Igor S Lukashevich · Haval Shirwan
Editors

Novel Technologies for Vaccine Development

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Editors
Igor S Lukashevich
Department of Pharmacology and Toxicology
University of Louisville
Louisville, Kentucky
USA

Haval Shirwan
Institute for Cellular Therapeutics and Department of Microbiology and Immunology
University of Louisville
Louisville, Kentucky
USA

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Introduction

At the beginning of the twenty-first century, the worldwide market for vaccines demonstrated a strong growth. Sales of innovative virus-like particles (VLP) vaccines, such as Prevnar against pneumococcal bacteria and Gardasil, the first preventive human papilloma virus cancer vaccines, contributed to this industry's success. According to industry forecasts, sales of preventive and therapeutic vaccine products will continue to grow and provide a lucrative market in the coming years for the pharmaceutical industry. To keep this trend intact, the vaccine manufacturers have to heavily rely on the development of innovative vaccines based on the recent progress in vaccinology (e.g., systems or cutting-edge "omics" approaches) and aggressively move from traditional manufacturing processes into novel approaches focusing on improving stability, updating formulation, and delivery methods. In the last few decades vaccine technologies have undergone fundamental changes, taking advantage of the profound understanding of the human immune system and its ability to mount protective and therapeutic immune responses against infections and malignancies. The development of novel cost-effective vaccine technologies with attractive risk-benefit ratio is fueling the pharmaceutical industry's interest in the vaccine market and will provide lucrative opportunities.

Vaccine technologies based on viral-vectored platforms continued to be very attractive for vaccine manufactures as exemplified by recent approval of IMOJEV®, the first yellow fever 17D-vectored vaccine to control Japanese encephalitis in Southeast Asian countries, and alipogene tiparvovec (Glybera®), an adeno-associated virus serotype 1 (AAV1)-based gene therapy for the treatment of patients with lipoprotein lipase (LPL) deficiency. In this book, we would like to focus mostly on viral-vectored vaccines as a prophylactic strategy to control emerging and reemerging human infections and as a therapeutic approach for cancer.

Replication-competent, "live-attenuated," antiviral vaccines (attenuated strains of poliovirus, yellow fever, measles, mumps, and rubella) are among the most cost-effective and widely used public health interventions. Currently, advances in molecular virology and creative design of replication-competent vaccines provide

new opportunities for rational design and development of the next generation of antiviral vaccines with optimally balanced safety and efficacy features. In the first chapter of the book, Yoshihiro Kawaoka and Gabriele Neumann, pioneers of reverse genetics approach for influenza research, provide their view on how this technology will improve safety and efficacy of the current influenza vaccines because "influenza vaccine protection is markedly lower than for most routinely recommended vaccines and is suboptimal." Notably, reverse genetics approaches are well positioned for the development of influenza vaccines again highly pathogenic A/H5N1 viruses and other potential pandemic influenza strains.

One of the obvious advantages of live-attenuated antiviral vaccines as well as viral-vectored vaccines is the expression of vaccine antigens in the context of natural viral infections, which activate innate immune responses and effectively process and present protective antigens to MHC molecules. In Chap. 2, Chad Mire and Thomas Geisbert describe current approaches to design vaccines against deadly Marburg and Ebola viruses. Unprecedented outbreak of Ebola hemorrhagic fever currently ongoing in West Africa, which the WHO has already declared a global public health emergency, and re-emerging of Ebola virus in the Democratic Republic of Congo, is a strong reminder that we are still poorly prepared to these devastating infections caused by highly pathogenic filoviruses. Although no vaccines or antiviral drugs for filoviruses are currently available for human use (WHO has recently backed treatment of Ebola patients with an experimental ZMapp drug, a mixture of 3 monoclonal antibodies against Ebola virus glycoproteins), remarkable progress has been made in developing preventive vaccines against Marburg and Ebola viruses in nonhuman primate models. Most of these vaccines are based on viral vectors including recombinant adenoviruses, alphaviruses, paramyxoviruses, and rhabdoviruses, and lead candidates are currently in early stage of clinical development.

Empirical methods which were used to develop very successful antiviral vaccines in the past (e.g., polio, yellow fever, mumps) are not currently applicable due to higher safety requirements raised by regulatory agencies. Although new generation of live-attenuated vaccines provides a very attractive risk-benefit ratio, safety is the major "inherent" weakness of these vaccines, and regulatory agencies express grave concerns regarding viral escape and reversion to wild type. In addition, public vaccine perception changed dramatically from the means of preventing devastating infectious diseases in the twentieth century to 100 % safe medical intervention in the twenty-first century. In Chap. 3, Peter Pushko and Irina Treryakova summarize current status of alphavirus-based replicon vaccines as an attractive alternative to live-attenuated platform. Alphavirus-vectored vaccines are replication-incompetent, cannot spread from cell-to-cell, and lack any DNA or nuclear stages during cytoplasmic expression of foreign antigens. Due to the presence of intrinsic RNA-dependent RNA polymerase activity, replicons are capable of self-amplification resulting in high levels of expression of antigen of interest. For vaccination or therapeutic purposes, replicons have to be packed or encapsidated into VLP vectors to target cells for antigen (transgene) expression. These vaccines stimulate innate immune responses, possess intrinsic systemic and mucosal

adjuvant activities, and practically have no anti-vector immunity concerns. The first clinical trials have shown excellent safety and immunological profiles. Several alphavirus replicon vaccine candidates are currently in clinical development as preventive measures to control infectious diseases and as therapeutic cancer vaccines.

Antiviral vaccines are among the most effective biomedical interventions as it was proved by global eradication of smallpox in 1979 and rinderpest disease in cattle in 2011. Notably, vaccinia virus, which replaced cowpox virus, as the smallpox vaccine is currently being explored for the development of viral-vectored approaches for preventive, immunotherapeutic, and oncolytic applications. Successful immunization against polio and measles is a promising move to global eradication of these infections as well. In Chap. 4, Konstantin Chumakov summarizes current status of polio eradication campaign which resulted in virtual elimination of the disease in almost the entire world with the exception of a few countries. Meanwhile, he provides arguments that polio eradication is a much more complex issue in comparison with eradication of smallpox. While the WHO eradication efforts may sooner or later result in complete cessation of wild poliovirus transmission, vaccination against poliomyelitis still has to be continued for the foreseeable future due to significant differences in the nature and epidemiology of polio and smallpox viruses. Notably, novel post-eradication polio vaccines with a new target profile will be required to maintain high population immunity and to ensure the global polio eradication.

New emerging and reemerging viral infections are attractive and challenging targets for novel vaccine technologies as it has been discussed in Chap. 2. Almost all newly discovered emerging pathogens are zoonotic infections. Among them, hantaviruses are an excellent example of how environmental changes affect natural virus-host balance and result in unexpected public health challenges. Hantaviruses are a fast-growing group of rodent-borne viruses causing two clinically distinct diseases, hantavirus pulmonary syndrome (HPS) in the Americas and more prevalent hemorrhagic fever with renal syndrome (HFRS) in Eurasia. The first hantavirus vaccines were rodent brain-derived inactivated extracts produced in China and South Korea. In Chap. 5, Eugeniy Tkachenko and coauthors overview current epidemiology of hantavirus infections and the development of new generation of cell culture-based vaccines against hantaviruses including expression of hantavirus proteins in different vectors, viral-vectored vaccines, VLPs, alphavirus replicons, and DNA immunization against hantaviruses.

Several DNA preventive vaccines against different targets are currently in early stages of clinical development. However, poor immunogenicity in humans is the major obstacle for these vaccines, and they are mostly used as a prime in a vector prime-protein boost immunization. Tremendous improvements in design of DNA vaccines and methods of their delivery provide hope that these vaccines will likely be commercially available in the nearest future in the area of therapeutic cancer vaccines (the second part of the book). In Chap. 6, Peter Pushko and coauthors have described novel vaccine technology that is based on an infectious DNA (iDNA) platform and combines advantages of DNA immunization and efficacy of

replication-competent vaccines. This technology is based on transcription of the full-length genomic RNA of the live-attenuated virus from plasmid DNA in vivo. The authors have provided proof-of-concept results using available attenuated and experimental vaccines, yellow fever 17D vaccine, Chikungunya (181/25), and Venezuelan equine encephalitis virus (TC-83), cloned in plasmid DNA under control of CMV promoter. Notably, only a few μg of iDNA was required to launch a live-attenuated virus and induce robust protective immune responses in mice. This approach eliminates many deficiencies of live-attenuated vaccines because iDNA is genetically and chemically stable, is easy to produce and control, and does not require cold chain. More importantly, DNA-launched vaccine is "manufactured" in vaccinated individuals and does not require traditional vaccine manufacturing. Further successful development of this technology has the potential to revolutionize the way we make live-attenuated vaccines.

In Chap. 7, Nikolai Petrovsky describes the various carbohydrate-based adjuvants and their potential to replace more traditional adjuvant such as aluminum salts and oil emulsions across a wide variety of human and veterinary vaccine applications using traditional antigens, viral vectors, or DNA to protect against infectious disease and for treatment of cancer and allergy.

The next five chapters in this book focus on the advancements in immunotherapies against chronic infections and cancer with particular focus on combinatorial immunotherapies involving viral-based vectors as treatment modalities. Unlike prophylactic vaccines against infections where vaccines are administered to healthy individuals with a competent and to a certain extent unbiased immune system, therapeutic vaccines are administered to diseased individuals whose immune system has lost battle against the targeted indication, and therefore either has been tolerized or severely compromised. Therefore, the efficacy requirements are quite different for prophylactic versus therapeutic vaccines. All that is required from prophylactic vaccines is to prime competent immune system of healthy individuals for the generation of high titers of neutralizing antibodies and/or cell-mediated immunity against targeted infection agent. With the exception of some challenging infections, prophylactic vaccines against many deadly infections for the most part have been quite successful. In contrast, the development of effective therapeutic vaccines against chronic infections and, most importantly, cancer has been a rather daunting task with a limited success, primarily due to the nature of vaccine formulations and the genetic and disease status of patients.

The genetic makeup of the patient will eventually dictate the efficacy of a vaccine. In particular, patients who have deficiency in genes required for mounting effective innate and/or adaptive immune responses will fail to respond to a vaccine formulation effective in individuals competent for these genes. For example, a significant portion of Caucasians are deficient for a functional toll-like receptor (TLR) TLR4 gene, which is important for the generation of both innate and adaptive immune responses. As such, these individuals will not benefit from a vaccine formulation that includes TLR4 as adjuvant. The negative impact of aging on the immune responses has also been well characterized and involves various arms of the immune system, including innate, adaptive, and regulatory immunity.

Although elderly individuals generate fewer naïve immune cells, they appear to have a normal number of lymphocytes, which composed of mostly memory B and T cells. Some of the age-related changes also include functional incompetence due to alteration of molecules involved in immune activation, expansion, and acquisition of effector function. As such, the therapeutic efficacy of vaccine will require a rational design based on not only the genetic makeup but also the age of the patient.

Standard treatments for cancer and the stage of cancer also play major roles in the efficacy of therapeutic vaccines. Patients with excessive chemo- or radiotherapy and large tumor burden will likely have compromised immune responses and, as such, manifest limited vaccine efficacy. In this context, patients in early stages of cancer who had a successful first-line therapy and minimal prior chemotherapy present an ideal population. Chemo- or radiotherapy administered at right dose and time may enhance the efficacy of the vaccine by reducing tumor burden and overcoming various immune evasion mechanisms. The formulation, dose, and frequency of vaccine administration are additional parameters that need to be considered based on the patient population and type and aggressiveness of the tumor being targeted. Finally and most importantly, effective vaccine formulations need to overcome various immune evasion mechanisms utilized by chronic infections and progressing tumor.

Advancements in molecular medicine have resulted in sophisticated tools to dissect the genetic makeup of individuals and the complexity of cellular and molecular nature of immune responses, requirements for effective immune activation, and, most importantly, the impact of infections on the evolution of the immune system. This accumulated knowledge has led to reevaluation of approaches for the development of therapeutic vaccines and, most importantly, raised awareness to refrain from applying the rules of prophylactic vaccines for the design of therapeutic vaccines. Without question, this is an exciting time for the vaccine field, and the enthusiasm and confidence in therapeutic vaccines have never been this high. However, given the complex interactions between the tumor and the immune system and the ability of individual tumor to evade the immune system by immune editing or generation of various immune evasion mechanisms, the full promise of therapeutic vaccines may only be realized through a combinatorial approach using vaccines with standard treatments that may work in synergy. In particular, the use of immune adjuvants in vaccine formulations will have the most significant impact on the efficacy of the vaccines. Adjuvants or adjuvant systems that target all three arms of the immune system, i.e., innate, adaptive, and regulatory, for the generation of the desired effector immune responses at the expense of immune evasion mechanisms have a great chance to succeed.

