated, and compared to wild type DEN viruses and YF 17D, which is not transmissible by mosquitoes [103]. The replication profile of the chimeric viruses in mosquito tissue was similar to that of YF 17D virus. In mosquitoes, the growth rate of each chimeric virus was similar whether it was a single serotype infection, or part of the tetravalent mix, with no interferences observed. The chimeric viruses replicated and disseminated to head tissue, but mean titers of all chimeric viruses were lower than that of IT-inoculated YF 17D virus. The ChimeriVax™-DEN viruses infected mosquitoes poorly via infectious blood meals compared to the wt DEN parent viruses, which indicates that the chimeric viruses are not able to infect and replicate in *Ae. aegypti* midgut tissue. Similar studies and conclusions were reported by Vanlandingham et al. [104].

To evaluate efficacy of the plaque-purified chimeras, studies were performed in monkeys inoculated SC with 5 $\log_{10}$ PFU of P10 monovalent chimeras or tetravalent vaccine [100, 101]. Serotype specific viremias were measured by a validated monoclonal immunofocus assay that detected the four different serotypes in a

mixture without interference between serotype viruses. Viremia in monkeys was similar to that induced by YF 17D and lower than those following inoculation with the wild-type parental dengue strains. All monkeys inoculated with the tetravalent vaccine developed neutralizing antibodies to all four serotypes (Tables 10 and 11). The predominance of DEN2/YF seen in earlier studies with uncloned viruses was not observed. Neutralizing antibody titers following tetravalent vaccine were robust, indicating that interference was not a problem (in the monkey model). There was no clear advantage in safety or immunogenicity in monkeys of formulations with different dose ratios of the four component viruses (Table 11). Low level viremias were observed to most of the individual components, but in some cases viremia to DEN1 and DEN2 was not detected. In general DEN3 and 4 chimeras tended to induce higher and longer viremia than DEN 1 or 2. Despite those observations, the vaccine elicited antibody responses to all four serotypes. Monkeys were divided into groups, and challenged with wild-type DEN viruses; all were protected against viremia following challenge except for one monkey in the 5,5,5,3 group challenged with DEN1 and one animal in the 3,5,5,3 group challenged with DEN4 [101]. Compared to unimmunized controls, viremias in these animals were abbreviated and delayed. The two monkeys in question had the lowest prechallenge neutralization titers (20 and 40, respectively). The formulations containing 5 or 3 $\log_{10}$ PFU of each component appeared to perform best; a formulation containing 3.1–3.7 $\log_{10}$ of each component was selected for the first clinical trial. An IND was filed in 2004 and a Phase 1 trial initiated.

Despite these encouraging results, there were some subtle signs for interactions (interference) between chimeric subtype vaccines in the monkey model. Viremias to all four subtypes were less frequently detected (or in the case of DEN2) and were of shorter duration when the viruses were inoculated in a tetravalent formulation

Table 10 Viremia and neutralizing antibody responses to monovalent and tetravalent plaque-purified ChimeriVax™-DEN vaccines (P10) in cynomolgus macaques

Virus	Dose ($\log_{10}$ PFU/ml)	Viremia			Antibody to virus inoculated	
		No. viremic/ tested	Mean peak viremia ($\log_{10}$ PFU/mL)	Mean duration (Days)	Seroconverted	GMT
ChimeriVax™-DEN1	5	1/3	2.7	6	3/3	1016
ChimeriVax™-DEN2	5	1/3	2.0	3	3/3	320
(GMP lot)	5	3/3	1.8	2.3	3/3	127
ChimeriVax™-DEN3	5	3/3	1.8	3	3/3	403
ChimeriVax™-DEN4	5	3/3	2.1	4.3	3/3	50
ChimeriVax™-tetravalent	(5,5,5,5)	3/3	1.9	3.7	DEN1 3/3 DEN2 3/3 DEN3 3/3 DEN4 3/3	254 403 806 2032
YF 17D (YF-VAX®)	5	3/3	1.7	2.3	NT	NT

Table 11 A second experiment in groups of 6 cynomolgus macaques inoculated SC with different mixtures of chimeric viruses; monkeys were randomized and subsequently challenged with wild-type dengue virus; modified from Guirakhoo et al. [101]

Formulation DEN 1,2,3,4 $\log_{10}$ PFU	ChimeriVax™-DEN serotype	Proportion viremic (%) by serotype	$PRNT_{50}$ GMT by serotype		Wild-type DEN Challenge (with serotype shown in column 2)	
			Day 31	Day 121 (prechallenge)	Viremic post challenge (mean peak[a], duration[b])	≥4-fold rise in antibody
5,5,5,5	1	5/6 (83%)	452	359	0/1	1/1
	2	0/6 (0%)	508	359	0/2	2/2
	3	6/6 (100%)	452	1,016	0/2	2/2
	4	6/6 (100%)	508	508	0/1	1/1
3,5,5,3	1	0/6 (0%)	90	101	0/1	1/1
	2	4/6 (67%)	285	452	0/1	1/1
	3	4/6 (67%)	254	160	0/1	1/1
	4	2/6 (33%)	10	29	1[c]/3 (1.7, 2.0)	3/3
5,5,5,3	1	4/6 (67%)	226	142	1[d]/2 (3.3, 4.0)	2/2
	2	5/6 (83%)	452	508	0/3	3/3
	3	5/6 (83%)	275	320	0/1	1/1
	4	5/6 (83%)	26	718		
3,3,3,3	1	4/6 (67%)	254	63	0/2	2/2
	2	4/6 (67%)	359	320		
	3	4/6 (67%)	285	254	0/2	2/2
	4	6/6 (100%)	452	34	0/2	2/2
Not vaccinated	1			<10	4/4 (3.2, 6.0)	4/4
	2			<10	4/4 (2.5, 4.5)	4/4
	3			<10	4/4 (2.2, 4.2)	4/4
	4			<10	4/4 (3.2, 5.2)	4/4

[a] $\log_{10}$ PFU/mL
[b] Days
[c] Prechallenge neutralization titer was 20
[d] Prechallenge neutralization titer was 40

compared to monovalent vaccination. These subtle effects in the monkey model did not predict the more intense interactions observed clinically.

The initial human trial of tetravalent ChimeriVax™-DEN (unpublished) provided the first insights into the phenomenon of interference between the live virus components. The randomized, double-blind, placebo controlled Phase I study evaluated the safety, tolerability and immunogenicity of live attenuated tetravalent ChimeriVax™-DEN vaccine and YF 17D vaccine (YF-VAX®) in 99 healthy adult volunteers in the US. In the first stage, 33 subjects received tetravalent ChimeriVax™-DEN (Group 1), 33 subjects received YF-VAX® (Group 2) and 33 subjects (Group 3) received placebo (saline). A second vaccination was performed 6 months later (open label). Subjects in all three groups received tetravalent ChimeriVax™-DEN containing 3.1–3.7 $\log_{10}$ of each component . The vaccines were well tolerated, with no safety signals attributable to the tetravalent chimeric vaccine. A higher

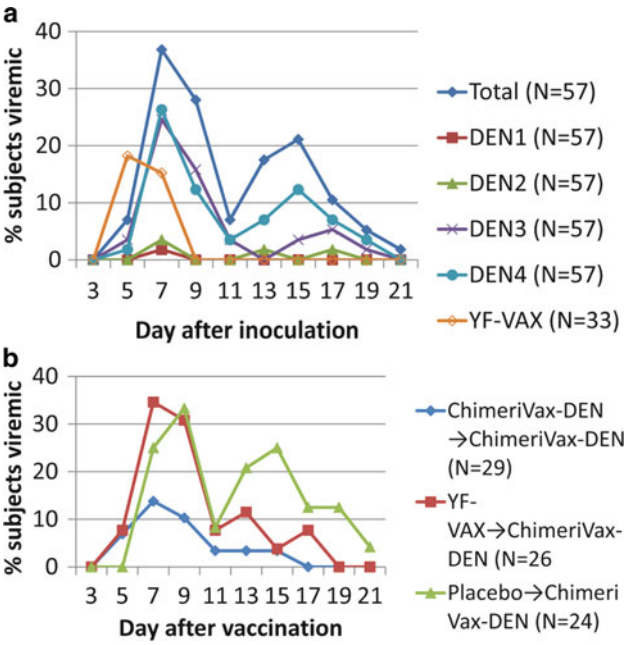


Fig. 8 Viremia in human subjects following tetravalent ChimeriVax™-DEN (mixture of 4 $\log_{10}$ of each virus) or YF-VAX®. (**a**). Viremia after a single dose of tetravalent ChimeriVax™-DEN showing total viremia and serotype specific viremia. Subjects in Group 1 after the first dose of chimeric vaccine and subjects in Group 3 who had previously received placebo and then ChimeriVax™-DEN were pooled for analysis. (**b**). Viremia following a second vaccination of tetravalent ChimeriVax™-DEN, or a single vaccination in YF-immune subjects 6 months after primary vaccination versus subjects receiving a primary vaccination with ChimeriVax at that time point

incidence of viremia and more prolonged viremia were observed after the first dose of chimeric vaccine than seen following YF-VAX® (Fig. 8a). As predicted from the studies of plaque-purified chimeric vaccines in monkeys [100, 101], viremia was principally caused by the DEN3 and DEN4 components of the vaccine, and rarely by DEN1 or 2. A biphasic pattern was also observed. When the chimeric vaccine was used to boost subjects at 6 months, viremia was blunted compared to subjects receiving primary vaccination (Fig. 8b), demonstrating a degree of protection. Prior YF vaccination did not affect the early phase of viremia but appeared to modulate viremia in the second week. All peak viremia levels were less than 2.2 $\log_{10}$ PFU/mL. These data, together with the viremia data from the Phase I study of monovalent ChimeriVax™-DEN2 [98] showing higher DEN2 viremias indicated that there was interference between the serotypes in the tetravalent mixture principally due to DEN3 and 4 which caused more active infections.

These observations were borne out by tests for antibody. Neutralizing antibodies were measured against the homologous (vaccine) virus and at least one different wild-type strain of each serotype (listed in the footnote to Table 12). Sera taken after the first dose of ChimeriVax™-DEN were tested against two wild-type strains

Table 12 Phase 1 clinical trial, tetravalent ChimeriVax™-DEN, percent of subjects seronegative at baseline and developing neutralizing antibodies (titer ≥10) to the homologous vaccine virus or at least one wild-type strain of each serotype 30 days after one or two doses (6 months apart) or one dose 6 months after YF 17D vaccination. The GMT is also shown by serotype

Statistic	Serotype	Group 1 ChimeriVax-DEN–> ChimeriVax-DEN		Group 2 YF-VAX–> ChimeriVax-DEN		Pooled single dose Chimeri-Vax™-DEN*
		30 days post primary ($N = 33$)	30 days post boost ($N = 29$)	30 days post primary ($N = 33$)	30 days post boost ($N = 26$)	30 days post primary ($N = 54$)
Seroconversion[a]	DEN1	21 (64%)	23 (79%)	6 (18%)	26 (100%)	38 (70%)
	DEN2	22 (67%)	17 (59%)	1 (3%)	25 (96%)	33 (61%)
	DEN3	32 (97%)	27 (93%)	3 (9%)	25 (96%)	47 (87%)
	DEN4	26 (79%)	23 (79%)	0 (0%)	20 (77%)	37 (69%)
GMT[a]	DEN1	25	57	9	102	34
	DEN2	26	42	5	183	29
	DEN3	225	87	5	163	152
	DEN4	100	44	5	45	61

[a]Sera were tested against the homologous virus (ChimeriVax) and 2 wild-type strains: DEN 1 16007 and Western Pacific 74; DEN2 16681 and S16803, DEN3 16562 and CH53489, and DEN4 1036 and TVP 360. Not all 30 day post boost sera were tested against the second wild-type dengue virus

of each serotype. This was important because, in contrast with the study of monovalent ChimeriVax™-DEN2 [98], some subjects seropositive for a wild-type strain were seronegative for the homologous virus. While respectable seroconversion rates were observed, antibody titers (particularly for DEN 1 and 2) were substantially lower and more variable than observed following monovalent DEN2 immunization [98]. The highest responses were seen against DEN 3 and 4, as expected from the viremia patterns (Fig. 8). Interestingly, in this study a booster dose at 6 months provided little increase in antibody titers. However, as noted in the monovalent vaccine trial, prior YF 17D immunization followed by tetravalent ChimeriVax™-DEN vaccine increased antibody responses to DEN 1 and 2. Overall, the results of the trial were encouraging and guided further vaccine development. However, the formulation for further development was changed to increase the dose of each component to 5 $\log_{10}$ PFU, and emphasis was placed on delivering multiple doses on an optimal schedule.

The tetravalent vaccine is now in advanced Phase II/III trials in Thailand, involving 4,000 children who will receive three doses of tetravalent ChimeriVax™-DEN vaccine or a control in a 2:1 ratio. Leading up to these trials, Sanofi Pasteur conducted Phase II studies in the US, Australia, Mexico and the Philippines in both adults and children. The results have not been published, but have been presented at meetings and summarized recently by Guy et al. [105]. Safety in over 880 subjects has been good, with no increase in adverse events over subjects receiving placebo or control vaccines. It is notable that no dengue-like adverse reactions (rash, transaminase elevations, neutropenia) have been observed, whereas these adverse events have been consistently observed following the empirically derived live, DEN vaccines as well as rationally designed mutagenized and chimeric

DEN vaccines (see below). Viremia levels have been consistently low and, importantly, were not enhanced in Mexican and Filipino children who were DEN-immune prior to vaccination. In a trial conducted in 66 flavivirus-naive adults, 18–40 years of age, in the US, subjects received three injections of vaccine or placebo at 0, 3–4 and 12 months. 100% seroconversion was noted after the third dose with GMTs of 67, 538, 122 and 154 against DEN 1–4, respectively. In Mexico, 72 children 2–11 years, 36 adolescents, and 18 adults received the same regimen outlined above except that a control vaccine (YF 17D) was given in lieu of placebo; the prevalence of dengue immunity at baseline was 8%. The proportion of subjects developing neutralizing antibodies increased after each successive injection of vaccine; after the third vaccine dose, 82% of subjects had developed neutralizing antibodies to three or more serotypes. The seroconversion rate was lowest to DEN 1 (79%) and highest to DEN 4 (92%). GMTs to the four serotypes (67, 538, 122 and 154) were higher than seen in the initial Phase 1 study in US adults. In the youngest age group (children 2–5 years) seroconversion was 94–100% for DEN types 2–4 and 88% for DEN 1. A trial in the Philippines had a design similar to that in Mexico, but there the prevalence of flavivirus (DEN and JE) immunity was 80%. The results were similar to those in Mexico, except that the background of natural immunity provided a booster effect and two doses of tetravalent vaccine (in the control group) evoked a similar response to three doses. These findings reinforce the conclusion that preexisting flavivirus immunity, whether due to DEN, JE or YF will be an important potentiating factor in the efficacy of tetravalent DEN vaccines.

The mechanisms underlying interference effects between serotypes in the tetravalent vaccine have not been elucidated, but probably involve competitive inhibition of replication in the skin and draining lymph nodes mediated by rapid innate immune responses , with monovalent components of the vaccine that replicate more rapidly shutting off sister components in the mixture. In vitro, absent immune mechanisms, interference is not observed even though the components of the tetravalent vaccine grow at different rates. The growth curves of single ChimeriVax™-DEN1–4 viruses in vitro have been compared to wild-type DEN viruses. The titer and growth rate of each chimeric serotype was similar, whether it was a single infection, or part of the tetravalent mix, (Fig. 9). No interference by one chimeric virus with a faster growth rate over a slower-growing serotype was observed. The interference effects in vivo are observed very early, during the viremic period. To wit, 64% of humans injected with 3 $\log_{10}$ PFU of monovalent ChimeriVax™-DEN2 developed detectable viremia (Table 9), whereas only 3.5% of subjects receiving tetravalent vaccine containing the same dose of ChimeriVax™-DEN2 developed viremia (Fig. 8). These observations support a role of innate immunity in the interference phenomenon.

To avoid the interference effects, several strategies have been investigated, including favoring the “weaker” viruses by adjusting the ratio of doses in the tetravalent formulation, or injecting bivalent vaccines at different anatomical sites, or spaced in time. Compared to simultaneous injection of all four serotypes in nonhuman primates, an improved response could be achieved by injecting a bivalent vaccine in different arms or at an interval of 2 months [105]. Priming with

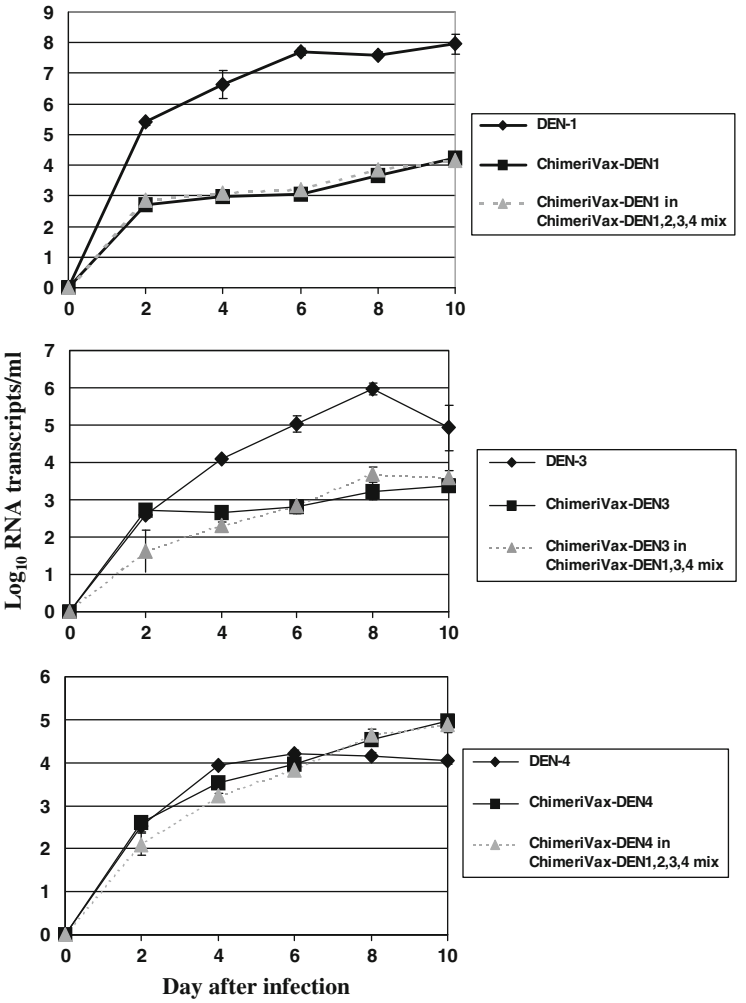


Fig. 9 Virus growth of ChimeriVax™-DEN alone or in a tetravalent formulation in C6/36 *Ae. albopictus* cells (MOI 0.01 PFU/cell). Virus titer shown is the mean titer ± SD from triplicate cell culture assays

a monovalent DEN vaccine (or YF 17D) also had the desired effect of stimulating a broad response after boosting with tetravalent vaccine. However, it would not be feasible to prime with a monovalent or bivalent dengue vaccine in human populations which may be placed at higher risk of developing severe DEN if exposed to wild-type dengue before the immunization regime could be completed. For practical reasons other approaches are being pursued, principally the use of multiple doses, spaced appropriately in time. It is important that booster doses not be given too early, since adaptive immune responses can interfere with the response to vaccination. The mechanism underlying short term cross-protection, observed by

Sabin to last about 6 months in early studies of dengue [106], is unknown, but could be IgM antibodies [105], antibodies that have not undergone affinity maturation, antibodies to cross-neutralizing epitopes on domains I and II that wane below protective titers, or cell mediated immunity. Cell-mediated immune responses to the tetravalent vaccine have been studied in some depth by Guy et al. [38]. Th1 oriented responses to DEN4 predominate after the first dose, but broaden after a boost.

A different approach being considered for avoiding interference of primary immunization is the construction of a virus with an E protein that contains neutralizing epitopes to all four DEN viruses. Such a protein can be generated by gene shuffling [107], [108] and naked DNA immunization with plasmids encoding the shuffled E protein has been shown elicit broad responses. Recombinant adenovirus carrying multiple DEN E genes have shown promise as well. It is uncertain whether a live chimeric virus with a shuffled E protein can be constructed and elicit strong tetravalent antibodies, but if so, use of a single virus could obviate interference phenomena.

3.2.4 Chimeric Dengue/Dengue and Deletion Mutant Vaccine Candidates Developed at NIAID

The development by several groups of live DEN vaccines using empirical passage has clearly demonstrated the feasibility of attenuating DEN virus. With the advent of infectious clone technology, an obvious strategy for rationale design would be to harness an attenuated DEN strain as a vector for insertion of the structural genes of other serotypes. One potential advantage of such an approach is that the entire chimeric virus would be composed of dengue genetic material, which might add to protective immunity and ensure long term immunological memory. However, a potential disadvantage of this approach is that heterologous cross-reactive T cell responses might favor DHF if neutralizing antibody responses were incomplete or not durable [42].

NIAID scientists developed DEN 4 virus to serve as the backbone for construction of intertypic chimeras. The first constructions utilized a cDNA clone of the wild type DEN4 strain 814669. The structural genes of DEN4 were replaced by the corresponding genes of another DEN serotype virus [11, 89]. Replacement of genes is facilitated by the fact that there is significant sequence conservation among the four DEN serotype viruses. Initially, the chimeras were constructed with all three structural genes (C-prM-E) of wild type DEN1, Western Pacific strain (WP), or the mouse-adapted DEN2, New Guinea C strain (NGC) and the remaining sequence from the DEN4 virus. A chimeric DEN3/DEN4 virus with the C-prM-E genes of the wild type DEN3 strain H53489 was also constructed [109]. The chimeras were attenuated for replication as the result of chimerization [89]. Construction of a chimeric DEN2/DEN4 virus that contains only the DEN2 prM-E genes was also constructed as an alternative strategy.

The biological behavior of the DEN4 (wild-type backbone) chimeras was explored in rhesus macaques inoculated by the SC route with DEN1/DEN4 (C-prM-E, Western Pacific), DEN2/DEN4 (prM-E, mouse adapted NGC), and the parental DEN4, DEN1, or DEN2 strains used to derive the chimeras [90]. Monkeys inoculated with the monovalent chimeras or a mixture of two chimeras developed respectable neutralizing antibody responses and were protected against challenge. These experiments were encouraging, but did not elucidate whether the chimeras were sufficiently attenuated for human use.

Mutations were subsequently introduced into the wild type DEN4 backbone to attenuate the virus, using biomarkers of reduced replication in vitro and reduced viremia in rhesus monkeys [21]. To avoid potential problems of reversion at point mutations, deletion mutants were preferred. A panel of mutant DEN 4 viruses with deletions ranging from 30 to 262 nucleotides (nt) in the 3′ NCR were investigated by Men et al. [23] A DEN4 virus with a 30-nt deletion (Δ30) in the 3′ NCR (nt 172–143 from the 3′ terminus in the TL2 stem-loop structure exhibiting reduced plaque size in C6/36 cells, reduced replication in LLC-MK2 cells and reduced viremia in rhesus monkeys elicited a moderate level of neutralizing antibodies. The virus was not restricted for growth in mosquitoes after intrathoracic inoculation, but was unable to establish disseminated infection of *Ae. aegypti* mosquitoes after oral feeding, and thus could not transmit the vaccine virus [110]. This virus was selected as a candidate DEN4 vaccine and intertypic chimeric vector.

Clinical grade recombinant DEN4 Δ30 (rDEN4Δ30) vaccine was manufactured in Vero cells and evaluated for safety, tolerability and immunogenicity in a Phase I clinical trial (Table 13) [111]. A single dose of 5 $\log_{10}$ PFU was administered by the SC route to 20 seronegative volunteers. A macular rash developed in 50% of the subjects; a transient mild-moderate elevation in liver enzymes (alanine aminotransferase, ALT) was observed in 25% of the subjects; and 15% of the subjects had mild and transient neutropenia. The rash was faint, difficult to discern, nonpruritic, and was seen only in subjects who had detectable viremia. Viremia occurred in 70% of subjects, but was low (mean peak 1.6 $\log_{10}$ PFU/mL). These side effects were not unexpected as similar experiences have been described in studies of empirically attenuated live dengue vaccines [117]. All 20 volunteers seroconverted to DEN 4 and developed high levels of neutralizing antibodies (GMT 580). The level of antibodies was similar in subjects who were viremic and those that were not. A subsequent controlled clinical trial was conducted in which lower doses (1,2 and 3 $\log_{10}$ PFU) were given to groups of 20 subjects (together with placebo in four subjects) to determine if the rash and transaminase elevations were less frequent at a lower vaccine doses [114]. While rash and neutropenia were observed at all dose levels, no ALT elevations were seen at doses of 1 or 2 $\log_{10}$ PFU and only one (5%) of subjects given 3 $\log_{10}$ PFU had ALT elevation. As with other live vaccines, including the JE and DEN/YF chimeras, there was no dose response for immunogenicity; 95–100% of subjects seroconverted and developed robust neutralizing antibody responses (GMT 139–380). The DEN4 genome with the 30-nt deletion in the 3′ NCR was considered an attractive vector for the construction of intertypic dengue chimeras expressing the antigenic regions of DEN1, DEN2, and DEN3 [118].

Table 13 Summary of clinical studies of DEN vaccines developed by NIH

Phase (Vaccine)	Location	Age (years)	Vaccine (No. subjects)	Dose (log_{10} PFU)	Adverse events		Viremia			Neutralizing antbodies to JE		Mutations in Δ30 region of virus recovered from serum	Reference
					Serious, related	Nonserious	Proportion viremic (%)	Duration, days	Peak, mean log_{10} PFU/mL[a]	GMT ($PRNT_{60}$[b]) Day 28–31	Seroconversion (%)[b] Day 28–31		
1 (DEN4)	US	18–45	rDEN4Δ30 ($N = 20$)	5.0	0	Minimal local reactions; no fever-arthraligia-headache syndrome (dengue fever); 50% maculopapular rash; 15% neutropenia; 20% ALT transient elevation	70%	4.4	1.6	567 (426–652[c])	100%	No	[111]
1 (DEN4)	US	18–50	rDEN4Δ 30-4995 ($N = 20$)	5.0		85% had min. rash at injection site; systemic AEs similar across active and placebo; 10% maculopapular rash; 5% neutropenia; 5% transient ALT elevation	0%	–	–	150	95%		[112]
			Placebo ($N = 8$)	–			0%	–	–	<10	0%		
1 (DEN4)	US	18–50	rDEN4Δ30-200,101 ($N = 20$)	5.0		No signif. Local reactions; systemic AEs similar across active and placebo; 5% mild fever; 20% maculopapular rash; 10% neutropenia (but also in 2/8 placebo); 0% ALT elevation	0%	–	–	100	100%		[113]
			Placebo ($N = 8$)	–			0%	–	–	<10	0%		
2 (DEN4)	US	18–50	rDEN4Δ30 ($N = 20$)	3.0	0	Minimal local reactions; no differences in AEs between active and placebo; 1.7% low-grade fever; 70% maculopapular rash; 23% neutropenia; 1.7% ALT transient elevation	35%	1.6	0.5	139	100%	No	[114]
			rDEN4Δ30 ($N = 20$)	2.0	0		55%	2.6	0.7	189	95%	No	
			rDEN4Δ30 ($N = 20$)	1.0	0		65%	1.8	0.6	380	100%	No	
			Placebo ($N = 12$)	_	0		0%	0	0	<10	0%		

1 (DEN1)	US	18–50	rDEN1Δ30 ($N = 20$)	3.0	0	Minimal local reactions; headache higher in	45%	2.8	1.0	444	95%	No	[115]
			Placebo ($N = 8$)	_	3	placebo, no other differences in AEs between active and placebo; 40% maculopapular rash;40% neutropenia;0% ALT elevation	0%	0	0	<10	0%		
1 (DEN2)	US	18–50	rDEN2/DEN4Δ30 ($N = 20$)	3.0	0	Minimal local reactions; no differences in AEs between active and	55%	3.2	0.6	147	100%	No	[116]
			Placebo ($N = 8$)	–	0	placebo, similar profile to other candidates above; no fever; 45% maculopapular rash; 35% neutropenia;15% ALT elevation[d]	0%	–	–	<10	0%		

[a]Mean peak for viremic subjects only
[b]Titers are to homologous strain (also see text); $PRNT_{50}$ = 50% plaque reduction neutralization test
[c]Nonviremic ($N = 6$) – viremic ($N = 14$) subjects
[d]2/3 subjects with ALT rise had elevated ALT on Day 0 (prevaccination) but had been normal on screening

It was thought, however, that the elevated liver enzymes associated with rDEN4Δ30 could be problematic as vaccine usage scaled up. In an attempt to abrogate this feature of the virus, two new vaccine candidates were constructed introducing two mutations at positions 200 and 201 in NS5 or a single mutation at position 158 of NS3 (rDEN4Δ30-4995) (Fig. 5). The rDEN4Δ30-200,201 and rDEN4Δ30-4995 candidates were more attenuated than the rDEN4Δ30 parent in monkeys and SCID mice reconstituted with human liver cells (HuH 7) [20, 119] but still elicited neutralizing antibodies. Clinical trials were performed with both vaccines (Table 13) [112, 113]. These studies showed that the vaccines were more attenuated than parental rDEN4Δ30, produced no viremia, little or no hepatotoxicity (0% of subjects for rDEN4Δ30-200,201; 10% for rDEN4Δ30-4995), and lower neutralizing antibody levels, and a lower incidence of neutropenia and generalized rash. The -4995 candidate was associated with a localized rash around the injection site in a high proportion of subjects. Sera from vaccinees in different trials who received rDEN4Δ30-4995, rDEN4Δ30-200,201 and rDEN4Δ30 in different trials were compared in a single neutralization test [112]. The rDEN4Δ30-200,201 candidate had a GMT only twofold lower than rDEN4Δ30. The two vaccine candidates are therefore being evaluated further in the context of tetravalent immunization.