The chapter by Anton V. Borovjagin et al. provides a brief overview of various types of recombinant vaccines and focuses on those involving viral vectors and their applications for prevention and treatment of infectious diseases and cancer. A special emphasis in the chapter is given to vaccine development strategies using human adenovirus (Ad) as a very popular antigen delivery and expression platform. Various genetic modification strategies allowing for infectivity enhancement and transductional or transcriptional retargeting of Ad-based vectors to target tissues are

discussed along with strategies to circumvent preexisting virus-specific immune responses and liver tropism, the major obstacles for Ad clinical applications. Strategies involving Ad-based delivery of antigens associated with infectious agents, cancer, or other diseases are discussed. The chapter also highlights the clinical milestones in vaccine development and summarizes recent and ongoing clinical trials with Ad-based cancer vaccines.

In Chap. 9, Kevin J. Harrington discuses the use of viral vectors engineered to express genes, with particular focus on sodium-iodide symporter (NIS), that drive uptake of radioisotopes into cancer cells for both detection and antitumor efficacy. The results of preclinical and clinical trials of various replication-competent and replication-deficient viral vectors expressing NIS genes are discussed. This chapter also addresses issues relating to combination of virotherapy and standard anticancer therapies, including external beam radiotherapy, cytotoxic chemotherapy, and novel agents.

The comprehensive chapter by Caroline Jochems and colleagues discusses the use of TRICOM poxvirus-based vaccines as off-the-shelf vaccine platform either alone or in combination with other standard-of-care regimens for the treatment of cancer. This concept involves co-expression of B7-1, ICAM-1, and LFA-3 costimulatory molecules for T cells in combination with selected tumor-associated antigens. Extensive preclinical and clinical data on the use of TRICOM poxvirus-based vaccines alone and in combination therapies have been presented. Furthermore, the potential of this vaccine concept with various other standard-of-care treatments, such as irradiation and chemotherapy, as well as other new treatment modalities, such as immune checkpoint blockers, has been discussed.

In Chap. 11, Tasha Hughes and Howard L. Kaufman discuss oncolytic immunotherapy for the treatment of cancer with particular focus on the herpesviruses. The chapter discusses the rationale behind oncolytic immunotherapy and various viruses used for this purpose and highlights the clinical data generated using oncolytic herpesvirus encoding GM-CSF.

The chapter by Rajesh K. Sharma et al. focuses on the importance of adjuvants for the development of therapeutic vaccines. The TNF ligand costimulatory molecules were presented as adjuvants with significant potential to confer therapeutic efficacy of vaccines against cancer and chronic infections. This is because of the demonstrated role of these ligands in inducing $CD8^+$ primary T-cell responses and establishing and maintaining long-term memory. Extensive discussion was devoted to a recombinant, novel form of 4-1BBL (SA-4-1BBL). Significant data in preclinical tumor models have demonstrated that SA-4-1BBL targets all three arms of the immune system and generates robust effector responses against the progressing tumor while overcoming various immune evasion mechanisms.

Finally, vaccines in general and viral-vectored vaccines in particular for preventive and therapeutic applications are rapidly evolving and complex. As such, we as editors attempted to gather experts with novel and innovative vaccine technologies to contribute to his book. It is hoped that the presented vaccine concepts and technologies will not only contribute to the development of this rapidly evolving field but also serve as an educational material for students, postdoctoral fellows,

junior researchers, and clinicians. The coeditors, Drs. Igor Lukashevich and Haval Shirwan, are grateful to the contributors for providing their comprehensive view on cutting-edge technologies included in this book. We also would like to thank Claudia Panuschka and Ursula Gramm for their superb technical help with this project and Springer for this opportunity.

Igor S Lukashevich
Haval Shirwan
Louisville, KY

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Part I
Preventive Viral-Vectored Vaccines

Chapter 1
Reverse Genetics Approaches for Rational Design of Inactivated and Live Attenuated Influenza Vaccines

Yoshihiro Kawaoka and Gabriele Neumann

Abstract Influenza viruses are a major cause of respiratory infections in humans. The disease caused by these viruses ranges from nonapparent to deadly infections with multiorgan failure. The severity of the disease is determined by the infecting strain, the immune and health status of the infected individual, and various genetic factors that are currently poorly defined. Inactivated and live attenuated vaccines are available for human use. Inactivated influenza vaccines are safe but their efficacy is suboptimal. Live attenuated influenza vaccines elicit stronger immune responses than inactivated vaccines and are more efficacious in children than inactivated vaccine preparations. In addition, some concerns exist over the biosafety of live attenuated influenza vaccines. Accordingly, the efficacy of current influenza vaccines needs to be improved. Reverse genetics is a technology for the generation of influenza viruses from cloned cDNA, which allows the introduction of mutations at will. This technology has been used extensively to develop novel live attenuated vaccine candidates that possess attenuating mutations in one or several viral proteins, elicit cross-protective antibodies, and replicate efficiently. Reverse genetics has also made possible the development of vaccines to highly

Y. Kawaoka
Department of Pathobiological Sciences, Influenza Research Institute, School of Veterinary Medicine, University of Wisconsin-Madison, 575 Science Drive, Madison, WI 53711, USA

ERATO Infection-Induced Host Responses Project, Japan Science and Technology Agency, Saitama 332-0012, Japan

Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan

Department of Special Pathogens, International Research Center for Infectious Diseases, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108-8639, Japan

Laboratory of Bioresponses Regulation, Department of Biological Responses, Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan
e-mail: kawaokay@svm.vetmed.wisc.edu

G. Neumann (✉)
Department of Pathobiological Sciences, Influenza Research Institute, School of Veterinary Medicine, University of Wisconsin-Madison, 575 Science Drive, Madison, WI 53711, USA
e-mail: neumanng@svm.vetmed.wisc.edu

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pathogenic influenza viruses. These vaccine viruses lack a multi-basic cleavage site in the viral hemagglutinin protein that is recognized by ubiquitous proteases; hence, these viruses can no longer cause systemic infections. The development of such vaccines could not have been achieved without reverse genetics. Here, we review the state of influenza vaccines and the use of reverse genetics to develop improved vaccine viruses.

Influenza viruses belong to the family *Orthomyxoviridae*, which comprises influenza A, B, and C viruses, Thogoto viruses, and Dhori viruses. Influenza viruses are typed into influenza A, B, and C viruses based on antigenic differences of the viral nucleoprotein (NP) and matrix protein (M1). Influenza A viruses are further subtyped into currently 18 hemagglutinin (HA, H1-H18) and 11 neuraminidase (NA, N1-N11) subtypes based on the antigenic properties of the two viral surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). With the exception of the H17N10 and H18N11 influenza viruses, which were recently identified in bats, influenza A viruses of all subtypes have been isolated from waterfowl, suggesting that aquatic birds serve as their natural reservoir. Influenza A and B viruses cause respiratory disease in humans with important public health consequences. Influenza C viruses cause mild respiratory infections in children but seldom severe illness and are not considered a threat to humans; for this reason, they will not be discussed here in detail. Influenza A viruses are also a great concern in veterinary medicine because they cause frequent outbreaks in poultry, pigs, horses, and dogs. Influenza A virus outbreaks in poultry can result in high levels of mortality (up to 100 %) and substantial financial losses. However, this article will focus on human influenza virus infections.

1.1 The Influenza Viral Life Cycle

The genomes of influenza A and B viruses consist of eight single-stranded RNAs of negative polarity, that is, the viral RNAs (vRNAs) are not infectious, unlike those of RNA viruses with a genome of positive polarity. The HA protein (encoded by the HA vRNA) is the major viral antigen and mediates virus binding to sialic acid-possessing receptors on host cells. The virus is internalized via receptor-mediated endocytosis. Acidification of the late endosome leads to an irreversible conformational change in HA, which triggers the fusion of the endosomal and viral membranes. The ion channel function of the viral M2 protein (which is encoded by a spliced mRNA derived from the M vRNA) leads to the acidification of the interior of the virion and the subsequent release of viral ribonucleoprotein (vRNP) complexes into the cytoplasm from whence they are transported to the nucleus. vRNP complexes, the functional units of influenza virus replication and transcription,

comprise vRNA; the three viral polymerase subunits PB2, PB1, and PA (encoded by vRNAs of the same name); and the nucleoprotein, which is encoded by the NP vRNA. Using vRNAs as a template, the viral polymerase complex first synthesizes viral mRNAs that are exported to the cytosol and translated by the host translation machinery. During replication, the viral polymerase complex also synthesizes a positive-sense copy of the vRNA, termed cRNA, which is then transcribed to yield large amounts of vRNAs. The vRNAs are exported from the nucleus in the form of vRNPs and transported to the plasma membrane, where newly assembled viruses pinch off. The NA protein (encoded by the NA vRNA) is important for efficient virus release because its enzymatic activity cleaves sialic acid and prevents the formation of large virus aggregates on the cell surface. In the virion, the M1 matrix protein (translated from an unspliced mRNA derived from the M vRNA) is the major viral structural protein that forms a shell underneath the host cell-derived membrane. The NS vRNA encodes two proteins, the NS1 interferon antagonist protein (derived from an unspliced mRNA) and the NS2 (=NEP, nuclear export protein, derived from a spliced NS mRNA), which mediates the nuclear export of vRNP complexes. Many influenza A viruses also encode the PB1-F2 protein (encoded by a second open reading frame in the PB1 vRNA), which plays a role in the regulation of apoptosis and the innate immune response. Influenza A viruses thus encode 10 or more proteins. The genome organization and coding strategies of influenza B viruses differ slightly from those of influenza A viruses; these differences will not be described here but are readily obtainable in textbooks on influenza viruses (Palese and Shaw 2007; Wright et al. 2007). Given the segmented nature of their genomes, influenza viruses can exchange gene segments ("reassort") in cells infected with two different influenza A or B viruses; however, reassortment does not occur between viruses of different types (i.e., reassortment does not occur between influenza A and B viruses). Reassortment plays a major role in influenza virus evolution; most significantly, several pandemics have been caused by reassortant viruses (see below).

1.2 Influenza Virus Epidemics and Pandemics

The burden of influenza virus infections on human health is huge. Influenza A and B viruses cause annual epidemics (i.e., local outbreaks) that affect 5–15 % of human populations and cause ~3,000–49,000 deaths in the USA per year (Thompson et al. 2010) and 250,000–500,000 deaths per year worldwide (http://www.who.int/mediacentre/factsheets/2003/fs211/en/). The annual costs of influenza epidemics in the USA are estimated at $71–167 billion due to work and school absenteeism, doctor's visits, and medical costs (http://www.who.int/mediacentre/factsheets/2003/fs211/en/). Epidemics are caused by "antigenic drift" variants that are no longer recognized by the majority of anti-influenza antibodies in humans, resulting in humans being reinfected with influenza A and B viruses throughout their lifetime. These antigenic drift variants possess point mutations in the epitopes

of HA that prevent virus neutralization by the antibodies present in humans. This constant change of viral antigenic properties requires the update of influenza vaccines every 1–3 years.

Influenza A, but not influenza B, viruses also cause worldwide outbreaks (i.e., pandemics) associated with high morbidity and increased mortality. Pandemics are caused by viruses to which humans are immunologically naïve. In 1918, the "Spanish influenza" was caused by an influenza virus of the H1N1 subtype that was transmitted to humans from birds either directly or indirectly via other animals. This pandemic had devastating consequences and killed ~50 million people. Descendants of the 1918 pandemic virus circulated in humans until 1957, when they were replaced by a human/avian reassortant virus that possessed H2 HA, N2 NA, and PB1 genes of avian virus origin, while its remaining genes were derived from the H1N1 viruses previously circulating in humans. This so-called "Asian influenza" claimed about one million lives worldwide. The H2N2 viruses circulated in humans for 11 years, before they were replaced in 1968 by a human/avian reassortant H3N2 virus that possessed H3 HA and PB1 genes of avian virus origin, whereas its remaining six genes were derived from the previously circulating human influenza viruses. In the USA, 30,000–40,000 deaths were attributed to the 1968 "Hong Kong influenza." In 1977, viruses of the H1N1 subtype (similar to those circulating in the mid-1950s) reappeared in humans and co-circulated in humans with H3N2 viruses for the next 32 years. In 2009, a reassortant H1N1 virus possessing genes of human, avian, and swine influenza virus origin caused the first pandemic of the twenty-first century. The death toll of the 2009 H1N1 pandemic was estimated at ~280,000 deaths worldwide (Dawood et al. 2012).