Multiple constructs were evaluated in the development of a chimeric DEN 1 vaccine, in which all structural genes (C-prM-E) or just the prME genes of DEN1 Puerto Rico/94 virus were inserted into either unmodified DEN4 or the DEN 4Δ30 vector (Fig. 5) [120]. The constructs were evaluated in the SCID-HuH-7 mouse model, rhesus monkeys and *Ae. aegypti* mosquitoes. The viruses grew to 6–7 $\log_{10}$ PFU/mL in Vero cells. During passage in Vero cells to produce virus stocks, 1–3 mutations appeared in all viruses, including mutations in NS4B likely to be an adaptation to growth in Vero (as was seen for ChimeriVax™-DEN). The Δ30 deletion restricted replication in mosquitoes. The viruses with unmodified DEN4 backbone were not attenuated for rhesus monkeys and the rDEN1 /4(prME) virus was not attenuated in the SCID-HuH-7 model when compared to wild-type DEN1 virus. The rDEN1/4Δ30 (C-prM-E) virus was over-attenuated, failed to elicit antibodies in monkeys or protect against wild-type DEN1 challenge. However, the rDEN1/DEN4Δ30 (prME) virus which retained the DEN4 capsid evoked a modest neutralizing response in 66% of monkeys and protected animals against challenge. This activity was considered to be insufficient for an effective human vaccine. An additional vaccine candidate had been developed, based on the clinical success of the rDEN4Δ30 candidate and the fact that the 3′NCR sequences are highly conserved across dengue serotypes – the Δ30 deletion was introduced into the 3′-NCR of DEN 1 [118]. The rDEN1Δ30 vaccine candidate was attenuated in the SCID-HuH7 mouse and showed reduced viremia in rhesus monkeys, while being immunogenic and protective against DEN1 challenge [121]. However, the virus was not restricted for growth in mosquitoes following intrathoracic inoculation.

Durbin et al. [115] evaluated rDEN1Δ30 at a dose of 3 $\log_{10}$ PFU in 20 healthy adult subjects; eight subjects received placebo in the double-blind study (Table 13). As observed for the rDEN4Δ30 candidate, asymptomatic rash (in 40%) and

neutropenia (in 40%) were associated with the rDEN1Δ30 vaccine, though none of the subjects developed clinical dengue fever and none had elevated liver enzymes (ALT). One subject had a transient, mild thrombocytopenia lasting 1 day. The rash was a faint maculopapular eruption, typically not self reported, rarely pruritic, and lasting up to a week. The investigators concluded that the vaccine candidate was well tolerated. Viremia was low (45% viremic; mean peak 1.0 $\log_{10}$ PFU/mL; mean duration 2.8 days), and antibody responses were robust, with Day 28 GMT of 444 and 95% seroconversion rate.

Attempts were made to develop DEN2 and three candidates were constructed with Δ30 mutations in the 3′-NCR analogous to the DEN1 and 4 candidates. Unfortunately, these constructs did not prove to be satisfactorily attenuated for monkeys [122, 123]. Development logically next focused on the construction of chimeric viruses using the DEN4Δ30 vector backbone [91, 122]. The DEN2 chimeric vaccine candidate was constructed by insertion of the prM-E genes of prototype New Guinea C (NGC) virus into the attenuated DEN 4Δ30 vector. The chimeric virus was attenuated in both SCID-HuH-7 mice and rhesus monkeys compared to wild-type DEN2 NCG virus. As for other Δ30 DEN viruses, the candidate vaccine failed to infect *Ae. aegypti* after oral feeding. A placebo controlled clinical trial was conducted with an identical design to the Phase I study of rDEN1Δ30 described in the preceding paragraph [116]. Overall the results of this study painted a consistent picture. Asymptomatic rash was seen in 45%, transient neutropenia in 35%, and mildly elevated ALT in 15% of the subjects. There were no cases of dengue fever, and the incidence of adverse events other than rash and the laboratory abnormalities did not exceed that in placebo treated subjects. Viremia was low (55% viremic; mean peak 0.6 $\log_{10}$ PFU/mL; mean duration 3.2 days). Neutralizing antibody responses were seen in 100% of subjects with moderate GMTs (147 and 237, on days 28 and 42, respectively).

The DEN3 chimeric vaccine [rDEN3(prME)/4Δ30] turned out to be more problematic. Preclinical evaluation in rhesus monkeys indicated that the virus was probably overattenuated (no viremia, low Day 28 GMT of 58), and indeed this proved to be the case in a clinical trial. Two dose levels of the vaccine (3 and 5 $\log_{10}$ PFU) were administered SC; only 5 (25%) of 20 subjects at the 5 $\log_{10}$ PFU dose and 6 (30%) of 20 subjects given 3 $\log_{10}$ PFU developed neutralizing antibodies in this study. Consequently, further development of rDEN3/4Δ30 was stopped. However, based on the original strategy of modifying the DEN 3 3′NCR, two additional candidates had been developed. In the first case, the Δ30,31 mutant, two deletions at nucleotides 173-143 and 258-228 in the DEN 3 Sleman/78 virus were made. A second construct replaced the entire DEN 3 3′-NCR with that of DEN4Δ30, designated rDEN3-3′DEN4Δ30 [26]. Both the rDEN3Δ30/31 and rDEN3-3′DEN4Δ30 mutant viruses were similarly attenuated as the successful rDEN4Δ30 vaccine for SCID-HuH-7 mice. In rhesus monkeys the new candidates were highly attenuated compared to wild type DEN3 and produced no viremia. The rDEN3Δ30/31 candidate elicited a moderately strong antibody response (Day 28 GMT 304) whereas immunogenicity of rDEN3-3′DEN4Δ30 was weaker (GMT 77). rDEN3Δ30/31 was also strongly attenuated for mosquitoes.

Both rDEN3Δ30/31 and rDEN3-3′DEN4Δ30 mutant viruses are being evaluated in clinical studies.

Several different tetravalent formulations were evaluated in rhesus monkeys and compared with a mixture of wild-type DEN viruses [124]. The vaccine formulations included: (1) a mixture of DEN1–4 each with a Δ30 mutation in the 3′NCR (recall that the DEN2 and 3 components when tested alone were not sufficiently attenuated); (2) a mixture of DEN1Δ30 and DEN4Δ30 mutants and two chimeras, rDEN2/4Δ30 and rDEN3/4Δ30 using the mutant DEN4 backbone; and (3) a mixture of DEN1Δ30, DEN2Δ30, DEN4Δ30 and a tenfold higher concentration (6 $\log_{10}$ PFU) of the chimera rDEN3/4Δ30 (recall that this construct had been shown to be overattenuated). None of these tetravalent formulations represent the current mix of vaccine candidates that is being pursued clinically.[3] In monkeys, all three formulations tested were highly attenuated compared to a tetravalent mix of wild-type DEN viruses. Tetravalent immunization was feasible, although a broad response with neutralizing antibody levels above 1:100 to all serotypes could not be achieved with a single dose of any of the three formulations. The least immunogenic of the formulations was number two above (having chimeric DEN2 and 3 viruses), which resulted in poor antibody responses to DEN2 and 3. However, when a booster dose of formulation 2 or 3 was given at 4 months, broad high titer responses and full protection against challenge was achieved. No booster effect was observed when the second vaccination was given at 1 month, possibly due to interference by cross-protective heterologous antibodies.

These studies illustrated once again the problem of interference between components of a tetravalent vaccine. The lower neutralizing antibody response to the chimeric DEN2 and 3 components in the tetravalent formulation were not predicted by studies of these candidates when tested as monovalent vaccines [91, 123]. When tested for replication in the SCID-HuH7 model, and compared with monovalent components, no interference between viruses in the tetravalent formulation was observed. This observation was consistent with the lack of interference between DEN/YF chimeric components for replication in mouse brain [101]. If interference is the result of innate immune responses in lymphoid tissues following peripheral inoculation, the very different SCID-HuH7 and mouse brain models may not reveal such effects.

At the present time, two candidates for inclusion in a tetravalent vaccine include those shown below (Whitehead S pers. comm. 2009). All components will be at a dose of 3.0 $\log_{10}$ PFU. The two formulations differ in the DEN4 component (rDEN4Δ30 in Tetravalent 1 and rDEN4Δ30-200,201, which was more attenuated in a trial of the monovalent component [113] in Tetravalent 2).

[3]However, the tetravalent formulations (described below) that are planned for clinical development are currently being tested in rhesus monkeys.

Tetravalent 1

Dengue type 1 rDEN1Δ30 3′NCR deletion in wild type DEN 1 Western Pacific
Dengue type 2 rDEN2/DEN4Δ30 Chimeric dengue 4 having Δ30 3′NCR deletion, with prM-E replaced by corresponding genes of wild-type DEN 2 New Guinea C
Dengue type 3 rDEN3-3′DEN4Δ30 wild-type DEN 3 Sleman/78 with DEN4Δ30 3′NCR
Dengue type 4 rDEN4Δ30 3′NCR deletion in wild type DEN 4 814669

Tetravalent 2

Dengue type 1 rDEN1Δ30
Dengue type 2 rDEN2/4Δ30
Dengue type 3 rDEN3-3′DEN4Δ30
Dengue type 4 rDEN4Δ30-200,201 additional mutations at NS5 200 and 2001

The safety and immunogenicity profiles in clinical studies with monovalent components for DEN1,2 and 4 at a dose of 3.0 $\log_{10}$ PFU are compared in Fig. 10. The two tetravalent formulations have been tested in rhesus monkeys in formulations

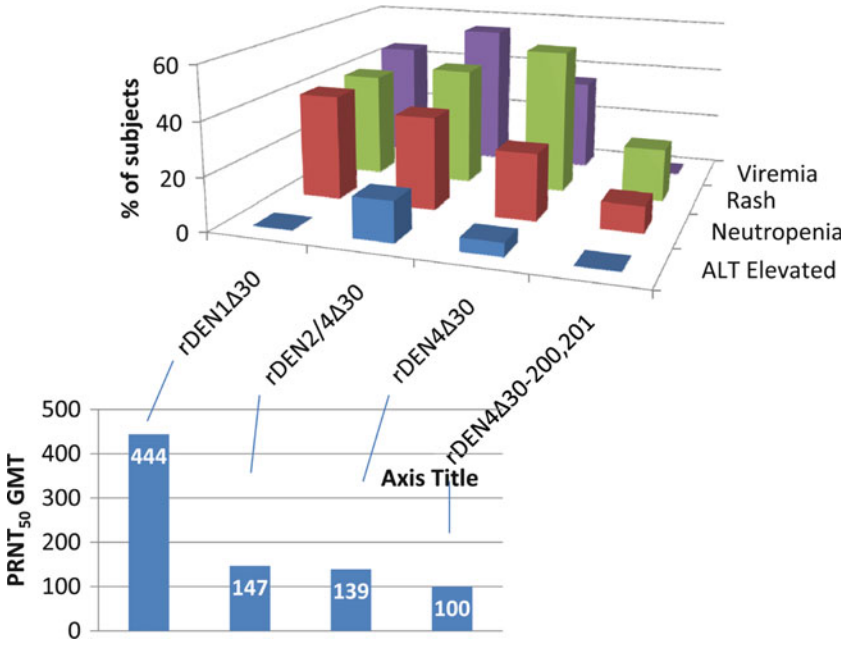


Fig. 10 Summary of clinical data with monovalent NIH dengue vaccines that will be formulated and tested in trials of tetravalent vaccines. Vaccines administered by the SC route at a dose of 3.0 $\log_{10}$ PFU. The DEN3 vaccine candidates are in ongoing clinical trials

containing 5 $\log_{10}$ PFU of each component and both showed seroconversion to all four viruses in 75–100% of the monkeys after a single dose and no significant differences between formulations (Whitehead S pers. comm. 2009). With respect to the choice of a DEN3 candidate for the first tetravalent trials, the outcome of clinical studies comparing rDEN3-3′DEN4Δ30 and rDEN3Δ30/31 are underway, and the latter vaccine will be substituted in the tetravalent formulations if immunogenicity and safety are superior to rDEN3-3′DEN4Δ30.

Interference phenomena in the monkey model were most apparent after the initial dose, and a booster dose of tetravalent vaccine formulations induced a broad response and higher antibody titers to all serotypes [124]. To achieve high antibody responses to all serotypes, it may be that two or more doses of the tetravalent vaccine will be required, but this decision must await clinical data. The conclusion that multiple doses would be required had been reached during development of the empirically derived live, attenuated vaccines at Mahidol University [125]. Preferably booster doses would be given at sufficiently long intervals (6 or 12 months) to avoid the transient cross-protection phenomenon [106] that would prevent "filling-in" of missing serotype immunity. A study in monkeys given the NIH tetravalent vaccine at 0 and 6 months showed, in contrast to the 0–1 month schedule, a boost in titer to all four serotypes, with respectable GMTs $>$ 100 for all four serotypes, with the highest rise in dengue 3 (which had the lowest level of antibody after primary immunization).

The NIH group also conducted studies with monovalent vaccine candidates in humans to investigate the ability of a second dose to boost homotypic immunity. Fifty subjects received 3 $\log_{10}$ PFU of the rDEN1Δ30 vaccine at an interval of 4–6 months. The first dose was followed by the expected viremia (in 67%), asymptomatic rash (27%), neutropenia (43%), seroconversion (92% on Day 42), GMT (98), titer range (19–844). The subjects were protected from side effects after the second dose with no cases of rash, no viremia and only 9% neutropenia. Interestingly, none of the subjects had a boost in antibodies after the second dose, indicative of sterile immunity. This was true even for subjects who developed very low titers after primary immunization, and contrasts with the results of the monkey experiment employing tetravalent vaccine described above. Possibly the presence of heterotypic antibodies, or a larger pool of B cells recognizing cross-reactive determinants explains the difference.

Some subjects that had participated in trials of monovalent vaccines were given a booster dose of a different (heterologous) monovalent vaccine. Enhanced clinical signs or disease were not observed after the heterologous boost, indicating that live attenuated DENV vaccines should be safe for use in populations with pre-existing DENV antibody. As might be expected from the studies of sequential YF and monovalent, bivalent or tetravalent YF/DEN chimeras [98, 105], heterologous prime-boost using the NIH vaccines resulted in a broad multivalent neutralizing antibody response and enhanced GMTs. In sum, these studies suggest that the second or third inoculations of tetravalent vaccine, at the appropriate interval (longer appears better), may result in a broad tetravalent neutralizing antibody response, a conclusion similar to that reached for the DEN/YF chimeras.

A meticulous effort has been invested in the construction and testing in non-human primates and humans of a series of monovalent vaccines with acceptable safety, tolerability and immunogenicity. The first clinical studies of the NIH tetravalent vaccine, using the best combinations of monovalent vaccines derived from this large body of work, are planned in 2010 in the US and also in Brazil at the Butantan Institute. The results are awaited with great interest.

3.2.5 Chimeric Dengue Vaccines Employing Attenuated Dengue Type 2 Vector

The chimeric DEN2 vectored vaccines were developed originally at CDC, Ft Collins and were licensed to Inviragen Inc. Inviragen has collaborated with Shantha Biotech (India) for manufacture and control of its vaccine. Inviragen recently merged with SingVax, a Singapore-based vaccine company. Inviragen plans to execute an initial proof of concept clinical study of a tetravalent formulation in the US in 2010. The Inviragen tetravalent vaccine is named DENVax™.

A live, attenuated DEN2 vaccine, DEN2 PDK53 was developed at Mahidol University, Thailand by empirical passage of the wild-type 16681 strain 53 times in primary dog kidney cells. The PDK53 vaccine candidate has small plaque phenotype, is temperature sensitive (ts), attenuated for infant mice inoculated IC, and has crippled replication in C6/36 cells. DEN2 PDK53 has been evaluated in various Phase I and II clinical trials as a monovalent vaccine [97, 125, 126] or in multivalent combinations [127]. DEN2 PDK53 had a good safety and tolerability profile and elicited moderately high $PRNT_{50}$ titers of 215–230 on day 60 in adult human subjects. PDK53 was therefore a suitable candidate as the backbone for construction of chimeric viruses having prM-E genes of heterotypic dengue viruses. DEN2 PDK53, like YF 17D and rDEN4Δ30, had the advantage that clinical data existed indicating safety and immunogenicity of the vector strain. Full-length cDNA clones were constructed from the DEN2 PDK53 virus and the parental DEN2 strain 16681 from which it was derived [128]. Sequence analysis showed that the PDK53 vaccine contains two variants designated PDK53-V and PDK53-E. PDK53-V contains all 9 NS gene mutations that occurred during derivation of the vaccine, while PDK53-E has these mutations except for a parental 16681 residue at NS3 250. PDK53-V is being pursued as the vaccine candidate for DEN2 and vector for intertypic chimeras.

The crippled replication of PDK53 virus in C6/36 cells and attenuation for mice are determined primarily by mutations in the 5′-NCR (5′-NCR57 C→T) and at NS153 G→D [129]. The ts phenotype of PDK-53 virus is caused by to the NS153 G→D and NS3250 E→V mutations. All three mutations contribute to the small-plaque phenotype of PDK53. Restoration of at least two of the three loci to the wild-type sequence was required to revert to the wild type characteristics of DEN2 16681 in vitro [129]. Genetic stability of the three attenuating mutations in PDK53 was

investigated by making multiple serial passages [130]. Instability of the 5′ NCR mutation was found but the other attenuating mutations were stable.

Since attenuation of the PDK53 virus depended on mutations in the NS gene backbone and 5′-NCR, a reasonable hypothesis was that the PDK53 backbone would confer an attenuated phenotype on chimeras constructed with wild-type DEN 1,3 and 4 structural gene sequences. To confirm this, chimeric viruses were constructed that contained the structural genes of an empirically derived attenuated DEN1 PDK13 vaccine or its parental wild-type DEN1 16007 strain in the NS gene background of either DEN2 PDK53 or the DEN2 16681 parental DEN2 virus [92]. DEN1 PDK13 had been assessed in clinical trials and was the most attenuated of all four live vaccine candidates, requiring 10,000 PFU to immunize 50% of subjects (compared to about 5 PFU for DEN2 PDK53 [131]. There are eight amino acid differences, five in the E protein, between the DEN1 PDK13 and its parental DEN1 16007 strain. The chimeric virus containing both donor and vector wild-type sequences [DEN1 (16007)/DEN2(16681)] grew less efficiently than either parental virus in C6/36 cells, demonstrating the attenuating effect of chimerization between heterologous dengue virus genes. The DEN1 (16007)/DEN2(PDK53) and DEN1 (PDK13)/DEN2(PDK53) chimeras retained the DEN2 PDK53 attenuation markers of small plaque morphology, ts in LLC-MK2 cells, and inefficient replication in C6/36 cells, as compared to wild type DEN1 16007. These findings validated the hypothesis that the PDK53 backbone would confer a fully attenuated phenotype when wild-type prM-E donors were used for construction of chimeras. The question was whether the chimeric constructs were overattenuated with respect to immunogenicity.

The immunogenicity of the DEN1 16007, DEN1 PDK13 and their derived chimeras was analyzed in 3-week-old outbred (ICR) mice [92]. Mice immunized with two doses (4 $\log_{10}$ PFU) of DEN1(16007)/DEN2(PDK53) or with parental 16007 virus developed high neutralization titers of 2,560–10,240. However, use of the attenuated DEN1 C-prM-E genes from PDK13 in the DEN2 PDK53 backbone evoked substantially lower $PRNT_{60}$ titers of 80–160 under the same conditions. The five amino acid changes identified in E separating the PDK13 from parental 16007 virus contributed to the reduced replication of the DEN1 (PDK13)/DEN2(PDK53) chimera not only in vitro (in LLC-MK_2 and C6/36 cells) but also in mice. The highly immunogenic DEN1 (16007)/DEN2(PDK53) chimera containing the C-prM-E genes of wild type DEN1 16007 was a promising vaccine candidate. The virus did not cause viremia, but stimulated DEN1 neutralizing antibodies and protected cynomolgus monkeys against viremia after challenge with wild-type DEN1 [132].

Multiple DEN virus chimeras were then constructed containing only the prME genes of wild-type DEN1 (16007), DEN3 (16562) or DEN 4 (1036) and the backbone of wild type DEN 2 (16681) and the attenuated dengue 2 PDK53-V and PDK53-E variants [133]. The chimeric viruses with prME elicited higher antibody titers in mice than constructs with C-prME. The DEN3/DEN2 (PDK53) chimera was mutagenized at residue E345 H→L to enhance growth in cell culture. At first, the DEN4/DEN2 (PDK53) chimeras could be recovered only from C6/36 mosquito but not mammalian cells. Adaptation in Vero cells was required for

adequate growth, and this was accompanied by mutations (at C100, E364, and E447) that were incorporated in vaccine constructs to ensure high yields in Vero cells used for manufacturing vaccine lots.

In summary, chimerization per se was insufficient to produce suitable vaccine candidates, as the constructs with the wild-type 16681 backbone did not have markers of attenuation (ts, reduced replication in vitro, and lack of neurovirulence for infant mice). In contrast, the intertypic chimeras with the DEN2 PDK53 backbone demonstrated attenuated phenotypes for all dengue serotypes. The DEN1/DEN2 (PDK53) chimera elicited higher neutralizing antibodies than the DEN 3 or 4 chimeras, and a two dose immunization schedule was required to generate high antibody titers in AG129 (interferon α/β and γ receptor deficient) mice. A tetravalent formulation was tested in AG129 mice, with antibody responses similar to those following monovalent vaccination. These responses were robust after a single dose against DEN 1, 2 and 3 and after two doses to all four serotypes. There were no differences between the –V and –E variants.

To prepare vaccine for clinical development, Inviragen has plaque-purified infectious clone-derived DEN2 PDK53 and DEN1, 3 and 4 chimeras in the DEN2 PDK53 backbone and prepared Master Virus Seeds at P8. Preclinical evaluation of tetravalent formulations at different doses (e.g., 5,5,5,5 and 3,3,3,3 $\log_{10}$ PFU) and at ratios favoring the less active components (e.g., 3,3,5,5 $\log_{10}$ PFU) of the different components has been performed in AG129 mice and nonhuman primates. In addition, a comparison of ID and SC inoculation was performed. A two dose schedule (Day 0, 42) was used in these experiments. The conclusions from these studies follow: (1) DEN2 PDK53 is associated with highest viremias and antibody responses, while the chimeric viruses are more attenuated; (2) among the four serotypes, the immune response to DEN4 was most severely inhibited on priming, but improved with the second (day 42) dose; (3) higher viremia was observed at lower doses (e.g., 3,3,3,3 vs. 5,5,5,5 $\log_{10}$ PFU); (4) partial protection against challenge was seen after one dose and full protection after two doses of tetravalent vaccine; (4) the ID route was more effective than SC without inducing higher viremia.

Inviragen's Phase 1 trial in healthy young subjects will investigate SC and ID routes of inoculation using a tetravalent formulation designed to limit interference by the DEN component (5,4,5,5 $\log_{10}$ PFU).

3.2.6 Single Vector Constructs That Induce Immunity to Multiple Dengue Serotypes

To avoid interference phenomena associated with simultaneous inoculation of four live viruses, it would be ideal to have protective epitopes of all DEN viruses in a single vector. Gene shuffling directed evolution techniques were employed to derive chimeric prM-E sequences containing segments of all for dengue serotypes [108]. Plasmids containing shuffled sequences express prME subviral particles in vitro and presumably in vivo. Mice immunized IM four times with tetravalent

chimeric DNA produced antibodies to all four DEN serotypes. Monkeys also developed low titers of neutralizing antibodies to all serotypes and exhibited partial protection to DEN1 (but not DEN2) challenge [107]. Ideally, the shuffled, tetravalent chimeric DNA could be used to construct a live vaccine by insertion in an appropriate vector such as YF 17Dm rDEN4Δ30, or DEN 2PDK53, which would be more likely than DNA immunization to elicit strong immune responses. However, the shuffled sequences may present some significant barriers to viral envelope assembly and replication. To date successful construction of replication competent viruses has not been reported.

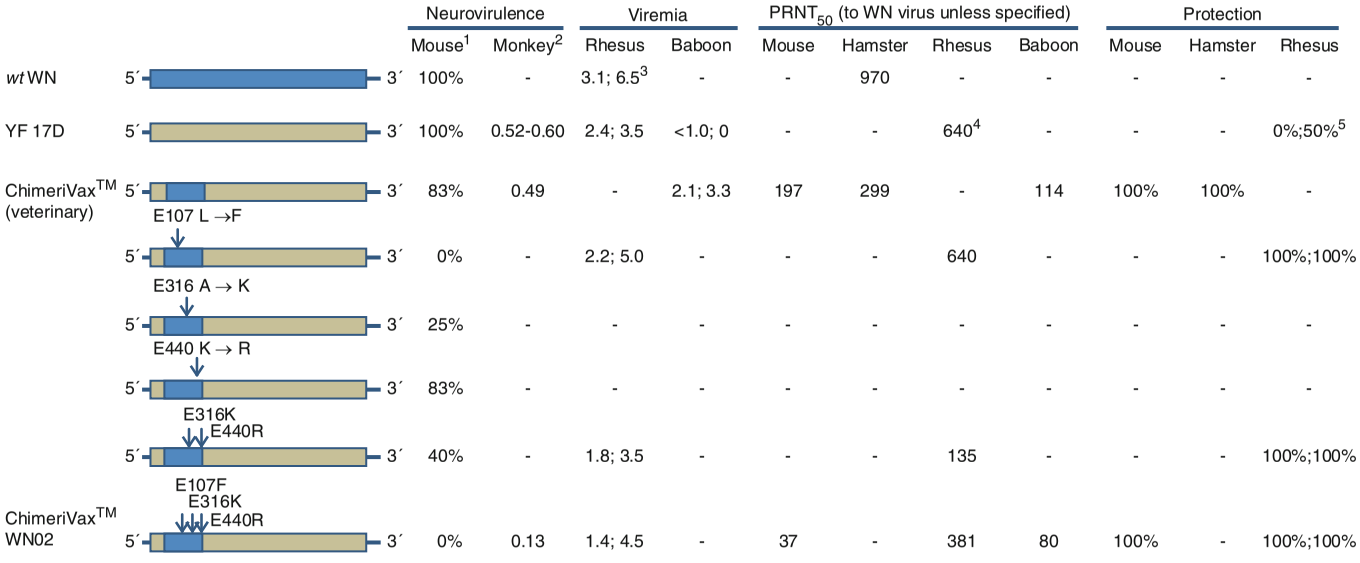
Khanam et al. designed a recombinant defective adenovirus type 5 virus with in-frame E protein domains III of both DEN2 and 4 [134] or all four serotypes [135]. The vector expressed all proteins and elicited modest levels of neutralizing antibodies in mice. This approach will be constrained by the usual issues surrounding anti vector immunity.

3.2.7 Vaccines Against West Nile

Chimeric West Nile/Yellow Fever 17D Vaccine

The strategy for development of vaccine against WN virus was similar to that described for JE and DEN. Development efforts at Acambis were initiated within months of the recognition of WN in North America in 1999 and a candidate vaccine entered preclinical studies in 2000 [136]. Initially, the prME genes of wild-type (383–99, isolated from a flamingo at the Bronx Zoo, 1999) WN virus were inserted into the YF 17D infectious clone. The construction was achieved using the standard two-plasmid system [137, 138]. The WN/YF chimera containing the wild-type prME WN sequence was less neurovirulent for 3–4 week-old mice than YF 17D, once again illustrating the attenuating effect of chimerization and showing the dominant influence of the YF 17D vector on phenotype of chimeras derived therefrom. Mice inoculated with 5.5 $\log_{10}$ PFU exhibited mortality ratios of 20% whereas mice given <1 $\log_{10}$ PFU of YF 17D showed 100% mortality. However, in rhesus monkeys inoculated IC neuropathologic scores were similar to those caused by YF 17D [17].

The WN/YF chimeric virus was immunogenic for mice [17] and hamsters [139] and immunized animals were protected against challenge with virulent WN virus (Fig. 11). Baboons inoculated SC with 5.2 $\log_{10}$ PFU developed viremias lasting 1–4 days ranging between 1 and 2.5 $\log_{10}$ PFU/mL, whereas YF 17D in this species caused no detectable viremia (Acambis, unpublished); the baboons all mounted strong WN neutralizing antibody responses. The results raised questions about the safety of the candidate, as it appeared to be marginally attenuated compared to YF 17D with respect to neurotropism for rhesus monkeys [17] and viscerotropism (viremia) in baboons. At this stage of the vaccine development program, it was decided to evaluate the chimera with a wild-type WN prME gene in horses, which



[1] 3-4 week-old mouse, survival ratio, ~ 4 $\log_{10}$ PFU IC
[2] Rhesus or cynomolgus macaque IC ~ 4 $\log_{10}$ PFU IC, mean combined lesion score at 30 days
[3] Mean peak ($\log_{10}$ PFU/ml); duration (mean days)
[4] Titer to yellow fever
[5] % animals protected against viremia; against death

Fig. 11 Site directed mutagenesis of a WN/YF chimeric infectious clone to develop a human WN vaccine candidate. The nonmutagenized virus was used as a veterinary vaccine for horses. Attenuation and immunogenicity are indicated. From [17, 139] and unpublished data

were impacted severely in the recurring summertime outbreaks in the US, but to further attenuate the vaccine candidate for use in humans.

The feasibility of immunizing horses was demonstrated in 2001 (Bowen R, Arroyo J, Monath TP, unpublished results). Horses given two doses of the WN/YF chimera (wild-type prM-E) failed to become viremic, but developed neutralizing antibodies and were protected against viremia and illness/death on challenge with WN virus introduced directly into the central nervous system by intrathecal inoculation. The development of this vaccine for veterinary use is described further below. Further attenuation of the vaccine (as proposed for humans) was not indicated since host restriction in the horse of the highly primate-adapted YF 17D vector limited replication, as evidenced by absence of vaccine viremia following SC inoculation and relatively low antibody responses.

The WN/YF chimera (wild-type prM-E) failed to infect *Culex (Cx.) tritaeniorhynchus* or *Cx. pipiens (p.) quinquefasciatus* by the oral route; in contrast, wild-type WN virus infected these mosquitoes and reached high titers of 5.8–6.7 $\log_{10}$ PFU/mosquito [140]. *Aedes* (the natural vector of wild-type YF virus) are somewhat more susceptible to infection with the chimeric or attenuated YF 17D strains. Fourteen days after blood feeding, a low proportion of *Ae. aegypti* and *Ae. albopictus* was infected with YF/WN or YF 17D virus; however, the virus titer was very low (1.6–1.8 $\log_{10}$ PFU/mosquito) and virus did not disseminate to head tissue. In contrast *Aedes* spp. ingesting a blood meal containing wild-type WN virus became infected, virus replicated to high titer and disseminated to head tissue.

3.3 Development of a Suitably Attenuated WN/YF Vaccine for Humans

To further enhance the safety profile of the WN/YF chimera, mutations in the E gene were inserted at sites predicted to attenuate the virus based on knowledge of the molecular structure of the closely related JE virus [17, 137, 138]. The objective was to introduce multiple independently attenuating mutations, so that reversion at one or two sites would retain the attenuation phenotype. The selection of nucleotides for mutagenesis was derived from studies of ChimeriVax™-JE [17]. The ChimeriVax™-JE prME genes were donated by an attenuated SA14-14-2 vaccine containing mutations in the E gene at 6 amino acid residues (E107, E138, E176, E279, E315, and E439) that play a role in attenuation. The WN and JE wild-type gene sequences are conserved at these residues, suggesting that mutations introduced at these sites in WN virus would have the same attenuating effects as they did in JE SA14-14-2.