Over the past century, only influenza A viruses of the H1N1, H2N2, and H3N2 subtypes have circulated widely in human populations. Occasionally, humans are infected by avian influenza viruses of other subtypes. The most prominent example are highly pathogenic avian influenza H5N1 viruses, which emerged more than a decade ago in Southeast Asia and are now enzootic in poultry populations in parts of Asia and the Middle East. As of January 24, 2014, these viruses have caused 650 confirmed human infections with a case fatality rate of nearly 60 % (http://www.who.int/influenza/human_animal_interface/EN_GIP_20140124CumulativeNumberH5N1cases.pdf?ua=1). Because these viruses may acquire the ability to efficiently transmit among humans (Herfst et al. 2012; Imai et al. 2012), which would inevitably cause a pandemic due to the lack of antibodies in humans to these viruses, considerable efforts have been made to develop vaccines to avian H5N1 viruses (see Sect. 1.7.2.1).

Humans have also been infected with avian influenza viruses of the H7 subtype. These infections are typically mild and self-limiting, but a highly pathogenic H7N7 virus caused a sizeable outbreak in humans in the Netherlands in 2003, which was likely associated with limited human-to-human transmission and caused the death of one individual (Fouchier et al. 2004; Koopmans et al. 2004). This event spurred the development of vaccines against viruses of this subtype. Since February of 2013, avian influenza viruses of the H7N9 subtype have infected >450 people in

China; more than >120 of these individuals succumbed to the infection (http://www.cidrap.umn.edu/sites/default/files/public/downloads/topics/cidrap_h7n9_update_080414.pdf) (China Mission Report 2013; Centers for Disease Control and Prevention 2013a; Gao et al. 2013). The high case fatality rate, the lack of antibodies in humans to this subtype of influenza viruses, and reports of potential human-to-human transmission of H7N9 viruses have created an urgent need for vaccines against these viruses.

Human infections have also been reported with avian influenza viruses of the H9 subtype, usually resulting in mild infections. Nonetheless, because humans lack protective antibodies to viruses of this subtype, candidate vaccines have been generated and tested [reviewed in Chen and Subbarao (2009), Keitel and Atmar (2009)].

1.3 Inactivated Influenza Vaccines

Protection against influenza virus infection is primarily achieved through antibodies to HA that prevent virus infection ("neutralizing antibodies"). Antibodies to NA do not prevent virus infection, but interfere with efficient virus release and spread. To elicit such antibodies, the currently approved influenza vaccines present the HA (or HA and NA) proteins to the human immune system in the form of inactivated vaccines, live attenuated vaccines, or recombinant HA protein (the latter will not be discussed here in detail).

Inactivated vaccines to human influenza viruses have been commercially available in the USA since 1945. Today, trivalent vaccines (composed of type A H1N1 and H3N2 viruses and a type B virus) and quadrivalent vaccines (composed of type A H1N1 and H3N2 viruses and two type B viruses that represent the two lineages of influenza B viruses that have co-circulated in humans in previous years) are available. For the influenza A virus components, reassortants are generated that possess at least the HA and NA genes of the recommended vaccine strain and the remaining genes of the A/Puerto Rico/8/34 (PR8; H1N1) virus. This virus was selected as a vaccine backbone because it confers efficient replication in embryonated chicken eggs, the most common substrate for influenza vaccine propagation. Two approaches exist for the generation of reassortant vaccine viruses. In "classical" reassortment approaches, cells are coinfected with PR8 and a recommended vaccine strain, and reassortants possessing at least the HA and NA genes of the recommended vaccine strain are selected. Given that coinfection of cells results in $2^8 = 256$ gene combinations, the selection of desired vaccine viruses can be cumbersome and may delay vaccine production. In an alternative approach ("reverse genetics," described in detail in Sect. 1.6), influenza viruses are generated from plasmids, allowing the desired gene combination to be engineered. Vaccine master strains generated through classical reassortment or reverse genetics are typically amplified in embryonated chickens eggs. Alternatively, Madin-Darby canine kidney (MDCK) cells can now be used in the USA and Europe for human influenza vaccine virus propagation; in Europe, African green monkey kidney (Vero) cells

are also used for this purpose. Amplified vaccine master strains are then chemically inactivated, purified, typically treated with detergents to generate "split" and "subunit" vaccines, mixed to generate trivalent or quadrivalent vaccines, and usually administered intramuscularly. For the influenza B virus component(s), the recommended wild-type strains are chemically inactivated. The generation of vaccines to highly pathogenic avian H5N1 influenza viruses faces additional safety challenges, which are discussed in Sect. 1.7.2.1.

The influenza vaccine manufacturing process requires 4–6 months; accordingly, vaccine viruses are recommended by the WHO each February for the following influenza season in the Northern Hemisphere and each August for the following influenza season in the Southern Hemisphere. Between these recommendations and the start of the influenza seasons, novel antigenically drifted variants may emerge that lead to an antigenic mismatch with the recommended vaccine strain. Such mismatches can markedly reduce the efficacy of the influenza vaccine and are a major challenge in influenza vaccine development.

1.4 Live Attenuated Influenza Vaccines

Live attenuated influenza vaccines (LAIV) were developed to mimic natural infection, which is believed to induce more efficacious immune responses than vaccination with inactivated viruses. The vaccine master strains currently used in the USA were derived in the 1960s by passaging the A/Ann Arbor/6/60 (H2N2) and B/Ann Arbor/1/66 viruses at successively lower temperatures in primary chicken kidney cell cultures (Maassab 1969). This strategy resulted in the accumulation of several mutations in several internal viral genes that confer the temperature-sensitive, cold-adapted, and attenuated phenotypes of the vaccine viruses. For annual vaccine production, the internal genes of the type A and B master strains are combined with the HA and NA genes of the recommended vaccine strains through classical reassortment or reverse genetics as described above. Live attenuated influenza vaccines are administered intranasally as a spray. They were licensed in the USA for human use in 2003 and are now also marketed in Great Britain and Germany.

The immunogenicity of LAIV would be expected to be superior to that of inactivated vaccines. Inactivated vaccines elicit primarily serum IgG responses, whereas LAIVs also stimulate mucosal IgA and T-cell responses (Belshe et al. 1998, 2000; Ghendon 1990), resulting in broader immune responses. On the other hand, inactivated vaccines have a better biosafety profile than LAIV, which could reverse to their wild-type phenotypes and/or undergo reassortment with circulating influenza viruses (however, no such events have been reported to date). As with inactivated vaccines, the development of LAIV against highly pathogenic avian H5N1 influenza viruses requires additional safety precautions (see Sect. 1.7.2.1).

1.5 Efficacy of Current Influenza Vaccines

Numerous studies have assessed the efficacy of trivalent inactivated and live attenuated influenza vaccines (Jefferson et al. 2007, 2010a, b, 2012; Osterholm et al. 2012a, b). A recent meta-analysis found that "during some influenza seasons vaccination offers substantially more protection for most of the population than being unvaccinated" (Osterholm et al. 2012a) but continued with the statement, "influenza vaccine protection is markedly lower than for most routinely recommended vaccines and is suboptimal" (Osterholm et al. 2012a). In children, live attenuated influenza vaccine is superior to trivalent inactivated vaccine (Jefferson et al. 2012). In the elderly, a paucity of data does not allow definite conclusions regarding vaccine efficacy (Jefferson et al. 2010a). In adults, low-to-high levels of protection may be achieved by trivalent inactivated and live attenuated vaccines, depending on the antigenic match between the vaccine strains and the strains circulating during the influenza season (Jefferson et al. 2007, 2010b). In addition, other factors clearly contribute to vaccine efficacy. For example, the efficacy of the H3N2 vaccine was low in the elderly in the 2012–2013 influenza season (Centers for Disease Control and Prevention 2013b), even though the recommended vaccine viruses were antigenically similar to the circulating strains that season. Taken together, there is a clear need to improve current influenza vaccines. Reverse genetics, which allows the artificial generation and modification of influenza viruses, may lead to vaccine improvements through the introduction of mutations into the viral genome.

1.6 Reverse Genetics for the Artificial Generation of Influenza Viruses

Reverse genetics, that is, the artificial generation of influenza viruses, requires that all eight influenza vRNAs and the polymerase and NP proteins are provided to initiate viral replication and transcription. The intracellular synthesis of influenza vRNAs in the nucleus of cells can be achieved with the use of cellular RNA polymerase I, which synthesizes ribosomal RNA in the nucleus of eukaryotic cells (Neumann et al. 1994). For influenza virus reverse genetics, cDNAs encoding the full-length viral RNAs are cloned between the RNA polymerase I promoter and terminator sequences (Neumann et al. 1999). The resulting eight plasmids are then transfected into human embryonic kidney (293T) cells, which have high transfection efficiency. The 293T cells are then cotransfected with four protein expression plasmids that yield the three viral polymerase proteins and NP. In cells transfected with all 12 plasmids, the cellular RNA polymerase I first synthesizes all eight vRNAs, which are then replicated and transcribed by the viral polymerase and

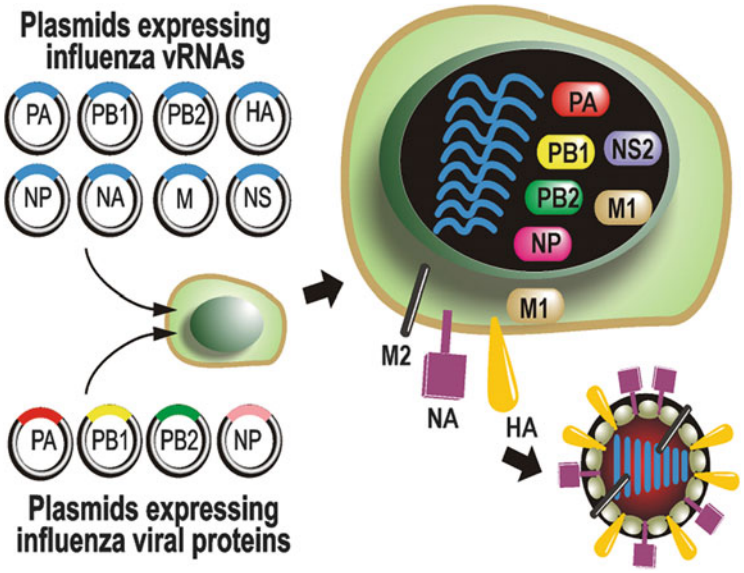

Fig. 1.1 Generation of influenza viruses from cloned cDNAs (reverse genetics). Cells are cotransfected with eight plasmids for the transcription of the eight influenza A viral RNAs and with four protein expression plasmids for the synthesis of the polymerase and NP proteins. Cellular RNA polymerase I synthesizes viral RNAs, which are replicated and transcribed by the viral polymerase and NP proteins, resulting in the expression of all viral proteins and the amplification of the viral RNAs, leading to influenza viruses. Figure modified after Neumann and Kawaoka, 2001, Virology 287:243–250

NP protein, resulting in authentic influenza viruses (Neumann et al. 1999) (Fig. 1.1). This system has proven to be very efficient, although it requires the simultaneous introduction of 12 plasmids into a single cell. Since the first report of influenza virus generation from plasmids, several modifications have been described that reduce the number of plasmids required, use different promoter or terminator sequences for the transcription of the vRNAs, or allow influenza virus generation in human, monkey, canine, and avian cells (note that the RNA polymerase I promoter is species-specific and that the human promoter originally used limited the system to human and closely related cells).

In vaccine virus generation, reverse genetics is now used to specifically generate desired gene constellations: cells are simply transfected with plasmids encoding the recommended HA and NA genes and the internal genes of PR8 or attenuated Ann Arbor viruses to generate master strains for inactivated vaccine or LAIV production.

1.7 Rational Design of LAIV

The ideal live attenuated influenza vaccine should (1) be genetically stable so that the wild-type phenotype is not restored, (2) replicate to high titers in embryonated chicken eggs and/or MDCK and Vero cells to ensure timely and cost-effective production of large quantities of vaccine, (3) efficiently stimulate humoral and innate immune responses, and (4) be sufficiently attenuated in humans to prevent adverse reactions to the vaccine. The production of LAIV against avian influenza viruses that may cause pandemics in humans (so-called pandemic viruses) presents additional challenges because these viruses pose significant risks to humans. Additional challenges lie in the development of LAIV that cannot reassort with circulating influenza viruses and provide protection against viruses of different HA subtypes (heterosubtypic cross-protection).