Mutagenesis of the WN residues E107, E280 (corresponding to E279 in JE virus), and E316 (corresponding to E315 in JE virus) caused further attenuation of neurovirulence. Mutation of the E protein at E440 (corresponding to E439 in JE virus) from K→R, a conservative residue change, also reduced neurovirulence

for mice. Surprisingly, while a mutation at E138 (E→K) was associated with attenuation of JE virus [15, 16], a corresponding mutation did not reduce the neurovirulence of the WN/YF virus. A construct with three mutations F, V, and R at positions E107, E316, and E440 respectively, designated ChimeriVax™-WN02, was selected as the candidate for human studies (Fig. 11). The mutated virus was significantly less neurovirulent than the original chimera with wild-type E sequence for infant and weanling mice inoculated IC [17]. On histopathological evaluation of weanling mouse brains following IC inoculation, the mutant vaccine had substantially lower neuronal degeneration and perivascular inflammation in the hippocampus (principal target area for mice) than the chimera with wild-type E gene and YF 17D (Xiao S, unpublished data, 2005). ChimeriVax™-WN02 was also attenuated for cynomolgus macaques following IC inoculation, causing significantly lower neuropathological scores than YF 17D and was thus distinguished from the original wild-type prME chimera [17].

During the manufacturing steps of the vaccine, it was noted that the Production Virus Seed (at P4 after electroporation of chimeric RNA) contained a population of approximately 10% of plaques with a small plaque phenotype. This virus was favored for growth in Vero cells, so that the next passage (the P5 vaccine lot) contained an equal number of large and small plaques. The small plaque population had a mutation (L→P) at M66 located in the C terminus of the M protein [17]. The two plaque variants were isolated by plaque purification and further characterized in mice and hamsters. Neither virus was pathogenic for hamsters (including hamsters immunosuppressed with cyclophosphamide) but the large plaque M66L variant caused a higher viremia in hamsters than the nonmutated small plaque virus (M66P). Both viruses were fully attenuated and not statistically different with respect to neurovirulence in the sensitive 8 day-old mouse test.

Key nonclinical experiments then focused on the monkey model. The vaccine candidate was evaluated in GLP studies for safety by inoculation of cynomolgus macaques by the SC route and by IC inoculation (using the WHO standard test for YF17D neurovirulence). Interestingly, viremia levels, while still low, were higher in monkeys inoculated SC with the ChimeriVax™-WN02 virus than with YF 17D, and a similar phenomenon was observed in hamsters (unpublished data, Acambis), a model for WN infection [141]. It will be recalled that viremia of the nonmutated WN/YF virus had been higher than YF 17D in baboons. Thus, while the mutated chimeric virus had significantly lower neurotropism, the ability to replicate in peripheral tissues and to cause viremia appeared to be enhanced, although without any pathological consequences as determined in a GLP toxicology study in monkeys. Since wild-type WN has been reported on one occasion to cause a hepatitis syndrome in humans, and YF17D can also in rare cases of vaccine-associated viscerotropic disease, it was of interest to compare the biodistribution of ChimeriVax™-WN and YF 17D in monkeys [39]. Both viruses replicated in skin at the site of inoculation, draining lymph nodes, and central lymph nodes, but were rarely found in visceral organs (and never in liver in the case of ChimeriVax™-WN02). ChimeriVax™-WN02 was cleared more rapidly from all sites than YF 17D. Thus, with the limitations of small sample size and uncertain relevance of the

Table 14 Immunization and challenge of rhesus macaques with YF 17D or YF/WN chimeric viruses having one, two or three E gene mutations

Vaccine	N	Vaccine viremia			PRNT50 (GMT) to WN		Challenge Wild-type WN NY99			
		% viremic	Mean peak	Mean duration	Day 30	Day 63	Viremia	Illness	Death	$PRNT_{50}$ (15 days)
None	2						100%	100%	100%	
YF-VAX®	4	100%	2.4[b]	3.5	NT	<40	100%	50%	50%	>640
YF/WN E107	4	100%	2.2	5.0	640	453	0%	0%	0%	2305
YF/WN E316/E440	4	100%	1.8	3.5	135	453	0%	0%	0%	1076
ChimeriVax™-WN02[a]	4	100%	1.4	4.5	381	190	0%	0%	0%	1280

The triple mutant virus (E107, 316, 440) is the human vaccine candidate (ChimeriVax™-WN02). Challenge (day 63) was performed by IC inoculation of 5.4 log10 PFU of wild-type WN virus (strain NY99-35262-11, flamingo, New York, 1999), a severe test of immunity. Data from [17]

[a]Mutations at all three sites E107, 316, 440

[b]$\log_{10}$ PFU/mL

Table 15 Viremia in humans following SC inoculation of 3.0 or 5.0 $\log_{10}$ PFU of ChimeriVax™-WN02

Parameter		Placebo ($n = 30$)	CV-WN02 5 $\log_{10}$ PFU ($n = 30$)	CV-WN02 3 $\log_{10}$ PFU ($n = 15$)	YF-VAX® ($n = 5$)
Cmax (PFU/mL)	Mean ±SD	0.0 ± 0.0	97.3 ± 159.2	187.3 ± 164.8	90.0 ± 81.9
AUC (D1 to 14) PFU.D/mL	Mean ±SD	0.0 ± 0.0	173.0 ± 251.8	311.7 ± 259.4	168.0 ± 156.4
Duration (days)	Mean ±SD	0.0 ± 0.0	5.1 ± 2.9	4.7 ± 1.9	3.6 ± 3.5
Number of days viremic	Mean ±SD	0.0 ± 0.0	4.0 ± 2.2	4.4 ± 1.8	3.2 ± 3.0
Number (percentage) of subjects viremic		0	27 (90%)	15 (100%)	4 (80%)

models to humans, the vaccine appeared to have biological properties of an acceptable vaccine.

To determine efficacy, a study was performed in rhesus macaques inoculated with YF 17D (control) or three different chimeric viruses containing one mutation (E107), two mutations (E316 and E440), or all three mutations (i.e., ChimeriVax™-WN) [17]. All animals developed neutralizing antibodies and were completely protected from clinical signs and death after IC challenge 2 months following vaccination with wild-type WN virus (Table 14).

A double-blind Phase 1 clinical trial was conducted in healthy young adults in the US [39]. A total of 75 subjects were randomized to four treatment groups and received a single SC inoculation [placebo ($N = 15$), YF-VAX® ($N = 5$), ChimeriVax™-WN02 high dose (5.0 $\log_{10}$ PFU) ($N = 30$) and low dose (3.0 $\log_{10}$ PFU) ($N = 15$)]. The chimeric vaccine was well tolerated and there were no differences

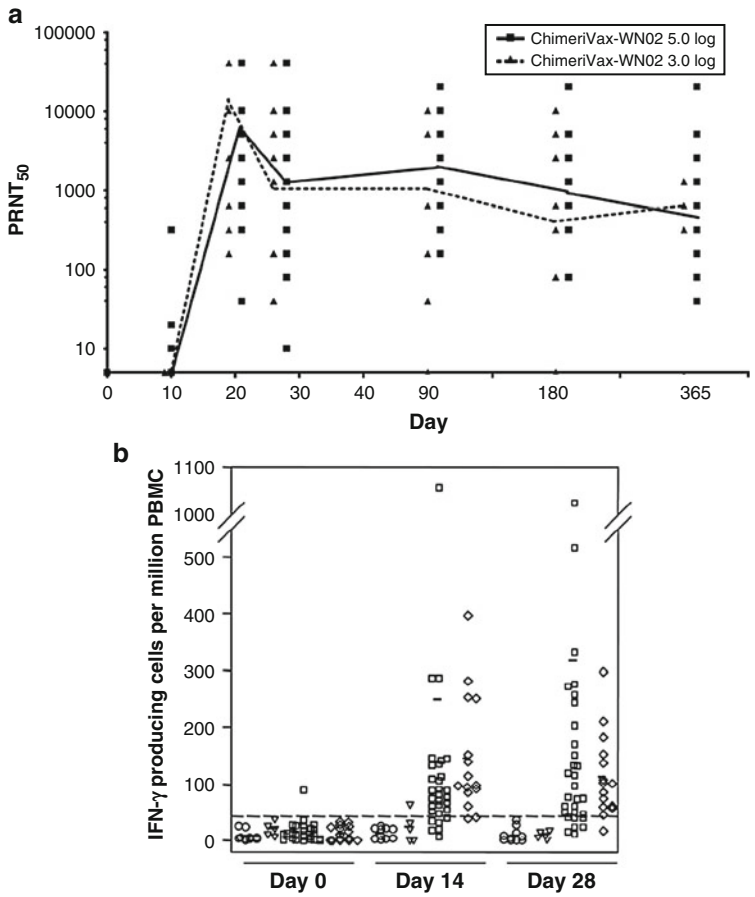


Fig. 12 (**a**). Neutralizing antibody levels in individual subjects, and GMT; clinical trial of ChimeriVax™-WN02. (**b**). T cell responses. From [39] with permission

in the incidence of adverse events across treatment groups. The observation of higher viremias in hamsters and cynomolgus monkeys associated with the chimeric vaccine was not recapitulated in humans (Table 15). High titers of neutralizing antibodies developed without relationship to vaccine dose, peaked at approximately 3 weeks and persisted for >1 year (Fig. 12a). Strong T cell responses were also documented (Fig. 12b).

The mutation at M66 (L → P) that was responsible for the small-plaque phenotypic change arose spontaneously during early passages after electroporation of RNA to prepare virus seeds. Since the large plaque variant was more viremogenic in animals than the small plaque virus, it was decided to attempt to remove it from the vaccine seed stock by plaque purification. New ChimeriVax™-WN02 SP (small plaque) master and production seeds were produced and vaccine lots manufactured at P13. This process produced a vaccine that was predominantly small plaque, and

the large plaque M66L virus was not detectable by consensus sequencing. However, it was evident that genetic instability at this locus was a feature of the vaccine virus and likely an adaptation to growth in Vero cells under serum free conditions. This finding was in concert with experience with ChimeriVax™-JE virus. During serial passages of that virus during manufacture of a new seed stock in serum-free Vero cells, the virus also developed a single mutation at a similar site in the carboxy terminus of the membrane protein M (an R to C change at residue M60). The M60 mutation was shown to significantly increase the rate of virus replication in serum-free Vero cells, but like M66 in the WN vaccine the M60 mutation did not increase neurovirulence of the ChimeriVax™-JE for suckling mice.

A sensitive MAPREC assay [142] was developed to monitor the presence of large plaque virus in batches of ChimeriVax™-WN02, which was <2% in the P13 vaccine lots. Despite a significant effort, it was not possible to entirely eliminate the large plaque variant; the virus was unstable at this locus, but the ratio of large and small plaque virus could be controlled during manufacture. It was expected that reduction of large plaque virus from 50 to <2% in the vaccine formulation would improve safety. Nonclinical tests on the ChimeriVax™-WN02 SP vaccine, including a GLP monkey neurovirulence test, confirmed that it was attenuated and efficacious. These studies in monkeys indicated that the small-plaque vaccine produced a similar level of neutralizing antibody response as that of mixed plaque vaccine which had been evaluated in monkeys and in the Phase 1 trial [39], but produced lower viremia levels than the mixed plaque vaccine.

A Phase II clinical trial was performed with the ChimeriVax™-WN02 SP vaccine. The results of this study have not yet been reported.

3.4 Development of a Veterinary Chimeric WN/YF Vaccine

West Nile is a severe disease with a 28–38% case fatality rate in horses, and large numbers of animals were affected across North America following introduction of the virus in 1999. In 2002, Acambis initiated a collaboration with, and then licensed the ChimeriVax™-WN vaccine to Intervet for veterinary indications. This collaboration led to commercialization of the vaccine for prevention of WN disease in horses in 2007 under the brand-name of Prevenile®. This collaboration between human and veterinary health companies is an outstanding example of the principles of the One Health Initiative [143].

As described above, Prevenile® was constructed by replacing the prME genes of YF 17D with the corresponding genes of the wild-type 383-NY WN without the addition of attenuating mutations. The natural host restriction for replication in horses of YF 17D and chimeric viruses derived therefrom was highly attenuating, as shown by initial exploratory studies in horses showing absence of viremia and lower neutralizing antibody levels than seen in primate hosts. Safety of the vaccine was exhaustively studied, with emphasis on transmission to nontarget animals. Horses were given an overdose of virus, euthanized and examined for virus in

tissues and pathological effects at 14 days postvaccination [144]. In addition, overdosed horses were evaluated for virus shedding and transmission to sentinel animals. Over 900 horses (including foals <4 months old) were vaccinated under field conditions and followed for adverse events. These studies showed that the vaccine was safe and well tolerated, was neither shed from mucosal surfaces nor infectious for contacts, and did not persist in tissues of the host. Immunogenicity of the vaccine was evaluated in multiple studies. All horses inoculated with vaccine (5 $\log_{10}$ PFU) developed neutralizing antibodies at titers of 4–64 (GMT ~10) within 7–14 days, and antibodies persisted for at least 12 months [145]. Given the low antibody levels following a single inoculation, it was important to employ a severe test of immunity to determine protection against virulent WN virus infection. Vaccinated and control horses were therefore challenged by intrathecal injection of a high dose (5 $\log_{10}$ PFU) of WN NY99 4132 virus (isolated from an infected crow in 1999) [145, 146]. Other studies used natural challenge by infected mosquitoes. Vaccinated horses were protected from viremia, fever, clinical signs, but developed high titers of neutralizing antibodies after challenge. Rarely (in <10%) mild clinical signs and brain histopathological lesions were observed in vaccinated-challenged horses, whereas in unvaccinated controls, WN NY challenge was 80% fatal and caused viremia in 90–100% of animals. Protection was observed when challenge was performed early after vaccination (10 days) or late (12 months). In a comparative study of horses vaccinated with two doses of Prevenile® or two other commercial WN vaccines (canarypox-vectored, Merial) and formalin-inactivated vaccine (Ft. Dodge), Prevenile® appeared superior to the inactivated vaccine in protecting animals against clinical signs following intrathecal WN virus challenge [146].

West Nile virus is highly pathogenic for crows and a number of other wild avian species, as well as valuable exotic zoo birds, for which artificial vaccination is indicated. The ChimeriVax™ vaccine (wild type prME) was tested but found not to be effective in an avian species (fish crows) [147]. The vaccine given at high dose to these birds elicited neutralizing antibodies in only 12% of birds. No evidence of protection against viremia or death was observed after SC challenge with virulent WN virus. Severe host restriction for replication, determined by YF 17D NS genes, precludes us of this vaccine for birds.

A chimeric DEN4/WN vaccine candidate was also restricted for growth in birds (see below).

3.4.1 Dengue Type 4 Δ30 Vectored Vaccine Against West Nile Virus

Chimeric viruses were constructed by inserting the prME genes of WN NY99 into the DEN4Δ30 vector [148–150]. Chimeric viruses constructed with wild-type DEN4 as well as the mutant DEN4 backbones were attenuated for mice, caused reduced viremia in monkeys, and had restricted ability to infect *Cx. tarsalis* and *Ae. aegypi* mosquitoes (though curiously grew in *Ae. albopictus*) [151]. Both constructs induced neutralizing antibodies and protected monkeys against viremia following

parenteral challenge with wild-type WN virus [151]. Several mutations appeared during the passages to make clinical grade vaccine using the DEN4Δ30 backbone, one in the E gene (E603 G→R) and three in the DEN4 backbone (one in NS2B, 2 in NS4B). The vaccine candidate was not neuroinvasive in weanling SCID mice and had markedly reduced neurovirulence and ability to replicate in brain tissue of 5-day-old mice inoculated IC [19]. The chimeric vaccine did not cause viremia in rhesus monkeys, but elicited high neutralizing antibody titers. Prior immunity to DEN did not modulate the anti-WN antibody response.

The chimeric vaccine was not infectious for and did not elicit antibodies in domestic geese (an economically important species that develops severe and fatal infection with WN virus) [150].

3.4.2 Dengue Type 2 PDK53 Vectored Vaccine Against West Nile Virus

This vaccine is under development by Inviragen Inc. The prME sequence of the NY99-35262 strain was inserted into the DEN2 infectious clone. Three backbones were constructed (wild-type 16681, and PDK53-E and –V). In addition, constructs were made with engineered mutations at M58 (M→L) and E 191 (E→A) to enhance growth in Vero cells [19]. The chimeras with PDK53 backbones were ts and restricted for growth in C6/36 cells. Chimeras with 16681 or PDK53 backbones were less neurovirulent for infant mice than WN99 and wild-type DEN2 16681. When inoculated IP into outbred mice, the chimeras elicited respectable neutralizing antibody titers after a single dose (GMT 40–108 for the PDK53 vectored vaccines) in 75–88% of the mice and protected 88–100% against lethal WN virus challenge. Two sequential immunizations substantially boosted titers (GMT 580–4,695) in all mice and fully protected against challenge.

3.4.3 Chimeric Vaccines Against Tick-Borne Encephalitis

Medically important members of the TBE antigenic complex include Russian spring–summer encephalitis virus (RSSE, the Far Eastern subtype), and Central European Encephalitis (CEE) virus, louping ill (LI), Kyasanur Forest disease (KFD), Omsk hemorrhagic fever (OHF), and Powassan (POW) viruses. These viruses have E gene homologies of 78% or greater and share protective epitopes [152], suggesting that a Jennerian approach to vaccine development might be employed, using a naturally attenuated member of the antigenic complex to construct a cross-protective vaccine against other TBE Complex viruses. The incidence of TBE in Europe has increased in countries that are not practicing routine vaccination against the disease. Two highly effective, purified, formalin-inactivated vaccines prepared from the CEE virus grown in chick embryo cells and adsorbed to alum are approved for use in Europe [153]. The vaccines are believed to cross-protect against RSSE virus. Since they require two doses for primary immunization and boosters at regular intervals, there has been interest in developing improved

vaccine strategies, described below. However, children are the principal target for vaccination in industrialized countries endemic for TBE, and any new vaccine must have extraordinary assurances of safety to displace the existing, highly effective inactivated vaccines.

3.4.4 Chimeric TBE/DEN4 Vaccine

The first chimeric vaccine investigated for immunization against TBE employed an unmodified, wild type DEN4 backbone into which either the C-prM-E or prM-E genes of a virulent CEE virus (Sofjin strain) were inserted [33]. As shown for the DEN2/DEN4 chimeras, the chimera with C-prM-E from TBE was less competent for replication in cell culture than the prM-E construct. The TBE (prM-E)/DEN4 chimera was significantly less virulent than parental TBE virus, which had an IP LD_{50} of 14 PFU, whereas the chimera was not lethal by IP injection. However, when inoculated IC, the chimera caused lethal encephalitis in suckling and adult mice showing that the prME genes of TBE conferred an neurovirulent phenotype. The introduction of mutations in prM or E or in the DEN4 NS1 gene of the chimera reduced neurovirulence for mice and also reduced replication in cell culture [154]. The chimera with or without attenuating mutations induced immunity and protected mice against lethal challenge with TBE [154, 155]. Given the safety concerns around use of pathogenic viruses in constructing a live vaccine, attention refocused on alternative strategies.

3.4.5 Chimeric LGT(TP21)/LGT and LGT(E5)DEN4 viruses

To improve upon the safety phenotype of DEN/TBE chimera, the prM-E genes from strains of the more attenuated Langat (LGT) virus (a member of the TBE antigenic complex originally isolated from ticks in Malaysia) were inserted into the wild-type DEN4 backbone. The prototype LGT TP21 strain had actually been evaluated on its own in Russia for Jennerian vaccination against TBE [155], but had been associated with serious adverse events (vaccine-associated encephalitis) [156]. A derivative, the E5 strain, had been attenuated by serial passage in eggs to a point where it was less neurovirulent for mice than TBE virus and was considered a candidate vaccine strain [157]. LGT E protein is approximately 88% homologous with RSSE virus and neutralizing antibodies cross protect but require antibody titers fourfold higher than antibody against homologous strains of TBE virus [154].

Chimeric viruses were constructed inserting the prME genes of the LGT TP21 or E5 viruses into DEN4 814669 [158]. The resulting chimeras were significantly less neurovirulent than the parental TP21 and E5 viruses by IC inoculation in suckling mice and were not neuroinvasive. Adult mice immunized IP with as little as 1 $\log_{10}$ PFU developed neutralizing antibodies and were protected against lethal IP challenge with 1,000 LD_{50} of homologous TP21.

The vaccine candidates were subsequently adapted for growth in Vero cells and investigated for their ability to protect against heterologous challenge with virulent CEE (Sofjin) as well as the RSSE (Absettarov) strain [159]. The chimeric viruses carrying LGT prME induced neutralizing antibodies to LGT, but were substantially less effective in eliciting heterologous protective immunity to Sofjin or Absettarov viruses. After a single vaccination, partial protection was achieved, while complete protection required two sequential vaccinations. Moreover, the chimera with prME derived from the more attenuated E5 strain was less effective than the LGT(TP21)/DEN4 chimera. The latter conferred complete protection after two inoculations, whereas only 67% of mice inoculated with two doses of LGT(E5)/DEN4 survived TBE challenge. These studies illustrated the predominant influence of the structural gene sequence, and the difficulty achieving a correct balance of attenuation and immunogenicity. They also raised questions about the use of a heterologous E gene in providing cross-protection against virulent TBE strains.

The results were nonetheless considered sufficiently promising to proceed with advanced preclinical evaluation. The chimeric LGT(TP21)/DEN4 vaccine prepared in Vero cells was tested in rhesus macaques [160]. The vaccine was highly attenuated in this host following SC inoculation. Only 1 of 12 monkeys in groups of four immunized SC with 3, 5 or 7 $\log_{10}$ PFU developed detectable viremia, whereas viremia was detected in all monkeys inoculated with 5 or 7 $\log_{10}$ PFU of parental LGT TP21 or 5 $\log_{10}$ PFU of DEN4 virus. Monkeys in the groups immunized with LGT(TP21)/DEN4 developed high $PRNT_{60}$ titers of 372–2,344 against LGT TP21 or 320–640 against heterologous TBE virus. When challenged 6 weeks after vaccination with 5 $\log_{10}$ PFU of LGT TP21, all were protected against viremia.

A comprehensive study in cell cultures, mice, and rhesus monkeys compared the safety and protective activity of TBE/DEN4, LGT(TP21)/DEN4, and a construct in which wild-type TBE (Sofjin) virus prME was inserted into the DEN4 backbone having the attenuating 3′ NCR Δ30 deletion [152]. The genomes of the TBE/DEN4 and TBE/DEN4Δ30 viruses differed at four amino acid residues in prM, NS3 and NS4B. The LGT(TP21)/DEN4 differed from the other chimeras and wild-type LGT virus in being restricted for growth in murine and human neuroblastoma cells. The virus was also significantly less neurovirulent for suckling mice (IC LD_{50} 2.4–3.2 $\log_{10}$ PFU) than either TBE/DEN4 or TBE/DEN4Δ30 chimeras (IC $LD_{50} <$ 1 $\log_{10}$ PFU), and none of the vaccine constructs were neuroinvasive. The safety and immunogenicity of a single dose of the chimeric viruses were compared with commercial inactivated TBE vaccine given as three sequential vaccinations on days 0, 7 and 21 (Table 16). The take-away messages were: (1) as determined by vaccine viremia and antibody response, the LGT(TP21)/DEN4 and TBE/DEN4Δ30vaccines were more highly attenuated than TBE/DEN4; (2) homologous antibody responses were ~5-fold higher than heterologous responses; (3) the TBE/DEN4Δ30 vaccine elicited very low antibody responses to heterologous LGT virus and to homologous TBE virus, was thus over-attenuated, and did not confer

Table 16 Viremia and immune response of rhesus monkeys following immunization with chimeric virus or inactivated TBEV vaccine and after subsequent challenge with wild –type LGT strain TP21 (From [152], with permission)

Immunizing virus	No. of monkeys	Response to immunization[a]							Response to challenge[d]				
		No. of viremic monkeys	Mean no. of viremic days per monkey	Mean peak virus titer (log_{10} PFU/ml)[b]	Geometric mean serum NT antibody titer (reciprocal dilution)[c] against:				No. of viremic monkeys after LGT challenge	Mean no. of viremic days per monkey	Mean peak virus titer (log_{10} PFU/ml)	Geom. mean NT antibody titer[c] on day 21 post challenge	
					αLGT		αTBEV/DEN4					αLGT	αTBEV/DEN4
					Day 0	Day 42	Day 0	Day 42					
LGT/DEN4	4	3	1.0	0.9	<10	177	<10	14	0	0	<0.7	2801	834
TBEV/DEN4	4	4	3.5	3.1	<10	227	<10	1125	0	0	<0.7	1372	6457
TBEV/DEN4 Δ30	4	3	0.8	0.7	<10	17	<10	59	1	0.25	0.7	767	2951
"Encepur"	4	0	0	<0.7	<10	454	<10	281	0	0	<0.7	1125	1804
Control	4	0	0	<0.7	<10	<10	<10	<10	4	2.0	1.9	357	97

[a]Groups of rhesus monkeys were inoculated SC with 10^5 PFU of indicated virus or L-15 medium (control) in a 1 mL dose on day 0. Another group of monkeys was inoculated SC with a formalin-inactivated TBEV vaccine "Encepur" in three doses (3 × 0.5 mL) on day 0, 7, and 21. Serum used to measure viremia was collected daily for 10 days

[b]Virus titer serum was determined by plaque-forming assay on LLC-MK_2 cells. The lower limit of detection was 0.7 log_{10} PFU/mL. For purpose of calculating the mean peak titer, a titer of 0.6 log_{10} PFU/mL was assumed for monkeys serum with undetectable viremia. Viremia was not detected in any monkey after day 4

[c]Plaque reduction (60%) neutralizing antibody titers were determined against wild-type LGT TP21 strain or chimeric TBEV/DEN4 virus as indicated. Serum for neutralization assay was collected on days 0

[d]On day 43, all monkeys were inoculated SC with 10^5 PFU of wild-type LGT TP21. Serum used to measure LGT viremia was collected daily for 10 days. LGT virus titer in serum was determined by plaque-forming assay on LLC-MK_2 cells, and viremia was not detected in any monkey after day 4 post-inoculation

complete protection against LGT TP21 challenge; (4) administration of three doses of inactivated vaccine induced broadly cross-reactive, high antibody titers and was fully protective against LGT TP21 challenge.

Tick-borne flaviviruses do not infect mosquitoes; thus it was of interest to determine whether chimeras of tick and mosquito-borne viruses would be competent for replication. The chimeric LGT (TP21)/DEN4, as well as parental LGT TP21 were incapable of infecting *Toxorrhynchites* mosquitoes after intrathoracic infection, while DEN4 virus replicated efficiently [158], showing that the chimera would not be transmissible by mosquitoes and that the prME genes from a tick-borne flavivirus determined specificity for arthropod infection.

Based on safety (reduced neurovirulence for suckling mice, lack of neuroinvasiveness in mice, and low viremia in monkeys) and immunogenicity for monkeys, the chimeric LGT (TP21)/DEN4 vaccine was selected for testing in a clinical trial [161]. The principal objectives of the study were to confirm the safety of this construct and to determine whether the vaccine would elicit antibodies to the LGT insert, and more importantly to TBE virus. Twenty healthy young adults received 3 $\log_{10}$ PFU of the chimeric vaccine by SC injection and eight received a placebo. The vaccine was safe and well tolerated, and, as predicted by monkey studies, highly attenuated, viremia being observed in only one subject (5%). In concert with this level of attenuation, immunogenicity was relatively poor. Only 80% of the vaccines seroconverted to LGT and 35% seroconverted to TBE. Neutralizing antibody GMT to LGT and TBE were low – 63 and 9, respectively.

Two subsequent studies cast a level of doubt on the safety of this vaccine construct, despite the attenuation observed in mice (inoculated IC) and in humans. Pripusova et al. [162] reported that while the LGT (TP21)/DEN4 vaccine had 40,000-fold reduced neurovirulence in mice compared to LGT TP21 or TBE (Absettarov) viruses, African green monkeys inoculated IC demonstrated virus replication, histopathological lesions and clinical signs of encephalitis. In a separate analysis in rhesus macaques inoculated IC, the TBE/DEN4Δ30 (which had shown overattenuation with respect to antibody response in the same species), was compared to YF 17D [58]. Interestingly, the TBE/DEN4Δ30 construct caused higher neuropathological lesion scores than YF 17D. These studies suggest that chimeras with structural genes derived from tick-borne viruses retain residual neurovirulence for primates. While the low viremias caused by these vaccines would likely prevent neuroinvasion in humans, from a regulatory perspective the residual neurovirulence is problematic. Moreover, these studies call into question the ability to correlate neurovirulence of virus vaccines in mice and monkeys [34].

Recent attempts have been made to develop mutagenized LGT E5 virus with reduced neurovirulence properties that could ultimately be used to construct more attenuated chimeras [163]. However, given the overattenuated phenotype of the chimera having unaltered TP21 prM-E it is unlikely that the answer to a successful construct lies in this direction.

3.4.6 Chimeric Vaccines Against Other Flaviviruses of Medical Importance

Chimeric viruses were constructed using prM-E genes donated from virulent (MSI-7) or naturally attenuated (CorAn9124) strains of St. Louis encephalitis (SLE) virus in the YF17D vector backbone [164]. The phenotype of these constructs was predictable based on previous work with related JE and WN chimeras. The chimera with the MSI-7 insert had reduced neuroinvasiveness for weanling mice compared to wild-type MSI-7 and was ~1,000 × less neurovirulent when inoculated IC. The chimera constructed with the naturally attenuated CorAn9124 genes was not neuroinvasive and was 100,000 × less neurovirulent compared to wild-type CorAn9124 parent. No evaluation has been performed of the utility of these constructs as vaccines.

As noted previously, cross-protection has been demonstrated between closely related viruses in the JE antigenic complex (including WN, SLE and MVE). It is likely that a live, chimeric vaccine against JE could be employed in an emergency to protect against SLE, WN or MVE, or conversely a vaccine against WN could be employed against JE or SLE. These applications could extend to both veterinary and human disease indications. Now that chimeric vaccines are becoming commercially available, studies of cross-protection are increasingly relevant.

4 Alphavirus Vaccines

Like the flaviviruses, alphaviruses (family *Togaviridae*) have single-strand, positive sense genomes and the viral RNA is infectious following transfection of cells in culture. The viral RNA of about 12 kb can be reverse transcribed and cDNA cloned for engineering in bacterial plasmids. It is relatively simple to insert coding regions for foreign genes, and several groups have explored the use of Sindbis (SIN), Semliki forest, or attenuated Venezuelan equine encephalitis (VEE) virus as vectors. Site directed mutagenesis has also been used to derive attenuated alphavirus strains of virus that could be used as vectors for foreign genes [165]. In this section, we consider the use of alphavirus vectors constructed with donor genes from another alphavirus to make live, replicating chimeric vaccines against alphaviruses of medical importance, such as VEE, EEE and WEE, respectively, RRV, and CHIK. The approach is thus analogous to that described for flavivirus vaccine development. Interestingly, WEE and all but one of the other New World members of the WEE antigenic complex originally evolved through a recombination event in nature between a SIN-like virus and EEE [166]. The E1 and E2 genes of WEE are derived from SIN, whereas the C and NS genes are derived from EEE. This natural experiment demonstrated in advance that viable chimeric constructs would be achievable in the laboratory.