1.7.1 Modifications of the NS Gene for Virus Attenuation

The NS1 protein serves as the major viral interferon antagonist that counteracts host cell innate immune responses triggered upon influenza viral infection. NS1 interferes with the induction of interferon beta (IFNβ) mRNA synthesis, IFNβ signaling, and the IFNβ-stimulated synthesis of antiviral proteins such as Mx, PKR, and RNaseL/OAS [reviewed in Ehrhardt et al. (2010), Hale et al. (2008)]. NS1 also interferes with the virus-induced activation of RIG-I, which leads to the activation of IRF-3 and NF-κB and hence the further stimulation of IFN responses [reviewed in Ehrhardt et al. (2010); Hale et al. (2008)]. Most influenza A virus NS1 proteins range in length from 217 to 237 amino acids; the first ~77 amino acids confer binding to RNA (Qian et al. 1995), while the remaining two-thirds of the protein (the so-called effector domain) mediate interaction with several host proteins.

The expression of full-length NS1 protein is not obligatory for the viral life cycle since viruses with NS1 C-terminal deletions of various lengths have been found in nature. Garcia-Sastre et al. (1998) and Talon et al. (2000) first demonstrated that a PR8 virus lacking the NS1 gene (except for the first 10 amino acids which overlap with NEP) replicated to appreciable titers in IFN-deficient systems such as Vero cells, $STAT^{-/-}$ mice, and 6-day-old embryonated chicken eggs but was significantly attenuated in IFN-competent systems including MDCK cells, 10- to 14-day-old embryonated chicken eggs, and wild-type (BALB/c or C57BL/6) mice. In mouse embryo fibroblasts and human 293T cells infected with an NS1-deleted virus, the IFNα- and IFNβ-mRNA levels were higher than those in cells infected with wild-type PR8 virus (Wang et al. 2000). Collectively, these studies indicated that the lack of wild-type NS1 results in high amounts of IFN in infected cells, which ultimately restricts virus replication; hence, NS1-deficient viruses may be attractive candidates for improved LAIV.

Because the attenuation level is vital for the development of live attenuated vaccines, NS1 proteins of different lengths have been tested, ranging from deletion constructs (encoding only the 10 amino acids shared with NEP) to truncated versions that express the N-terminal 73, 99, or 126 amino acids of NS1 (among other candidates tested) (Chambers et al. 2009; Egorov et al. 1998; Ferko et al. 2004; Garcia-Sastre et al. 1998; Hai et al. 2008; Kappes et al. 2012; Maamary et al. 2012; Mueller et al. 2010; Park et al. 2012; Pica et al. 2012; Quinlivan et al. 2005; Richt et al. 2006; Romanova et al. 2009; Solorzano et al. 2005; Steel et al. 2009; Talon et al. 2000; Vincent et al. 2007; Wacheck et al. 2010; Wang et al. 2008; Wressnigg et al. 2009a, b; Zhou et al. 2010). These *C-terminally truncated* LAIV candidates have been tested in the background of PR8 virus (Egorov et al. 1998; Ferko et al. 2004; Garcia-Sastre et al. 1998; Maamary et al. 2012; Mueller et al. 2010; Park et al. 2012; Pica et al. 2012; Talon et al. 2000), "seasonal" human influenza A and B viruses (i.e., influenza viruses currently circulating in humans) (Baskin et al. 2007; Hai et al. 2008; Wacheck et al. 2010; Wressnigg et al. 2009a, b), pandemic 2009 H1N1 viruses (Zhou et al. 2010), and highly pathogenic H5N1 viruses (Maamary et al. 2012; Park et al. 2012; Romanova et al. 2009; Steel et al. 2009). In addition, several LAIV candidates have been tested as veterinary vaccines for the protection of swine (Kappes et al. 2012; Richt et al. 2006; Solorzano et al. 2005; Vincent et al. 2007), horses (Chambers et al. 2009; Quinlivan et al. 2005), or poultry (Wang et al. 2008).

Viruses possessing C-terminally truncated NS1 proteins are replication competent but attenuated in IFN-competent systems. Typically, the level of attenuation increases with the length of the C-terminal deletion, although exceptions have been noted. In animal models including mice (Ferko et al. 2004; Hai et al. 2008; Maamary et al. 2012; Mueller et al. 2010; Pica et al. 2012; Quinlivan et al. 2005; Steel et al. 2009; Talon et al. 2000; Wressnigg et al. 2009a; Zhou et al. 2010), ferrets (Zhou et al. 2010), macaques (Baskin et al. 2007), pigs (Kappes et al. 2012; Loving et al. 2013; Richt et al. 2006; Solorzano et al. 2005; Vincent et al. 2007), horses (Chambers et al. 2009; Quinlivan et al. 2005), and poultry (Steel et al. 2009; Wang et al. 2008), these LAIV candidates were attenuated to various extents, induced cell-mediated and humoral immune responses, and/or provided protective immunity against challenges with homologous influenza viruses (Baskin et al. 2007; Hai et al. 2008; Kappes et al. 2012; Maamary et al. 2012; Pica et al. 2012; Richt et al. 2006; Steel et al. 2009; Vincent et al. 2007; Wang et al. 2008; Wressnigg et al. 2009a; Zhou et al. 2010). Some studies also reported cross-reactive immunity against antigenically mismatched challenge viruses (Hai et al. 2008; Kappes et al. 2012; Richt et al. 2006; Steel et al. 2009; Vincent et al. 2007; Wang et al. 2008). In addition to NS1 C-terminally truncated LAIV candidates, a virus possessing an internal deletion of five amino acids (amino acid positions 196–200) in NS1 (which also leads to an internal deletion of amino acid positions 39–43 of the NEP protein) was tested (Zhou et al. 2010). This deletion was found in a highly pathogenic H5N1 virus isolated from pigs (A/swine/Fujian/1/2003) and attenuated another highly pathogenic H5N1 virus (A/swine/Fujian/1/2001) in chickens (Zhu et al. 2008). The LAIV candidate possessing this internal deletion in NS1 was

highly attenuated in mice and ferrets but elicited strong neutralizing antibody responses and protected animals from challenge with homologous virus (Zhou et al. 2010).

Influenza A and B LAIV candidates that *lack the entire NS1 protein* (except for the 10 amino acids that overlap with NEP) have been tested in the genetic background of PR8 virus (Ferko et al. 2004; Garcia-Sastre et al. 1998; Mueller et al. 2010), seasonal human influenza A and B viruses (Hai et al. 2008; Wacheck et al. 2010; Wressnigg et al. 2009b), and highly pathogenic H5N1 viruses (Park et al. 2012; Romanova et al. 2009). These viruses are essentially replication incompetent in IFN-competent systems, although an influenza B virus lacking NS1 replicated to detectable titers in MDCK cells and C57BL/6 mice (Hai et al. 2008). NS1-deficient vaccine candidates will therefore need to be amplified in IFN-incompetent systems. Alternatively, cell lines expressing NS1 could be used to overcome the growth restriction of NS1-deficient viruses in IFN-competent systems (van Wielink et al. 2011). Seasonal H1N1 and highly pandemic H5N1 NS1-deficient vaccine candidates did not cause disease symptoms in mice, ferrets, and/or macaques and were not shed from these animals (Park et al. 2012; Romanova et al. 2009; Wressnigg et al. 2009b). They, however, elicited enhanced IFN responses, strong B- and T-cell-mediated immune responses, and protective immunity against homologous (Park et al. 2012; Romanova et al. 2009; Wacheck et al. 2010; Wressnigg et al. 2009b) and heterologous (Romanova et al. 2009; Wacheck et al. 2010) as well as heterosubtypic (Park et al. 2012) challenge viruses. An NS1-deficient seasonal H1N1 vaccine candidate was tested in phase I and II clinical trials and found to be safe and well tolerated with adverse events similar to those observed in a control group (Wacheck et al. 2010). The vaccine candidate also elicited virus-specific antibodies in a dose-dependent manner.

In addition to its role in IFN suppression, NS1 is also involved in the regulation of viral RNA synthesis and viral mRNA splicing and translation. Interestingly, some C-terminally truncated NS1 proteins reduce the levels of HA mRNA and protein expression (Falcon et al. 2004; Maamary et al. 2012; Solorzano et al. 2005) but have lesser or no effect on other viral gene segments. Given that HA is the major viral antigen, reduced levels of this protein may lower vaccine immunogenicity. Reverse genetics was therefore employed to introduce two mutations (G3A and C8U) into the NS vRNA promoter (Maamary et al. 2012); these mutations had previously been shown to increase viral replication/transcription (Neumann and Hobom 1995). The resulting virus retained the attenuating phenotype conferred by the truncated NS1 protein but expressed increased levels of HA compared with a control virus that lacked the promoter mutations (Maamary et al. 2012).

Most of the NS1 vaccine candidates have been generated by inserting a stop codon at the desired position in the NS1 gene. One study reported revertants that eliminated the stop codon, resulting in the expression of wild-type NS1 or mutant NS1 lacking 4 or 57 internal amino acids (Brahmakshatriya et al. 2010). These mutations emerged after only five passages of the candidate LAIV in chickens, and the resulting mutants were more pathogenic than the candidate virus

(Brahmakshatriya et al. 2010). Hence, the genetic stability of all LAIV vaccine candidates must be thoroughly assessed and monitored.

1.7.2 Modifications of the HA Gene for Virus Attenuation

1.7.2.1 Modification of the Multi-basic Cleavage Site for the Development of Vaccines Against Highly Pathogenic Influenza Viruses

The fusion of the viral and endosomal membranes (an event that releases the virus genetic material into the cytoplasm) is initiated by the HA fusion peptide (formed by the N-terminus of the HA2 subunit), which becomes exposed after HA is cleaved into HA1 and HA2. This cleavage event is therefore essential for efficient virus replication. Highly pathogenic influenza viruses possess a hallmark cleavage site sequence that comprises several basic amino acids. Because this cleavage motif is recognized by ubiquitous proteases, highly pathogenic avian influenza viruses can spread systemically. By contrast, viruses of low pathogenicity possess a single basic amino acid at the cleavage site and are restricted to organs in which proteases that recognize such a motif are expressed (i.e., the respiratory tract of mammals or the respiratory and intestinal tracts of avian species).

The development of vaccines to highly pathogenic influenza viruses faced biosafety obstacles because these viruses pose a considerable risk to the public and therefore had to be handled in biosafety level 3 laboratories. Large-scale vaccine production at this biosafety level is costly. In addition, because highly pathogenic influenza viruses kill chicken embryos, high titers of these viruses cannot be obtained in embryonated chicken eggs. Reverse genetics was, therefore, used to generate vaccine strains in which the multi-basic HA cleavage site was replaced with a single basic amino acid; conventional methods would not have allowed the development of safe vaccines against highly pathogenic influenza viruses. The resulting vaccine candidates possess the internal genes of PR8, the wild-type NA segment of a highly pathogenic H5 or H7 virus, and the modified HA segment of a highly pathogenic H5 or H7 virus [reviewed in Keitel and Atmar (2009)]. These vaccine viruses maintained their antigenic properties against the highly pathogenic virus but can be efficiently propagated in embryonated chicken eggs and safely handled in biosafety level 2 containment. In the USA, an inactivated H5N1 vaccine was approved for use in humans in 2007 (http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm112838.htm). The rapid evolution of highly pathogenic H5N1 viruses and their diversification into several clades and subclades requires constant reevaluation of vaccine strains; to date, a number of candidate vaccines against different H5N1 (sub)clades have been developed, which could be used for expedited vaccine production in the event of a pandemic.

Inactivated vaccines to highly pathogenic H5N1 viruses are of poor immunogenicity in humans, likely because of the lack of prior exposure to viruses of this subtype [reviewed in Keitel and Atmar (2009)]. LAIV candidates possessing the H5N1 virus-derived NA and modified HA genes have therefore been evaluated [reviewed in Chen and Subbarao (2009)]. However, no LAIV H5N1 vaccine has been approved to date, primarily due to biosafety concerns.

Candidate vaccines have also been developed for viruses of the H7 (Cox et al. 2009; de Wit et al. 2005; Jadhao et al. 2008; Jiang et al. 2010; Joseph et al. 2007, 2008; Min et al. 2010; Pappas et al. 2007; Park et al. 2006; Szecsi et al. 2006; Toro et al. 2010; Whiteley et al. 2007) and H9 subtypes (Atmar et al. 2006; Cai et al. 2011; Hehme et al. 2002; Karron et al. 2009; Pushko et al. 2005, 2007; Stephenson et al. 2003), and live attenuated H7N3 (Talaat et al. 2009) and H9N2 (Karron et al. 2009) viruses have been evaluated in clinical trials. However, no vaccine against H7 or H9 influenza viruses has yet been approved for human use.

1.7.2.2 Is a Universal Vaccine Attainable?

Current influenza vaccines provide limited protection against antigenic drift variants and little to no protection against influenza viruses of other subtypes. The constant changes in the antigenic properties of these viruses and our inability to predict the HA subtype that will cause the next pandemic leave us in a constant need to catch up with a fast-evolving virus. "Universal" vaccines that protect against viruses of all subtypes, or at least against antigenic drift within a subtype, are therefore the "holy grail" of influenza vaccinology.

Phylogenetic and sequence analyses can be used to infer ancestral HA sequences that are located at the base of phylogenetic lineages or clades. Using such analyses, a candidate vaccine has been generated that expresses the ancestral, inferred HA protein at the base of clade 2 avian H5N1 viruses (Ducatez et al. 2011). This computationally designed HA was stable, and the resulting virus was immunogenic; however, it did not provide a significant increase in cross-protection compared with vaccine viruses based on the natural clade 2 avian H5N1 HA proteins (Ducatez et al. 2011).

Over the past years, several studies have documented the presence of broadly cross-protective antibodies in humans and mice naturally or experimentally infected with influenza viruses. Almost all of these antibodies bind to the HA stalk region, which is much more conserved among the different subtypes than the highly variable head region, which possesses the antigenic epitopes. HA proteins can be divided into two large phylogenetic clusters that include H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, and H17 (group 1) or H3, H4, H7, H10, H14, and H15 (group 2) HA proteins. Several stalk-specific antibodies have now been identified that cross-react within group 1 (Corti et al. 2010; Ekiert et al. 2009; Okuno et al. 1993; Sui et al. 2009; Tan et al. 2012; Throsby et al. 2008) or group 2 (Corti et al. 2011; Ekiert et al. 2011; Wang et al. 2010) HAs; one of these

antibodies reacts with HAs of both phylogenetic groups (Corti et al. 2011). One study also identified a stem-specific antibody that provides protection against influenza A and B viruses (Dreyfus et al. 2012). Broadly cross-reactive antibodies have now also been identified that bind to the HA head region (Dreyfus et al. 2012; Ekiert et al. 2012); however, these antibodies appear to be generated at low frequencies, in contrast to the more numerous antibodies against immunodominant epitopes in the HA head. Hence, the challenge is to develop vaccines or vaccination strategies that elicit robust, neutralizing antibody responses to the HA stalk while suppressing immunodominant responses to the major epitopes in the HA head.

Toward this goal, "headless" HAs were tested that lacked the immunodominant head region (Steel et al. 2010). In mice, headless HAs elicited antibody responses that were more cross-reactive than those obtained with full-length HA. Moreover, vaccination with headless HAs provided some (although not complete) protection from homo- and heterosubtypic virus challenge (Steel et al. 2010). In another approach, chimeric HAs have been tested that possess the stalk region of one subtype (e.g., the H1 HA stalk region) but the head region of a different subtype (e.g., the H9 or H6 head region) (Hai et al. 2012; Krammer et al. 2013; Margine et al. 2013). The underlying idea was that by repeated immunization with the same HA stalk domain, but different HA head domains, stalk-specific antibodies would be boosted. In fact, sequential immunization of mice with viruses expressing H9/H1 (H9 head + H1 stalk) and H6/H1 (H6 head + H1 stalk) HAs protected mice against homologous and heterologous H1 virus challenge, demonstrating that neutralizing stalk-specific antibodies can be elicited (Krammer et al. 2013). Importantly, these mice were also protected against heterosubtypic H5N1 challenge (Krammer et al. 2013), proving the cross-protective potential of stalk-specific antibodies. In initial experiments, the chimeric HAs were expressed from DNA plasmids or provided as soluble protein; however, a recent study expressed the chimeric influenza A virus HA from a reverse genetics-engineered virus (Krammer et al. 2013). Because live virus infection induces more cross-protective antibodies than does vaccination with inactivated vaccine (Margine et al. 2013), the use of reverse genetics to develop live attenuated vaccines that elicit protective, stalk-specific antibodies may be vital to develop broadly cross-protective influenza vaccines.

1.7.2.3 Increased HA Stability for Higher Vaccine Yield and Long-Term Potency

In the acidic environment of the late endosome, HA undergoes an irreversible conformational change that leads to membrane fusion and the release of vRNPs into the cytosol (see Sect. 1.1). The optimum pH for fusion differs among HAs (Galloway et al. 2013; Reed et al. 2010; Scholtissek 1985a, b; Zaraket et al. 2013b), which likely affects the infectivity of the particular viruses. The heat stability of HA proteins at 50 °C serves as a reliable surrogate for HA stability. Moreover, the thermal stability of HA in itself is an important factor because it can directly affect vaccine potency. In fact, for vaccines against the pandemic 2009 H1N1 virus, a

decrease of HA antigen content over time was noticed that led to revised vaccine expiration dates (http://www.hc-sc.gc.ca/ahc-asc/media/advisories-avis/_2010/2010_54-eng.php; http://www.health.state.mn.us/divs/idepc/immunize/mnvfc/bfaxarchive/bf15dec09.pdf; http://ama.com.au/seasonal-flu-vaccine-and-young-children). This decline in potency correlated with the low thermal stability of the HA protein in the vaccine preparation (Farnsworth et al. 2011; Robertson et al. 2011). Moreover, ferret-transmissible H5 viruses possessed mutations in HA that affected thermal and acidic stability and compensated for the loss of thermal stability that resulted from mutations in the receptor-binding pocket (Herfst et al. 2012; Imai et al. 2012) (our unpublished data). Together, these findings renewed interest in the identification of mutations that increase HA stability and could affect vaccine virus yield and potency over time.

Reed et al. (2009) identified several mutations in HA that affect the optimum pH for fusion. Reverse genetics was subsequently used to demonstrate that a virus possessing one of these mutations possessed increased resistance to heat inactivation and low-pH treatment (Krenn et al. 2011). This virus replicated efficiently in mice (Krenn et al. 2011) and ferrets (Zaraket et al. 2013a) and elicited higher antibody titers in mice than did a control virus (Krenn et al. 2011). Reverse genetics thus provides an ideal platform to identify mutations that affect HA stability and fusogenicity and to test mutations such as these in the background of vaccine strains for increased virus yield and/or stability.

1.7.3 Modifications of Other Viral Genes for Virus Attenuation

Many attenuating mutations have been described in viral proteins other than HA and NS1. However, most of these mutations reduce virus growth in cultured cells and/or eggs and may thus not allow efficient vaccine production. This hurdle could be overcome by using cell lines that express the respective wild-type protein, resulting in efficient vaccine virus growth in such helper cells. Cell lines expressing the viral polymerases (Ozawa et al. 2013), the PB2 polymerase subunit (Ozawa et al. 2011; Victor et al. 2012), NS1/NS2 (Portela et al. 1986), or the M2 protein (Hatta et al. 2011; Octaviani et al. 2010; Watanabe et al. 2009) have been established to date.

The M1 matrix protein is the major structural component of the virus. It also executes a critical role in the nuclear export of vRNP complexes, presumably by forming a bridge between vRNP complexes and the viral nuclear export protein. A lab-adapted human influenza A virus (A/WSN/33, H1N1; WSN) with mutations in the nuclear localization signal of M1 replicated comparably to wild-type virus in MDCK cells but was attenuated in mice (Xie et al. 2009). It elicited a strong antibody response and protected mice against not only homologous WSN virus but also against challenges with viruses possessing the HA and NA genes of a

human H3N2 virus or the modified HA and NA genes of a highly pathogenic H5N1 virus (Xie et al. 2009).

Mutations in the putative zinc finger motif of M1 did not affect the replication kinetics of WSN virus in MDCK cells (Hui et al. 2003) or embryonated chicken eggs (Hui et al. 2006). Interestingly, however, such viruses were attenuated in murine and human cells, and in mice (Hui et al. 2006), suggesting a host-specific role of the zinc finger motif in M1. Mice immunized with an M1 zinc finger mutant were protected against lethal challenge with WSN virus (Hui et al. 2006), demonstrating the potential of M1 zinc finger mutants as LAIV.

The ion channel M2 protein allows proton influx from acidic endosomes into the interior of the virion, resulting in the destabilization of interactions between M1 and vRNP complexes, allowing these complexes to be released into the cytoplasm. M2 possesses a long cytoplasmic tail of 54 amino acids. Viruses encoding truncated M2 tails are viable but usually attenuated in cell culture (Hatta et al. 2011; Iwatsuki-Horimoto et al. 2006; McCown and Pekosz 2005, 2006; Watanabe et al. 2008, 2009). However, deletion of 11 amino acids from the C-terminus of the cytoplasmic tail attenuated the resultant virus in mice, but not in cell culture (Hatta et al. 2011; Watanabe et al. 2008). An H5N1 vaccine candidate encoding this truncated M2 and an avirulent-type HA cleavage site (see Sect. 1.7.2.1) protected mice against challenge with lethal doses of homologous and heterologous H5N1 viruses (Watanabe et al. 2008). A virus lacking the transmembrane and cytoplasmic domains of M2 grew as efficiently as wild-type virus in a cell line expressing M2 and protected mice against challenge with a lethal dose of wild-type virus (Watanabe et al. 2009).

The temperature-sensitive (*ts*) phenotype of the A/Ann Arbor/6/60 (H2N2) master donor virus has been mapped to five mutations in the viral replication complex (PB2-N265S, PB1-K391E, -E581G, -A661T, and NP-D34G) (Jin et al. 2003). Transfer of these PB2 and PB1 mutations to an avian H9N2 virus resulted in insufficient attenuation in chickens (Song et al. 2007); however, an additional mutation in PB1 (an HA-tag epitope at the C-terminus of PB1) resulted in further attenuation (Hickman et al. 2008; Song et al. 2007). These changes were also introduced into turkey and swine viruses that share the polymerase genes with the pandemic 2009 H1N1 virus (Pena et al. 2011; Solorzano et al. 2010), resulting in attenuated replication at elevated temperatures of 39 and 41 °C. An LAIV candidate possessing the pandemic 2009 H1N1 HA and NA genes and pandemic 2009-like internal genes with the attenuating mutations in PB2 and PB1 was highly attenuated in mice and pigs but protected animals against challenge with a lethal dose of pandemic 2009 H1N1 virus (Pena et al. 2011). In another study, six mutations in the PB2 protein of a human H3N2 influenza virus (PB2-E65G, -P112S, -N265S, -W552F, -W557F, and -W564F) conferred a *ts* phenotype, were genetically stable, attenuated in mice and ferrets, and significantly reduced the replication rates of homologous challenge virus in mice and ferrets (Parkin et al. 1997).

Attempts have also been made to attenuate influenza virus by rearranging the viral genes. In one example, the NEP open reading frame (ORF) was cloned

downstream of the PB1 ORF, separated by a foot-and-mouse-disease virus (FMDV) 2A cis-acting cleavage sequence (Pena et al. 2013). In the original location of the NEP ORF, a foreign gene (such as a second HA gene of a different subtype) could now be inserted (Pena et al. 2013). However, due to the modified PB1 gene, the resulting H9–H5 bivalent virus was significantly affected in its replicative ability, limiting its usefulness for large-scale vaccine virus production (Pena et al. 2013).

1.7.4 Modifications of Viral Genes for High-Growth Backbones

The high replicative ability of a vaccine candidate in its respective growth substrate (embryonated chicken eggs or cultured cells) is important to produce large vaccine quantities in a short period of time. High-growth capability is particularly important in the event of a severe pandemic, when there will be an overwhelming demand for vaccine to the novel virus. Attempts have therefore been made to improve the growth characteristics of the current vaccine backbone (i.e., PR8 virus). One study compared the growth properties of the PR8 Cambridge isolate [termed PR8(Cambridge); one of the isolates used for vaccine production] with another isolate [PR8 (UW)], which was found to replicate more efficiently in embryonated chicken eggs than PR8(Cambridge) when tested with the HA and NA genes of a highly pathogenic H5N1 viruses (Horimoto et al. 2007). Similar findings were made in MDCK cells (Murakami et al. 2008). Further testing showed the highest virus titers for a vaccine virus possessing the H5N1 HA and NA genes; the PB2, PB1, PA, NP, and M genes of PR8(UW) virus; and the NS gene of PR8(Cambridge) virus (Murakami et al. 2008). This effect was mediated by a tyrosine residue at position 360 of PB2 and a glutamic acid residue at position 55 of NS1 (Murakami et al. 2008).