Live, attenuated vaccines have been developed against VEE [167] and CHIK [168] using empirical passage. TC-83 has been used as an investigational vaccine in over 8,000 humans [169], but is highly reactogenic and fails to immunize about 18% of subjects. The VEE vaccine (TC-83) has also been approved for use in Equidae. A candidate VEE vaccine (V3526) was developed by introducing mutations in the PE2 furin cleavage site of the virus, and this candidate has been tested in rodent models and horses [170]. An inactivated VEE vaccine (C-84) is not protective in animals against respiratory challenge. It is used only to immunize TC-83 vaccinees who failed to seroconvert but requires multiple doses and frequent boosters to achieve adequate titers. The live CHIK vaccine (181/25) appears to be highly immunogenic, but transient arthralgia was observed in ~9% of subjects [171], and there are concerns about genetic stability of the virus. A formalin inactivated CHIK vaccine has also been evaluated [172]. Investigational, formalin inactivated vaccine against WEE and EEE are in limited use in laboratory workers. Improved vaccines against all these alphaviruses are required.

The genome organization of alphaviruses differs from flaviviruses in that the four NS proteins encoding transcriptases and replicases are translated from the 5′ two-thirds of the full-length 42S messenger RNA, whereas the structural genes encoding capsid (C) and envelope proteins (usually two E1 and E2, but sometimes three) are translated from a 26S subgenomic messenger RNA transcribed from the 3′ one-third of the genome. Viable, replication-competent chimeric viruses are constructed using the 5′ and 3′ noncoding regions and polyA tail, subgenomic promoter and NS genes of the vector and the 5′ noncoding region of the 26S subgenomic RNA and structural protein genes (capsid, E1 and E2) from the virus against which immunization is desired. Some variations on this scheme have been used and will be described below. As for flaviviruses, the absence of structural genes of the vector may preclude interference due to antivector immunity; however, this has not been assessed empirically, for example by sequential immunization with different chimeras sharing the same backbone vector. It is known that prior immunization with EEE or WEE interferes with subsequent vaccination with live VEE vaccine [173, 174], so similar effects could apply to the use of chimeric viruses.

The chimerization process itself significantly attenuates the resulting virus, and use of attenuated gene donor and vector strains or introduction of mutations can further optimize biological phenotype. Limited experience to date suggests that chimeric alphavirus vaccines are attenuated even when two virulent viruses are used in their construction. A chimeric virus containing the structural genes of EEE virus and the nonstructural genes of WEE virus was attenuated compared to the two parental strains in mice [175]. Thus, the principles of use are similar to those employed for flaviviruses. The live, attenuated alphavirus vaccines would be expected to induce strong innate and adaptive immune responses, immunize with a single dose, produce long-lived immunity, would be unlikely to revert to virulence, and wound be in expensive to manufacture.

4.1 Chimeric Vaccine Candidates Using Sindbis Virus as a Vector

Sindbis (SIN) and several closely related subtypes are mosquito-borne alphaviruses distributed widely in Europe, Africa and Australia. These viruses are human pathogens, causing endemic and epidemic illness characterized by a self-limited fever–rash–arthritis syndrome (variously named Okelbo disease, Pogosta disease, and Karelia fever in Scandanavia and Russia) [176]. Given the pathogenic potential of SIN virus, and the lack of an animal model for the natural disease syndrome in humans, use as a vector requires demonstrated reduction in other virulence markers in animals and, ultimately proof of safety and tolerability in nonhuman primates and humans. SIN is naturally attenuated for many laboratory animals, so that it is difficult to judge whether a chimeric vaccine is more attenuated than the vector. However, wild-type SIN virus is neurovirulent for infant mice and attenuation of chimeric viruses can be assessed in this model. In addition, a model of lethal SIN infection in interferon-α/β and interferon-γ receptor deficient mice [177] provides a possible approach to the investigation of attenuation of chimeric vaccines. These mice develop a hemorrhagic diathesis, which is atypical for human SIN infections, although rare cases have been reported [178]. Wild-type SIN virus is not known to cause disease in domestic animals and thus it is a vector of interest for veterinary vaccines against VEE, EEE and WEE.

A number of studies using SIN as a vector for vaccines against VEE, EEE, WEE and CHIK viruses have been reported (Table 17). Viable chimeras of RR and SIN were also constructed [179] but have not been evaluated for immunogenicity. The largest body of work on SIN vectors has been conducted by Scott Weaver and his collaborators at the University of Texas Medical Branch, Galveston. A VEE/SIN chimera with structural genes of the live, attenuated TC-83 strain of VEE inserted in the SIN (Toto1 101) vector was shown to be attenuated, immunogenic and protective in mouse models [180]. The parental TC-83 vaccine was neurovirulent and neuroinvasive for infant (6-day-old) mice, whereas the VEE(TC-83)/SIN chimera was not. The chimera elicited high titers of neutralizing antibodies and protected mice against lethal SC challenge with VEE subtype IC as well as the more distantly related VEE subtype ID. The work was extended to investigate various chimeric vaccines in which structural genes were derived from TC-83, the virulent parental (TrD) strain, or VEE ID virus [181]. In addition, a construct was prepared with 3 point mutations in the SIN backbone that are present in SAAR86, a wild-type SIN strain that is unusual in being virulent for mice by the peripheral route of injection [185]. None of the chimeras killed adult mice after IC inoculation, and they were attenuated for neurovirulence and neuroinvasiveness in the 6-day-old mouse model compared to TC-83 (Table 17). A single SC inoculation elicited neutralizing antibodies in mice and there was little or no rise in antibodies after a booster dose given at 8 weeks, indicating sterile immunity (Table 18). The VEE (ID)/SIN and VEE(TrD)/SIN vector with three SAAR86 mutations were more immunogenic than the other constructs. However all mice were protected against

Table 17 Biological features, immunogenicity and protective activity of live, chimeric alphavirus vaccine candidates

Target	Designation	Structure	Replication in vitro	Virulence/attenuation		Neutralizing antibody		Protection vs. challenge	Reference
				Neurovirulence (mice), mortality (%)	Neuroinvasiveness, Other mice (SC or IP) (mortality (%)	Seroconversion rate	PRNT mean or range of titers 4 wks		
Ross River	RR/SIN chimera	RR structural genes, SIN backbone	Replicates to high titer. Tenfold lower growth in Vero, CEF but higher growth in C6/36 than parental SIN. Growth in C6/36 similar to RRV	0/22 (0%)[a]	0/28 (0%)				[179]
	SIN	parental strain derived from infectious clone		0/9 (0%)	0/9 (0%)				
	RR	parental strain derived from infectious clone		23/23 (100%)	28/28 (100%)				
VEE	VEE(TC83)/SIN[b]	VEE structural genes (from attenuated TC-83 vaccine), SIN (Toto1 101) backbone	Replicates to high titer. Tenfold lower growth in Vero and BHK than TC-83, but to similar or higher titer than SIN	0%[c]	0%		60–960[d]	100% protected vs. challenge SC with 6 log10 LD50 of either VEE ID (ZPC738) ($N = 5$) or VEE IC (SH3) ($N = 5$)	[180]
	VEE TC-83	Vaccine strain		100%	20%		480	100% protected vs. challenge SC with 6 log10 LD50 of either VEE ID (ZPC738) ($N = 5$) or VEE IC (SH3) ($N = 5$)	

VEE	VEE(TC83)/SIN[b]	VEE structural genes (from attenuated TC-83 vaccine), SIN (Toto1 101) backbone		0%[e]	0%	~5 $\log_{10}$ PFU/g[f]		see Table 15	see Table 15	[181]
	VEE(TrD)/SIN	VEE structural genes (from virulent Trinidad donkey subtype IAB), SIN backbone		20%	0%	~7 $\log_{10}$ PFU/g		see Table 15	see Table 15	
	VEE(ZPC)/SIN	VEE structural genes (from ZPC738 subtype ID), SIN backbone		40%	0%	~7.7 $\log_{10}$ PFU/g		see Table 15	see Table 15	
	VEE(TrD)/SIN (SAAR, mutated)	VEE structural genes (from virulent Trinidad donkey subtype IAB), SIN backbone contains three attenuating mutations in 5′ NCR, nsP1, nsP3/nsP4 present in a virulent strain of SIN (SAAR86)		30%	0%	~6.5 $\log_{10}$ PFU/g		see Table 15	see Table 15	
	VEE TC-83	vaccine strain		100%	100%	~9.5 $\log_{10}$ PFU/g		see Table 15	see Table 15	
EEE	EEE (NA)/SIN	EEE structural genes (North American subtype), SIN Ar339 nonstructural genes	All 3 viruses grew to 6–7 $\log_{10}$ PFU/mL. Replication of the EEE (SA)/SIN chimera and parental SIN in Vero and C7/10 mosquito cells was	100%[g] Prolonged AST	No viremia in 8 week-old mice inoculated SC	~8.3 $\log_{10}$ PFU/g[h]	70–100%[i]	125–660 [20–50 vs. EEE (SA)]	80–100% protected across three dose groups[i]; all sham immunized controls died	[182]
	EEE (SA)/SIN	EEE structural genes (Sorth American subtype), SIN Ar339 nonstructural genes		90% Prolonged AST	No viremia in 8 week-old mice inoculated SC	~7 $\log_{10}$ PFU/g	50–100%	28–308 [20–36 vs. EEE (NA)]	100% protected; all sham immunized controls died	

(*continued*)

Table 17 (continued)

Target	Designation	Structure	Replication in vitro	Virulence/attenuation			Neutralizing antibody		Protection vs. challenge	Reference
				Neurovirulence (mice), mortality (%)	Neuroinvasiveness, mice (SC or IP) (mortality (%)	Other	Seroconversion rate	PRNT mean or range of titers 4 wks		
	SIN	Ar339	similar; EEE (NA)/SIN grew to ~10-fold higher titer.	100%		~7 $\log_{10}$ PFU/g				
	EEE	North American subtype (FL93-939)		100%		~9 $\log_{10}$ PFU/g				
	EEE	South American subtype (BeAr436087)		100%		~8 $\log_{10}$ PFU/g				
WEE	WEE(CO92)/SIN	WEE (CO92-1356) structural genes, SIN Ar339 nonstructural genes[j]	All 3 chimeric viruses grew to $\geq$7 $\log_{10}$ PFU/mL and had similar growth curves in Vero and C7/10 mosquito cells	80%[k]	30%[k]	6.2 $\log_{10}$ PFU/g[l]	50–100%[m]	60–190[m]	50–100% protection; all sham animals died[m]	[183]
	WEE(McM)/SIN/SIN	WEE (McMillan) structural genes except for amino-terminal domain of C gene of SIN Ar339, SIN nonstructral genes[j]					100%	600–604	100% protection	
	WEE(McM)/EEE/SIN	WEE (McMillan) structural genes except for amino-terminal domain of C gene of EEE (436087), SIN nonstructral genes[j]		100%	100%	8.1 $\log_{10}$ PFU/g	80–100%	416–420	100% protection	
	WEE	CO92-1356 *Culex tarsalis* Colorado 1992		100%	100%	8.5 $\log_{10}$ PFU/g				
	WEE	McMillan Human, Canada, 1941		100%	100%	9.4 $\log_{10}$ PFU/g				

	SIN	Ar339		100%		~7 $\log_{10}$ PFU/g				
CHIK	CHIK/SIN	CHIK (LR) structural genes, SIN (Ar339) nonstructral genes	All viruses grew to high titer. CHIK/VEE and CHIK/	0%[k]	0%	Low viremia, no replication in knee joint or	100%[o]	43[o]	100% protection[p]	[184]
	CHIK/VEE	CHIK (LR) structural genes , VEE (TC-83 vaccine strain) nonstructral genes	EEE grew to titers ~4-fold higher than CHIK/SIN in	0%	0%	brain 2–10 days after SC inoculation of 3–4 day-old mice[n]. No	100%	136[o] 224–260[p]	100% protection[p]	
	CHIV/EEE	CHIK (LR) structural genes , EEE (SA subtype BeAr436087) nonstructral genes	Vero cells.	0%	0%	viremia or illness in 3 week-old C57/ Bl6 or Swiss mice inoculated SC with 5.3–5.8 $\log_{10}$ PFU	100%	116[o] 112–200[p]	100% protection[p]	
	CHIK	LR, human, La Réunion, 2006		45%	0%	Higher viremia, replication in knee and brain of suckling mice (see above)				
	SIN	Ar339		100%						
	VEE	TC-83		100%						
	CHIK	(181/25 attenuated vaccine)			0%	Higher viremia, replication in knee and brain of suckling mice (see above)	100%	144[p]		

[a]CD1 mice 7 days of age inoculated with 3 $\log_{10}$ PFU IC or (neuroinvasiveness study) SC in footpad
[b]Author's designation is SIN-83
[c]Mice (unspecified strain and number), 6 days old, inoculated IC or (neuroinvasiveness) SC with 6.3 $\log_{10}$ PFU
[d]Mice inoculated SC at 6 days old (publication may be in error and corectly mean 6-weeks old) were tested for antibody at day 21 prior to challenge
[e]Mice (NIH Swiss), 10/group, 6 days old, inoculated IC or (neuroinvasiveness) SC with 6.7 $\log_{10}$ PFU
[f]Brain virus titer, peak
[g]NIH Swiss mice 6 days of age inoculated IC with 5 $\log_{10}$ PFU and assessed for illness and death

(*continued*)

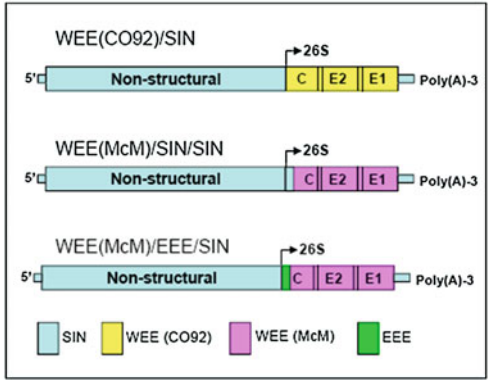
Table 17 (continued)

Target	Designation	Structure	Replication in vitro	Virulence/attenuation			Neutralizing antibody		Protection vs. challenge	Reference
				Neurovirulence (mice), mortality (%)	Neuroinvasiveness, mice (SC or IP) (mortality (%)	Other	Seroconversion rate	PRNT mean or range of titers 4 wks		

[h]Brain tissue virus titers 1–2 days after IC inoculation (6 day old mice)

[i]NIH Swiss mice 8 weeks of age inoculated SC with 3.7–3.8, 4.7–4.8, or 5.7–5.8 log10 PFU, bled for antibody at 4 weeks and challenged IP with 6 $\log_{10}$ PFU of EEE(NA) challenged

[j]



[k]Mice (NIH Swiss) 6 or 4 days of age inoculated, respectively IC (neurovirulence) or SC (neuroinvasiveness) with ~5 $\log_{10}$ PFU

[l]Brain virus titer 1–2 days after IC inoculation of 6 day old mice

[m]Neutralizing antibody mean $PRNT_{80}$ titers 6 week-old mice 4 weeks after a single SC immunization with 4.8 or 5.8 $\log_{10}$ PFU; mice challenged IN at 4 weeks with 5.3 log10 PFU WEE TBT235