As briefly described earlier, vaccine viruses are typically generated by coinfecting cells with the PR8 virus and the circulating virus to which the vaccine should provide protection, followed by negative selection with antibodies to PR8. Retrospective analyses demonstrated that an appreciable number of past vaccine viruses possessed a 5 + 3 gene constellation in which the HA, NA, and PB1 genes were derived from the wild-type virus, while the remaining five genes originated from PR8 (Cobbin et al. 2013; Fulvini et al. 2011; Ramanunninair et al. 2013). In addition, replacement of PR8-PB1 with wild-type PB1 (derived from the strain recommended for vaccine production) improved the growth of PR8-based vaccine candidates (Rudneva et al. 2007; Wanitchang et al. 2010). Based on these observations, a follow-up study identified five amino acid changes in PR8 PB1 (G180E, S216G, S361R, Q621R, N654S) that increased the replication properties of PR8 and a vaccine candidate possessing the HA and NA genes of a seasonal H3N2 virus (Plant et al. 2012). Similar to the PB1 gene, replacement of the PR8 M gene with that of a wild-type strain affected viral growth properties and improved the HA content of the vaccine candidate (Abt et al. 2011). In another approach, an influenza

B virus was serially passaged in embryonated chicken eggs, resulting in high-growth variants with mutations in the PB2 and NP proteins, in addition to mutations in HA (Lugovtsev et al. 2005).

Collectively, several strategies have been explored to improve the replicative ability of the PR8 vaccine backbone. Systematic studies utilizing reverse genetics will be needed to identify changes in the PR8 and Ann Arbor virus backbones that increase growth properties in embryonated chicken eggs, MDCK cells, and/or Vero cells.

1.7.5 Vectored (Bivalent) Vaccines

Several RNA viruses have been tested as vaccine vectors, including Newcastle disease virus (NDV), parainfluenza virus 5 (PIV5), and Sendai virus (see below). These viruses can infect humans but cause no or only mild disease in infected people. With few exceptions, humans lack antibodies to these viruses, thus triggering strong immune responses upon infection. DNA virus vectors such as vaccinia or adenoviruses are not discussed here because the challenges they face, such as genome integration, differ from those of RNA virus vectors.

NDV is an avian paramyxovirus that can infect poultry populations with high rates of mortality, depending on the virulence of the infecting strain. Lentogenic strains (i.e., those of low virulence in chickens) are used in poultry as live attenuated vaccines against velogenic strains (i.e., those causing high mortality in chickens). Humans exposed to NDV may develop mild conjunctivitis. A reverse genetics system was established for NDV in 1999 (Peeters et al. 1999), which opened the door to exploit NDV as a vaccine vector. Several live NDV vaccine candidates expressing an influenza viral protein have since been tested for their potential use in humans (Cornelissen et al. 2012; DiNapoli et al. 2007, 2010; Ge et al. 2007; Nakaya et al. 2001). Most of these vaccine candidates express the influenza virus HA protein from an additional open reading frame in the NDV genome. A live attenuated NDV expressing the influenza HA protein of a highly pathogenic H5N1 virus elicited serum IgG and mucosal IgA antibodies in African green monkeys (DiNapoli et al. 2007, 2010) and provided protection against a lethal dose of H5N1 challenge virus (DiNapoli et al. 2010). Numerous studies have also tested various influenza HA-vectored NDV vaccines (using different NDV strains and inserting HA at different positions in the NDV genome) as poultry vaccines to influenza and have found these vaccines overall safe and efficacious (Cornelissen et al. 2012; Ferreira et al. 2012; Ge et al. 2010; Lardinois et al. 2012; Lee et al. 2013; Nayak et al. 2009; Niqueux et al. 2013; Ramp et al. 2011; Romer-Oberdorfer et al. 2008; Sarfati-Mizrahi et al. 2010; Schroer et al. 2009, 2011; Swayne et al. 2003; Veits et al. 2006, 2008). To assess the potential of these viruses as bivalent vaccines, most studies also tested the protective efficacy against NDV infection and found various levels of protection (Ge et al. 2007, 2010;

Lee et al. 2013; Nayak et al. 2009; Park et al. 2006; Sarfati-Mizrahi et al. 2010; Schroer et al. 2009, 2011; Steel et al. 2008; Swayne et al. 2003; Veits et al. 2006).

In another approach, a recombinant influenza virus expressing an H5 HA served as a vector for the expression of the NDV hemagglutinin/neuraminidase protein (Park et al. 2006). This virus was attenuated in primary normal human bronchial epithelial (NHBE) cells and protected mice against challenge with an H5 HA-expressing virus (Steel et al. 2008). In chickens, this bivalent vaccine candidate elicited high levels of protection against NDV and H5N1 virus (Steel et al. 2008).

PIV5 (formerly known as simian virus 5, SV5) is a paramyxovirus that infects many different cell types; replicates efficiently in cultured cells, including Vero cells; infects humans but does not cause disease in them; and can be genetically modified (Murphy and Parks 1997) to express additional genes. Different PIV5 constructs have been tested that express influenza virus H3N2 HA (Tompkins et al. 2007) or H5N1 HA (Li et al. 2013a, c; Mooney et al. 2013) or the influenza virus NP (Li et al. 2013b) from different positions of the PIV5 genome. PIV5 vectors expressing HA triggered serum and mucosal antibody responses, as well as T-cell responses to HA in mice, and protected against (lethal) challenge with influenza viruses (Li et al. 2013a, c; Mooney et al. 2013; Tompkins et al. 2007). PIV5 vaccine candidates expressing NP elicited humoral and T-cell responses to this protein; in addition, they provided partial protection against H5N1 challenge virus and complete protection against challenge with an H1N1 influenza virus (Li et al. 2013b).

A bivalent vaccine was also designed to protect against human parainfluenza and influenza virus (Maeda et al. 2005). In this approach, an influenza virus was modified to express the ectodomain of the hemagglutinin/neuraminidase protein of human parainfluenza virus instead of the influenza virus neuraminidase protein (Maeda et al. 2005). Such a virus can be efficiently amplified in eggs but is attenuated in mice. More importantly, vaccinated mice developed antibodies and were protected against challenge with both human parainfluenza and influenza viruses (Maeda et al. 2005).

Sendai virus, a member of the family *Paramyxoviridae*, infects mice, rats, guinea pigs, and hamsters. An attenuated live Sendai virus expressing the HA protein of PR8 from an additional open reading frame induced influenza virus-specific IgG and IgA antibodies in mice and provided immunity against lethal challenge with PR8 virus (Le et al. 2011).

Recently, bivalent influenza vaccine candidates have been generated that express HA proteins of two different subtypes. In one study, the overlapping open reading frames of the M1 and M2 genes of an influenza H9N2 virus were separated into two gene segments, and the extracellular domain of M2 was substituted with the HA1 coding region of PR8 HA (Wu et al. 2010). The resulting nine-segment virus (expressing the PR8 HA head domain and the transmembrane and intracellular domains of M2 from the additional viral RNA) was genetically stable, elicited antibodies to the H9 and H1 HAs, and protected against challenges with H9N2 and H1N1 viruses (Wu et al. 2010). Another study replaced the PB2 open reading frame with a "foreign" H1 or H5 HA, so that two different HAs were

expressed by the virus (Uraki et al. 2013). This virus lacks PB2 but can be propagated efficiently in cells expressing this protein (see Sect. 1.7.3). In mice, this bivalent virus provided protection against the vector virus (PR8) and against the pandemic 2009 H1N1 or highly pathogenic H5N1 viruses, respectively, from which the additional HAs were derived (Uraki et al. 2013).

1.8 Conclusion

Reverse genetics has had a substantial impact on influenza virus vaccine generation and development. It is now used to generate annual influenza vaccines, has allowed the development of vaccines to highly pathogenic influenza viruses, and is extensively used to develop novel, improved influenza vaccines.

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Chapter 2
Viral-Vectored Vaccines to Control Pathogenic Filoviruses

Chad E. Mire and Thomas W. Geisbert

Abstract For more than 35 years the filoviruses, Marburg virus and Ebola virus, have caused sporadic outbreaks of hemorrhagic fever that result in severe and often fatal disease in humans and nonhuman primates. Pathogenic Marburg and Ebola viruses are endemic in resource-poor regions in Central Africa and are also of concern as they have the potential for deliberate misuse. Although no vaccines or antiviral drugs for filoviruses are currently available for human use, remarkable progress has been made in developing candidate preventive vaccines against Marburg and Ebola viruses in nonhuman primate models. Most of these vaccines are based on viral vectors including recombinant adenoviruses, alphaviruses, paramyxoviruses, and rhabdoviruses. Because of the remote geographic locations of most filovirus outbreaks, a single-injection vaccine is an important goal in vaccine development. Among the prospective viral-vectored vaccines that have demonstrated efficacy in nonhuman primate models of filoviral hemorrhagic fever, two candidates, one based on a replication-defective adenovirus serotype 5 and the other on a recombinant vesicular stomatitis virus (rVSV), were shown to confer complete protection to nonhuman primates when administered as a single injection. Notably, the rVSV-based vaccines have also shown utility when used as postexposure treatments for filovirus infections.

2.1 Introduction

Marburg virus (MARV) and Ebola virus (EBOV), the causative agents of Marburg and Ebola hemorrhagic fever (HF), comprise the family *Filoviridae* (Feldmann et al. 2013). The taxonomy of filoviruses has become complicated and controversial. For the purposes of this chapter, the most commonly used terms in the

C.E. Mire • T.W. Geisbert (✉)
Galveston National Laboratory, University of Texas Medical Branch, 301 University Blvd., Galveston, TX 77550-0610, USA

Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX, USA
e-mail: twgeisbe@utmb.edu

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published literature will be used to best ensure clarity. The MARV genus contains two lineages: one represented by a number of strains including Angola, Ci67, and Popp and a second lineage represented by the Ravn strain. The EBOV genus is comprised of five distinct species: (1) *Sudan ebolavirus* (SEBOV), (2) *Zaire ebolavirus* (ZEBOV), (3) *Ivory Coast ebolavirus* (ICEBOV) (also referred to as *Cote d'Ivoire ebolavirus or Tai Forest ebolavirus*), (4) *Bundibugyo ebolavirus* (BEBOV), and (5) *Reston ebolavirus* (REBOV) (Feldmann et al. 2013). MARV, ZEBOV, SEBOV, and BEBOV are important human pathogens with case fatality rates frequently ranging up to 90 % for MARV and ZEBOV, around 50–55 % for SEBOV, and 40–48 % for BEBOV [reviewed in Feldmann et al. (2013)]. ICEBOV caused mortality in chimpanzees and a severe nonlethal human infection in a single case in the Republic of Cote d'Ivoire in 1994 (Le Guenno et al. 1995). REBOV is highly lethal for cynomolgus macaques but has not been associated with disease in humans (Feldmann et al. 2013). An outbreak of REBOV was reported in 2008 in pigs in the Philippines; however, it is unclear whether the disease observed in the pigs was caused by REBOV or other agents shown to be coinfecting the animals, especially porcine reproductive and respiratory syndrome virus (Barrette et al. 2009).

Filoviruses are filamentous enveloped non-segmented negative-sense RNA viruses with genomes approximately 19 kb in length. These viruses encode seven gene products: the nucleoprotein (NP), virion protein (VP)35, VP40, glycoprotein (GP), VP30, VP24, and polymerase (L). In addition, the EBOV species express two additional nonstructural proteins from the GP gene referred to as soluble (s)GP and small soluble (ss)GP [reviewed in Feldmann et al. (2013)].

Currently, there are no FDA-approved vaccines or postexposure treatments available for preventing or managing EBOV or MARV infections; however, there are at least seven different vaccine systems that have shown promise in completely protecting nonhuman primates (NHPs) against EBOV and four of these have also been shown to protect macaques against MARV infection (Hevey et al. 1998; Sullivan et al. 2000, 2003, 2006, 2011; Jones et al. 2005; Daddario-DiCaprio et al. 2006a; Bukreyev et al. 2007; Warfield et al. 2007; Geisbert et al. 2008a, 2009, 2010a, 2011; Swenson et al. 2008a, b; Qiu et al. 2009; Pratt et al. 2010; Hensley et al. 2010, 2013; Falzarano et al. 2011; Blaney et al. 2013; Marzi et al. 2013; Richardson et al. 2013; Mire et al. 2013). A preventive vaccine would be important for several populations: (1) the general population during filovirus outbreaks in endemic areas in sub-Saharan Africa or related to imported cases of filovirus infection in humans or NHPs, (2) healthcare workers and family members involved in patient care and management in endemic regions, (3) personnel involved in outbreak response missions, (4) laboratory workers conducting research on filoviruses, and (5) military and other service personnel susceptible to the use of filoviruses as biological weapons.

The requirements for a filovirus vaccine may vary based on the diversity of the affected populations. While multidose vaccine regimens would be feasible for laboratory and healthcare workers and some military personnel in stable settings with defined risk, an outbreak setting or a case of deliberate release would require

rapidly conferred protection with a single administration. The durability of protection required by each group may also vary. Laboratory or healthcare workers and military personnel rotating through high-risk situations for fixed periods may not require an extended duration of protection, perhaps as short as a year, while long-term protective efficacy is desirable for those with more chronic exposure. The ideal vaccine meeting all needs would confer long-term protection with little or no filovirus viremia against SEBOV, ZEBOV, BEBOV, and the diverse strains of MARV with a single inoculation.

2.2 Animal Models

Guinea pigs, mice, and hamsters have been used as animal models of filoviral HF (Bechtelsheimer et al. 1971; Zlotnik 1971; Ryabchikova et al. 1996; Bray et al. 1998, 2001; Connolly et al. 1999; Geisbert et al. 2002; Warfield et al. 2009; Ebihara et al. 2013). However, filovirus isolates derived from humans or NHPs do not typically produce severe disease in rodents upon initial exposure. Lethal infection requires serial adaptation with up eight or more passes in rodents in some cases. Guinea pigs and mice have served well as early screens for evaluating antiviral drugs and candidate vaccines, with genetically engineered mice providing a platform for dissecting out specific host-pathogen interactions. However, the disease pathogenesis seen in rodent models is far less faithful in portraying the human condition than disease observed in NHPs (Bray et al. 2001; Geisbert et al. 2002). Some examples include the following: the coagulation disorders that are hallmark features of disease in filovirus-infected humans and NHPs are not present in filovirus-infected mice or guinea pigs (Bray et al. 2001; Geisbert et al. 2002), and while the bystander death of large numbers of uninfected lymphocytes due to apoptosis has been reported in filovirus-infected humans (Baize et al. 1999), NHPs (Geisbert et al. 2000), and mice (Bradfute et al. 2007), the morphology and process of lymphocyte apoptosis in primates and mice are not similar (Bradfute et al. 2007). A recently described hamster model of ZEBOV infection showed more similarity with primate disease than mice or guinea pigs (Ebihara et al. 2013), and further studies need to be conducted to fully assess the utility of this model. As data derived from studies using rodents may not correlate with human disease and as it is uncertain whether studies performed in rodents would be suitable for supporting applications for licensure of filovirus vaccines, this review focuses on vaccine studies performed in NHPs.

2.3 Viral-Vectored Vaccines

Recent efforts to develop vaccines for the filoviral HFs have focused on the use of various recombinant vectors expressing filovirus proteins to induce protective immunity (Tables 2.1, 2.2, 2.3, 2.4, 2.5 and 2.6). The delivery systems used for

Table 2.1 Preventive Marburg virus vaccines in nonhuman primates (NHP)

System	Gene product (strain)	Vaccine dose	No. of doses	NHP species	Challenge strain	Survivors/ total	Viremic/ Total	Illness/ Total	References
VEEV replicon	GP (Musoke)	10^7	3	Cynomolgus	Musoke[a]	3/3	0/3	0/3	Hevey et al. (1998)
VEEV replicon	GP (Musoke) + NP (Musoke)	10^7	3	Cynomolgus	Musoke[a]	3/3	0/3	0/3	Hevey et al. (1998)
VEEV replicon	NP (Musoke)	10^7	3	Cynomolgus	Musoke[a]	2/3	3/3	3/3	Hevey et al. (1998)
VEEV replicon	GP (Musoke)	10^7	3	Cynomolgus	Ravn[a]	0/3	NR	3/3	Lundstrom (2003)
VEEV replicon	GP (Musoke) + NP (Musoke)	10^7	3	Cynomolgus	Ravn[a]	0/3	NR	3/3	Lundstrom (2003)
DNA prime	GP (Angola) for both	4 mg	3	Cynomolgus	Angola[a]	4/4	0/4	2/4	Geisbert et al. (2010a)
Ad5 boost		10^{11}	1						
Ad5	GP (Angola)	10^{11}	1	Cynomolgus	Angola[a]	4/4	0/4	0/4	Geisbert et al. (2010a)
Ad5	GP (Z) + NP (Z) + GP (S) + GP (Ci67) + GP (Ravn)	10^{10}	2	Cynomolgus	Musoke[a]	5/5	NR	1/5	Swenson et al. (2008a)
VSV	GP (Musoke)	10^7	1	Cynomolgus	Musoke[a]	4/4	0/4	0/4	Jones et al. (2005)
VSV	GP (Musoke)	10^7	1	Cynomolgus	Musoke[a]	1/1	0/1	0/1	Daddario-DiCaprio et al. (2006a)
VSV	GP (Musoke)	10^7	1	Cynomolgus	Ravn[a]	3/3	0/3	0/3	Daddario-DiCaprio et al. (2006a)
VSV	GP (Musoke)	10^7	1	Cynomolgus	Angola[a]	3/3	0/3	0/3	Daddario-DiCaprio et al. (2006a)

VSV	GP (Musoke)	10^7	1	Cynomolgus	Musoke[b]	4/4	0/3	0/3	Geisbert et al. (2008a)
VSV	GP (Z) + GP (S) + GP (M-Musoke)	10^7	1	Cynomolgus	Musoke[a]	3/3	0/3	0/3	Geisbert et al. (2009)

Ad5 adenovirus serotype 5, *GP* glycoprotein, *NP* nucleoprotein, *NR* not reported, *S Sudan Ebola virus*, *VEEV* Venezuelan equine encephalitis virus, *VSV* vesicular stomatitis virus, *Z Zaire Ebola virus*

[a]Intramuscular

[b]Aerosol

Table 2.2 Preventive adenovirus-based Ebola virus vaccines in nonhuman primates (NHP)

System	Gene product (species)	Vaccine dose	No. of doses	NHP species	Challenge species	Survivors/ total	Viremic/ total	Illness/ total	References
DNA prime	GP (Z) + GP (S) + GP (IC) + NP (Z)	4 mg	3	Cynomolgus	Zaire[a]	4/4	1/4	0/4	Sullivan et al. (2000)
Ad5 boost	GP(Z)	10^{10}	1						
DNA prime	GP (Z) + GP (S)	4 mg	4	Cynomolgus	Bundibugyo[a]	4/4	1/4	1/4	Hensley et al. (2010)
Ad5 boost	GP (Z)	10^{11}	1						
Ad5	GP (Z) + NP (Z)	10^{12}	1	Cynomolgus	Zaire[a]	4/4	0/4	0/4	Sullivan et al. (2003)
Ad5	GP (Z) + NP (Z)	10^{12}	2	Cynomolgus	Zaire[a]	4/4	0/4	0/4	Sullivan et al. (2003)
Ad5	GP (Z) + NP (Z)	10^{12}	1	Cynomolgus	Zaire[a]	4/4	0/4	0/4	Sullivan et al. (2006)
Ad5	GP (Z) + NP (Z)	10^{11}	1	Cynomolgus	Zaire[a]	3/3	0/3	0/3	Sullivan et al. (2006)
Ad5	GP (Z) + NP (Z)	10^{11}	2	Cynomolgus	Zaire[a]	3/3	0/3	0/3	Sullivan et al. (2011)
Ad5	GP (Z) + NP (Z)	10^{10}	1	Cynomolgus	Zaire[a]	6/6	0/6	0/6	Sullivan et al. (2006)
Ad5	GP (Z) + NP (Z)	10^{9}	1	Cynomolgus	Zaire[a]	0/3	3/3	3/3	Sullivan et al. (2006)
Ad5	GP (Z) + GP (S)	10^{10}	2	Cynomolgus	Zaire[a]	1/1	0/1	0/1	Geisbert et al. (2011)
Ad5	GPΔTM (Z) + NP (Z)	10^{12}	1	Cynomolgus	Zaire[a]	2/3	1/3	1/3	Sullivan et al. (2006)
Ad5	GPΔTM (Z) + NP (Z)	10^{11}	1	Cynomolgus	Zaire[a]	1/3	2/3	2/3	Sullivan et al. (2006)
Ad5	GP (Z) E71D + GP (S) E71D + NP (Z)	10^{10}	1	Cynomolgus	Zaire[a]	1/3	2/3	2/3	Sullivan et al. (2006)
Ad5	GP (Z) E71D + GP (S) E71D	10^{10}	1	Cynomolgus	Zaire[a]	3/3	0/3	0/3	Sullivan et al. (2006)
Ad5	GP (Z) E71D + NP (Z)	10^{10}	1	Cynomolgus	Zaire[a]	2/3	1/3	1/3	Sullivan et al. (2006)
Ad5	GP (Z) + NP (Z) + GP (S) + GP (M-Ci67) + GP (M-Ravn)	10^{10}	2	Cynomolgus	Zaire[a]	5/5	NR	0/5	Swenson et al. (2008a), Pratt et al. (2010)

Ad5	GP (Z) + NP (Z) + GP (S) + GP (M-Ci67) + GP (M-Ravn)	10^{10}	2	Cynomolgus	Sudan[a]	5/5	NR	0/5	Pratt et al. (2010)
Ad5	GP (Z) + NP (Z) + GP (S)	10^{10}	1	Cynomolgus	Zaire[b]	3/3	NR	0/3	Pratt et al. (2010)
Ad5	GP (Z) + NP (Z) + GP (S)	10^{10}	1	Cynomolgus	Sudan[b]	2/3	NR	2/3	Pratt et al. (2010)
Ad5	GP (Z) + NP (Z) + GP (S)	10^{10}	2	Cynomolgus	Sudan[b]	3/3	0/3	1/3	Pratt et al. (2010)
Ad5/Ad-IFNα	GP (Z)	10^{10}	1	Cynomolgus	Zaire[a]	3/3	NR	0/3	Richardson et al. (2013)
Ad5/Ad-IFNα	GP (Z) (i.n. + i.t.)	10^{10}	1	Cynomolgus	Zaire[a]	2/3	NR	3/3	Richardson et al. (2013)
Ad26	GP (Z) + GP (S)	10^{12}	1	Cynomolgus	Zaire[a]	3/4	1/4	1/4	Geisbert et al. (2011)
Ad35	GP (Z)	10^{10}	1	Cynomolgus	Zaire[a]	1/6	5/6	6/6	Geisbert et al. (2011)
Ad35	GP (Z)	10^{11}	1	Cynomolgus	Zaire[a]	0/3	3/3	3/3	Geisbert et al. (2011)
Ad26/Ad35	GP (Z) + GP (S)	10^{11}	2	Cynomolgus	Zaire[a]	4/4	0/4	0/4	Geisbert et al. (2011)

Ad5 adenovirus serotype 5, *E71D* substitution of aspartic for glutamic acid at position 71 of ZEBOV GP, *GP* glycoprotein, *GPΔTM* recombinant GP lacking the transmembrane anchor region, *IC Ivory Coast Ebola virus*, *NP* nucleoprotein, *NR* not reported, *S Sudan Ebola virus*, *Z Zaire Ebola virus*

[a]Intramuscular

[b]Aerosol

Table 2.3 Preventive adenovirus-based Ebola virus vaccines in adenovirus-immune nonhuman primates (NHP)

System	Gene product (species)	Vaccine dose	Vaccine route	NHP species	Challenge species	Survivors/ total	Viremic/ total	Illness/ total	References
Ad5	GP (Z)	10^{10}	i.m.	Cynomolgus	Zaire[a]	0/3	3/3	3/3	Geisbert et al. (2011)
Ad26	GP (Z) + GP (S)	10^{10}	i.m.	Cynomolgus	Zaire[a]	0/4	4/4	4/4	Geisbert et al. (2011)
Ad26	GP (Z) + GP (S)	10^{11}	i.m.	Cynomolgus	Zaire[a]	2/4	2/4	2/4	Geisbert et al. (2011)
Ad35	GP (Z)	10^{10}	i.m.	Cynomolgus	Zaire[a]	1/3	2/3	2/3	Geisbert et al. (2011)
Ad5/Ad-IFNα	GP (Z)	10^{10}	i.m.	Cynomolgus	Zaire[a]	0/3	NR	3/3	Richardson et al. (2013)
Ad5/Ad-IFNα	GP (Z)	10^{10}	i.n./i.t.	Cynomolgus	Zaire[a]	3/4	NR	4/4	Richardson et al. (2013)

Ad5 adenovirus serotype 5, *Ad26* adenovirus serotype 26, *Ad35* adenovirus serotype 35, *NR* not reported, *S Sudan Ebola virus*, *Z Zaire Ebola virus*
[a]Intramuscular

Table 2.4 Preventive vesicular stomatitis virus-based Ebola virus vaccines in nonhuman primates (NHP)