[n]3–4 day old Swiss mice inoculated SC with 5 $\log_{10}$ PFU

[o]3 week old Swiss ($N = 10$) vaccinated SC with 5.3–5.8 $\log_{10}$ PFU,bled 3 weeks after immunization for antibody

[p]3 week old C57/Bl6 ($N = 5$) vaccinated SC with 3.9, 4.9 or 5.9 $\log_{10}$ PFU,bled 3 weeks after immunization for antibody and challenged IN with 6.5 $\log_{10}$ PFU CHIK (Ross)

Table 18 Viremia, immune responses, and protective activity of chimeric vaccines using SIN virus as a vector for structural genes of VEE virus. Data from Paessler et al. [181]

Virus[a]	Age	Dose/route (N)	Viremia (peak)	PRNT80 mean, vs. TC-83 virus				Mice given single inoculation of vaccine, challenged at 8 wks with VEE subtype ID (ZPC738) 5.3–6 log10 PFU		
				Single immunization ($N = 6$)		Booster ($N = 6$)		Illness rate (Mortality) by challenge route		
				4 weeks p.i.	8 weeks p.i.	4 weeks p.i.	8 weeks p.i.	SC ($N = 5$)	IN ($N = 5$)	IC ($N = 5$)
VEE(TC83)/SIN	6 weeks	5.7 log10 PFU SC ($N = 12$ for immunization; $N = 15$ for challenge)	0	55	73	100	160	0% (0%)	20% (0%)	40% (20%)
VEE(TrD)/SIN			0	37	57	50	73	0% (0%)	0% (0%)	0% (0%)
VEE(ZPC)/SIN			0	187	253	253	487	0% (0%)	0% (0%)	0% (0%)
VEE(TrD)/SIN(SAAR, mutated)			0	126	167	160	152	0% (0%)	0% (0%)	0% (0%)
Mock								100% (100%)	100% (100%)	100% (100%)

[a]See Table 15

challenge with virulent VEE ID virus by various routes, including the respiratory route. Hamsters were also vaccinated with the chimeric viruses or with TC-83. The animals given TC-83 developed a self-limited illness and had viremia ~1,000-fold higher than those caused by the chimeras. All animals survived lethal SC challenge with VEE ID. Overall, these results indicate that the chimeric constructs had an improved safety profile compared to TC-83.

Chimeric EEE/SIN viruses were evaluated by Wang et al. [182]. The structural genes were derived from North American and South American subtypes of EEE virus, the latter being a naturally attenuated virus and antigenically distinct from North American virus. In the 6-day-old mouse model, the chimeras were more attenuated than parental EEE virus strains based on survival times (though mortality ratios were high) (Table 17). The chimeras did not cause viremia in adult mice, but elicited high neutralizing antibody titers to the homologous virus and protected against challenge. Although these viruses are interesting, considerable work will be required to assess safety, and it is likely these viruses are insufficiently attenuated. The authors included some benchmarks in their evaluation, including TC-83 and the V3526 [170] VEE vaccine candidate. The EEE/SIN chimeras were intermediate in neurovirulence compared to these vaccines. The chimeras were substantially restricted for disseminated infection after oral feeding in one mosquito vector of EEE virus (*Ae. taeniorhynchus*) compared to parental EEE and SIN viruses, but disseminated infection occurred in another species (*Ae. sollicitans*) [186]. It was concluded that chimeric alphaviruses may have selective competence for mosquito vectors. This would be a potential issue if viremia levels in target hosts for vaccination are sufficient for mosquito infection, since recombinational events could result in the arthropod vector.

SIN was also investigated as a vector for WEE structural genes [183]. A first generation construct was made using the SIN (prototype Ar339 strain) backbone and structural genes from a recent mosquito isolate of WEE (CO92-1356). Two second generation chimeric viruses were also constructed in which the amino-terminal portion of the C gene which contains the RNA-binding domain was replaced with the corresponding sequence of SIN or EEE virus (Table 17). The chimeras were less attenuated than similar constructs with other structural genes (e.g., from CHIK or VEE TC-83), showing that the structural gene inserts determined the virulence phenotype. In suckling mice, the WEE(CO92-1356)/SIN chimera was somewhat more attenuated than the other constructs, but still killed 80% and 30% of the mice after IC or SC inoculation, respectively. It replicated to lower titer in mouse brain, with titers similar to parental SIN. The lower neuroinvasiveness in suckling mice compared to parental WEE (CO92-1356) showed that chimerization per se was attenuating. Unfortunately the mortality ratio for the SIN Ar339 vector in infant mice inoculated SC was not presented, so that it is difficult to determine whether addition of WEE structural genes in the chimera enhanced neuroinvasiveness compared to SIN virus. The less attenuated chimeras with the amino-terminal C proteins from SIN or EEE elicited higher neutralizing antibody responses and afforded higher grade

protection against a severe IN challenge with WEE virus than the more attenuated WEE(CO-1356)/SIN chimera.

Finally, a chimeric SIN virus with structural genes from CHIK has also been evaluated in mice [184]. Unlike the other alphavirus chimeras, the CHIK/SIN virus was nonpathogenic in infant mice. This high level of attenuation is likely due to the fact that the wild-type CHIK structural gene donor is relatively attenuated (45% mortality ratio in suckling mice inoculated IC). Chimeric viruses were also constructed using VEE TC-83 and the South American subtype of EEE as vectors. The chimeras containing CHIK structural genes were also not neurovirulent, whereas parental gene donor strains were highly lethal, showing that the CHIK genes modulated virulence in these backbone viruses as well. Attenuation of the chimeras was compared to CHIK in suckling mice after SC inoculation. Wild-type CHIK replicates in skeletal muscle and joints as well as brain. All chimeras were attenuated compared to parental CHIK for replication in these tissues. The constructs induced modest neutralizing antibody levels in Swiss and C57/Bl6 mice; however, the CHIK/SIN construct was least immunogenic. Vaccinated mice resisted challenge with CHIK viruses.

The results of these investigations lead to the following conclusions: (1) Chimeric viruses using SIN as a vector can be readily constructed and are replication efficient in cells that could be used for manufacturing vaccines; (2) While SIN is one of the less pathogenic alphaviruses for humans, and no fatal human cases are recorded, its use as a vector is challenging due to lack of biomarkers for attenuation for humans; (3) chimeric viruses in which structural genes of heterologous alphaviruses are inserted have variable attenuation profiles in mouse models; (4) in general, such chimeras are more attenuated than the donor structural gene parent, but may be more virulent than the SIN backbone parent; (5) the chimeras are highly immunogenic and protect against severe challenge infection.

The observation that both the structural and nonstructural genes play a role in determining virulence is supported by several investigations of VEE chimeras, in which genes of low-virulence enzootic strains (less frequently associated with equid or human disease) were combined with genes from epizootic, virulent viruses [187, 188]. This observation comes as no surprise, based on the more extensive experience with flavivirus chimeras described in this chapter, and it simply emphasizes the requirement in vaccine development to carefully characterize the biological phenotype of each construct not only in mice, but in other models. The use of a benchmark vaccine virus as the backbone vector with known biological characteristics in humans (or domestic animal species if they are the target for vaccination), such as TC-83 [169] or possibly VEE V3526 [189] or CHIK 181/25 [171], will facilitate development. This is the principle that was used in the case of YF 17D, as it was useful to show that even when donor genes from a virulent virus like JE (Nakayama) or WN (NY99) were inserted in the YF 17D backbone, virulence was attenuated or at least similar to that of the 17D vaccine strain.

5 Use of Chimeric Viruses in Diagnostic Tests

The plaque reduction neutralization test is the most specific serological assay and the presence of neutralizing antibodies (or a rise/fall in titer between paired sera) provides the most precise means of differentiating past infections with close antigenic relatives and sympatric viruses (like SLE and WN); moreover neutralizing antibodies are the best immune correlate of protective immunity in vaccine studies. Unfortunately, use of the test is limited by a number of factors, including poor plaquing efficiency of some viruses (e.g., dengue), select agent status (JE), and the need for high-level biocontainment of pathogenic viruses, such as JE, SLE, and WN. In contrast, the corresponding ChimeriVax™ viruses plaque very well, and, being designated as BSL-2 agents, do not require high level laboratory practices and containment. For this reason, they have been deployed by the Centers for Disease Control and a number of State Health Laboratories for use in serological tests. Several reports document their use for surveillance of human and equid cases and infection in wildlife caused by St. Louis and Japanese encephalitis and West Nile viruses [164, 190, 191].

Similar use of chimeric alphaviruses as safer diagnostic reagents was reported by Ni and coworkers [192]. They compared attenuated VEE/SIN chimeras with parental VEE viruses in a variety of serological tests, including PRNT, hemagglutination-inhibition and complement fixation using subtype specific sera and found that it functioned as well as the parental viruses. The study included the chimeras incorporating structural genes from VEE IAB (TrD or TC-83), and VEE ID (ZPC738) [177], as well as new chimeras constructed with enzootic VEE subtypes IE and IF.

6 Recombination Events and Mutagenesis: Cause for Concern?

Some authors have raised concerns about the potential for recombination of chimeric flavivirus vaccines in hosts or arthropod vectors undergoing dual or sequential infections, for reversion of attenuation as a result of mutation, or for serious adverse events in individuals with genetic or acquired susceptibility to infection [193]. Some of the critics are conflicted by virtue of their own work, which is aimed at nonreplicating vaccine development [194]. Similar concerns could be raised for chimeric alphaviruses, but these vaccines have not reached a point of development that attracts adversarial commentary. The positive side of such criticism is that it has stimulated a healthy debate and scientific exploration to determine the likelihood and impact of untoward events. It is, of course, impossible to prove a negative, so the probability and consequences of recombination, reversion, and spread (arthropod infection) must be assessed and then put into context considering not only risks but also the benefits of vaccination. The debate has been framed in

published responses to the theoretical concerns [194, 195], and will not be reiterated here in full. The conclusion that may be drawn is that the risks of virus mutation (or recombination) or host-dependent susceptibility to adverse events associated with the chimeric Flavivirus vaccines are certainly no greater than (and are likely far less than) those associated with use of nonrecombinant live vaccines which have long been in use [191, 192].

Several studies were designed to investigate the phenotypic consequences of a hypothetical "worst-case" recombination event resulting in the substitution of genes from a highly virulent virus strain with genes from an attenuated chimeric vaccine based on the YF 17D vector. The results are summarized in Fig. 13. To investigate the outcome of a recombination event with a wild-type Australian flavivirus and ChimeriVax™-JE (which was being tested clinically in Australia), a virulent Kunjin (KUN) prME cassette was inserted into the YF17D backbone and the SA14-14-2 prME genes of ChimeriVax™-JE were inserted into the KUN backbone. The resulting chimeric viruses were somewhat less attenuated than the original ChimeriVax™-JE virus but were similar to or more attenuated than parental YF 17D. Both chimeras were attenuated compared to wild-type KUN virus. A similar examination of potential worst-case recombination was conducted by McGee and colleagues [30], who made chimeras incorporating virulent wild-type

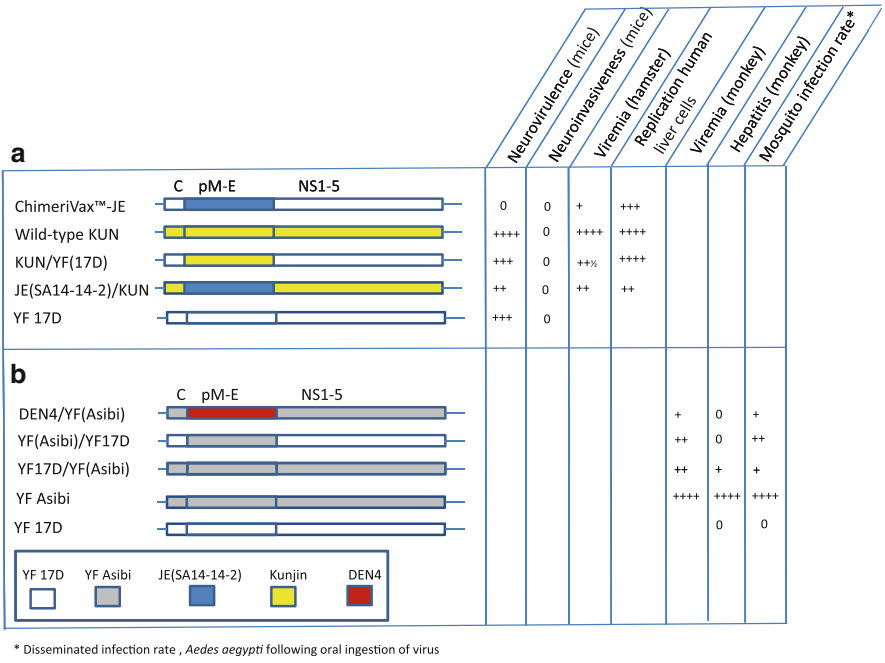


Fig. 13 Construction of "worst-case" chimeras representing hypothetical recombination events with virulent flaviviruses or strains. (**a**). Chimeras representing intertypic recombinants between ChimeriVax™-JE and Kunjin virus (from Pugachev et al. [32]). (**b**). Chimeras representing recombinantional events between ChimeriVax™-DEN and YF Asibi (from McGee et al. [30, 31])

YF Asibi prME genes on the YF17D backbone, or conversely inserted YF17D prME into YF Asibi (Fig. 13). In addition, wild-type DEN4 prME was inserted into the YF Asibi backbone. The constructs were injected SC into cynomolgus macaques. The YF17D/YF (Asibi) and YF (Asibi)/YF17D chimeras, as well as the DEN4/YF (Asibi) chimera were highly attenuated compared to YF Asibi . The only finding of note was minimal to mild liver inflammation and transient proinflammatory cytokine elevation in monkeys given the YF17D/YF(Asibi) virus. Overall, these studies showed that even in the unlikely event of an intertypic recombination event involving an exchange of a full structural or NS gene set with a highly virulent virus, the attenuating effects of the structural or nonstructural genes contributed by the vaccine, together with the chimerization effect, would result in an attenuated phenotype of the resulting virus.

An important feature of YF17D virus is its inability to cause disseminated infection of *Aedes* mosquitoes after oral ingestion of a virus-containing blood meal [196], whereas wild-type YF viruses are efficiently transmitted by these mosquitoes. A number of chimeric vaccines with heterologous prME genes in the YF 17D backbone were shown to be highly restricted for mosquito transmissibility [77, 103, 140, 197], due, in part to multiple mutations in the YF 17D backbone that occurred during passages during the development of 17D. Indeed, substitution of YF Asibi prME genes in the YF17D backbone does not restore high grade infectivity for mosquitoes [31, 198]. These findings, coupled with the very low viremia levels in the vaccinated host that would preclude infection, as well as the resistance of cells to superinfection with flaviviruses, makes recombination highly unlikely and the outcome of such an event innocuous.

Less is known about the potential for recombination of chimeric alphavirus vaccines, and little work has been done to assess the capacity for mosquito transmission [180] or the molecular determinants underlying susceptibility of mosquitoes to infection. Whereas intertypic recombination of flaviviruses in nature resulting in a viable virus has not been described, recombination as an evolutionary theme among alphaviruses is well documented [166]. The live, attenuated VEE TC-83 vaccine virus was isolated from mosquitoes following a vaccination campaign in horses [199], indicating the potential for transmission and recombination of a vaccine that is not severely restricted for viremia and/or mosquito infection.

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