System	Gene product (species)	Vaccine dose	No. of doses	NHP species	Challenge species	Survivors/ total	Viremic/ total	Illness/ total	References
VSV	GP (Z)	10^7	1	Cynomolgus	Zaire[a]	4/4	0/4	0/4	Jones et al. (2005)
VSV	GP (Z)	10^7	1	Cynomolgus	Zaire[a]	4/4	0/4	0/4	Marzi et al. (2013)
VSV	GP (Z)	10^7	1	Cynomolgus	Zaire[b]	3/3	0/3	0/3	Geisbert et al. (2008a)
VSV	GP (Z) + GP (S) + GP (M-Musoke)	10^7	1	Cynomolgus	Zaire[a]	3/3	0/3	0/3	Geisbert et al. (2009)
VSV	GP (Z) + GP (S) + GP (M-Musoke)	10^7	1	Cynomolgus	Sudan[a]	2/2	0/2	0/2	Geisbert et al. (2009)
VSV	GP (Z) + GP (S) + GP (M-Musoke)	10^7	1	Cynomolgus	Ivory Coast[a]	3/3	0/3	0/3	Geisbert et al. (2009)
VSV	GP (Z)	10^7	1	Cynomolgus	Sudan[a]	0/1	1/1	1/1	Geisbert et al. (2009)
VSV	GP (Z) + GP (S) + GP (M-Musoke)	10^7	2	Rhesus	Sudan[a]	3/3	0/3	0/3	Geisbert et al. (2009)
VSV	GP (Z) − oral	10^7	1	Cynomolgus	Zaire[a]	4/4	NR	0/4	Qiu et al. (2009)
VSV	GP (Z) − IN	10^7	1	Cynomolgus	Zaire[a]	4/4	NR	0/4	Qiu et al. (2009)
VSV	GP (IC)	10^7	1	Cynomolgus	Bundibugyo[a]	1/3	3/3	3/3	Falzarano et al. (2011)
VSV	GP (Z)	10^7	1	Cynomolgus	Bundibugyo[a]	3/4	4/4	4/4	Falzarano et al. (2011)
VSV	GP (B)	10^7	1	Cynomolgus	Bundibugyo[a]	3/3	0/3	0/3	Mire et al. (2013)

(continued)

Table 2.4 (continued)

System	Gene product (species)	Vaccine dose	No. of doses	NHP species	Challenge species	Survivors/ total	Viremic/ total	Illness/ total	References
VSV	GP (Z) + GP (S)	10^7	1	Cynomolgus	Bundibugyo[a]	1/3	3/3	3/3	Mire et al. (2013)
VSV	GP (Z) + GP (S)	10^7	2	Cynomolgus	Bundibugyo[a]	3/3	0/3	0/3	Mire et al. (2013)

GP glycoprotein, *B Bundibugyo Ebola virus*, *IC Ivory Coast Ebola virus*, *NR* not reported, *S Sudan Ebola virus*, *VSV* vesicular stomatitis virus, *Z Zaire Ebola virus*

[a]Intramuscular

[b]Aerosol

Table 2.5 Other preventive Ebola virus vaccines in nonhuman primates (NHP)

System	Gene product (species)	Vaccine dose	No. of doses	NHP species	Challenge species	Survivors/ total	Viremic/ total	Illness/ total	References
Vaccinia	GP (Z)	10^7	3	Cynomolgus	Zaire[a]	0/3	3/3	3/3	Geisbert et al. (2002)
VEEV replicon	GP (Z)	10^7	3	Cynomolgus	Zaire[a]	0/3	3/3	3/3	Geisbert et al. (2002)
VEEV replicon	NP (Z)	10^7	3	Cynomolgus	Zaire[a]	0/3	3/3	3/3	Geisbert et al. (2002)
VEEV replicon	GP (Z) + NP (Z)	10^7	3	Cynomolgus	Zaire[a]	0/3	3/3	3/3	Geisbert et al. (2002)
VEEV replicon	GP (S)	10^{10}	1	Cynomolgus	Sudan[a]	6/6	0/6	6/6	Herbert et al. (2013)
VEEV replicon	GP (S) + GP (Z)	10^{10}	1	Cynomolgus	Sudan[a]	3/3	0/3	0/3	Herbert et al. (2013)
VEEV replicon	GP (S) + GP (Z)	10^{10}	1	Cynomolgus	Zaire[a]	3/3	0/3	0/3	Herbert et al. (2013)
VEEV replicon	GP (S)	10^{10}	1	Cynomolgus	Sudan[b]	0/3	2/3	3/3	Herbert et al. (2013)
VEEV replicon	GP (S)	10^{10}	2	Cynomolgus	Sudan[b]	3/3	1/3	2/3	Herbert et al. (2013)
HPIV3	GP (Z)	10^6	1	Rhesus	Zaire[c]	4/4	0/4	1/4	Bukreyev et al. (2007)
HPIV3	GP (Z)	10^7	1	Rhesus	Zaire[c]	2/3	2/3	2/3	Bukreyev et al. (2007)
HPIV3	GP (Z) + NP (Z)	10^6	1	Rhesus	Zaire[c]	1/2	1/2	2/2	Bukreyev et al. (2007)
HPIV3	GP (Z)	10^7	2	Rhesus	Zaire[c]	3/3	0/3	0/3	Bukreyev et al. (2007)

(continued)

Table 2.5 (continued)

System	Gene product (species)	Vaccine dose	No. of doses	NHP species	Challenge species	Survivors/ total	Viremic/ total	Illness/ total	References
RABV-RC	GP (Z)	10^7	1	Rhesus	Zaire[a]	4/4	1/4	4/4	Blaney et al. (2013)
RABV-RD	GP (Z)	10^7	1	Rhesus	Zaire[a]	2/4	3/4	4/4	Blaney et al. (2013)

GP glycoprotein, *NP* nucleoprotein, *S Sudan Ebola virus*, *RABV-RC* replication-competent rabies virus, *RABV-RD* replication-defective rabies virus, *VEEV* Venezuelan equine encephalitis virus, *Z Zaire Ebola virus*

[a]Intramuscular

[b]Aerosol

[c]Intraperitoneal

Table 2.6 Postexposure filovirus vaccines in nonhuman primates (NHP)

System	Gene product (species or strain)	Vaccine dose	No. of doses	Time postexposure	NHP species	Challenge species or strain	Survivors/ total	Viremic/ total	Illness/ total	References
VSV	GP (MARV-Musoke)	10^7	1	20–30 min	Rhesus	MARV-Musoke	5/5	0/5	0/5	Daddario-DiCaprio et al. (2006b)
VSV	GP (MARV-Musoke)	10^7	1	1 day	Rhesus	MARV-Musoke	5/6	1/6	4/6	Geisbert et al. (2010b)
VSV	GP (MARV-Musoke)	10^7	1	2 days	Rhesus	MARV-Musoke	2/6	5/6	6/6	Geisbert et al. (2010b)
VSV	GP (Z)	10^7	1	20–30 min	Rhesus	ZEBOV	4/8	8/8	8/8	Feldmann et al. (2007)
VSV	GP (S)	10^7	1	20–30 min	Rhesus	SEBOV	4/4	2/4	4/4	Geisbert et al. (2008b)

GP glycoprotein, *S Sudan Ebola virus*, *VSV* vesicular stomatitis virus, *Z Zaire Ebola virus*

these purposes include vaccinia viruses, Venezuelan equine encephalitis virus (VEEV) replicons, adenoviruses, rhabdoviruses vesicular stomatitis virus (VSV) and rabies virus (RABV), and human parainfluenza virus type 3 (HPIV3).

2.3.1 Recombinant Vaccinia Viruses

Vaccinia virus has been the most extensively studied live recombinant vaccine vector [reviewed in Jacobs et al. (2009)]. While recombinant vaccinia viruses have shown utility as vaccine vectors against a number of infectious agents, there have been very few studies which have evaluated this platform against filovirus infection. When this platform was evaluated in the cynomolgus macaque ZEBOV model, the recombinant vaccinia viruses expressing the ZEBOV GP were unable to prolong survival or protect cynomolgus monkeys from lethal ZEBOV infection (Geisbert et al. 2002) (Table 2.5).

2.3.2 Venezuelan Equine Encephalitis Virus Replicons

Alphaviruses have a broad host range and replicate in multiple vertebrate and invertebrate cells. The alphavirus genome contains a single-stranded, positive-sense RNA divided into two open reading frames, one encoding the nonstructural proteins responsible for transcription and replication and the second encoding the structural proteins, which are responsible for encapsidating the viral RNA and final assembly into enveloped virions. Alphaviruses can be employed as vaccine vectors by cloning the gene of interest in place of the alphavirus structural genes. These vectors are commonly called "replicons" and have the ability to replicate but do not make virus particles in the absence of the alphaviral structural proteins. Therefore, alphavirus replicons are single-cycle, non-replicating vectors that cannot spread from cell to cell. Three different expression vectors have been constructed based on alphavirus replicons, including VEEV and Semliki Forest and Sindbis viruses (Rayner et al. 2002; Schlesinger 2001; Lundstrom 2003).

VEEV replicons expressing MARV-Musoke strain (MARV-Musoke) GP either alone or in combination with NP were used to vaccinate cynomolgus macaques against MARV (Hevey et al. 1998) (Table 2.1). The experiment consisted of a regimen of three VEEV replicon injections 28 days apart followed by a high-dose i.m. MARV challenge 35 days after the final immunization. Animals vaccinated with either combination were completely protected against a homologous MARV challenge. NP alone protected against death but did not prevent disease in two of three monkeys, and all three animals became viremic. A similar strategy did not protect against challenge with the heterologous MARV-Ravn strain (MARV-Ravn) (Hevey et al. 2001a), which raises the question about the degree of cross-protection of candidate vaccines for the diverse strains of MARV using vaccines expressing

the GP and/or NP proteins. For EBOV, results in NHPs have been inconsistent. Vaccination of cynomolgus monkeys with multiple injections of VEEV replicons expressing either ZEBOV GP, NP, or both GP and NP at doses in the 10^7 pfu range failed to protect any animals from a lethal i.m. ZEBOV challenge (Geisbert et al. 2002) (Table 2.5). Subsequent studies have employed higher doses of VEEV replicons (Table 2.5). Specifically, it was recently shown that a single injection in cynomolgus macaques with blend of 10^{10} VEEV replicons expressing the ZEBOV GP and SEBOV GP was able to protect animals from high-dose (1,000 pfu) i.m. challenge with ZEBOV or SEBOV (Herbert et al. 2013). However, the same test conditions do not appear to afford complete protection against SEBOV if the challenge virus is administered by the aerosol route even when the challenge virus dose is reduced 10-fold (Herbert et al. 2013). In this study, a single injection of 10^{10} VEEV replicons expressing the ZEBOV GP and SEBOV GP was unable to protect any cynomolgus monkeys against an aerosol exposure of 100 pfu of SEBOV. Altering the vaccination regimen to two injections was able to provide protection of macaques against death but not clinical illness.

Currently, the VEEV replicon system faces a number of challenges as an ideal vaccine candidate against filovirus infection due to the inconsistency in studies against ZEBOV and inability to provide cross-protection between strains of MARV, along with protection against homologous MARV requiring a series of three injections over 17 weeks. It does appear that increasing the vaccine dose in the EBOV studies improves protection. However, even with the low dose of vaccine used in the MARV studies (10^7 pfu), the NHPs developed VEEV-neutralizing antibodies after two injections (Hevey et al. 2001b) which raises doubts about the possibility of reusing this system even if the problem of cross-protection against diverse MARV strains can be solved.

2.3.3 Adenoviruses

Adenoviruses are attractive vaccine vectors for gene therapy because of their high transduction efficiency, broad tropism, and ability to induce both innate and adaptive immune responses in mammalian hosts. Though there have been setbacks, including the death of a patient in 1999 from adverse effects associated with the administration of adenovirus vectors (Lehrman 1999), the interest in their use as a vaccine has remained high, and efforts have focused on developing vectors that have low or no immunogenic toxicities.

Replication-defective adenoviruses, such as the recombinant adenovirus serotype 5 (rAd5), are the most commonly used platform (Hitt and Gauldie 2000). The common feature of all replication-defective rAd vectors is deletion of the viral E1 region that is essential for the regulation of adenovirus transcription and viral replication. Additionally, the E3 region, which is not essential for production of the rAd vectors, is often deleted. The E4 region can also be deleted to increase

capacity for gene inserts and to reduce host responses in vivo; however, it must be provided in *trans* for production of recombinant virus (Hitt and Gauldie 2000).

In NHPs, the rAd5 platform has shown remarkable success for filoviral HFs (Sullivan et al. 2000, 2003, 2006, 2011; Swenson et al. 2008a; Pratt et al. 2010; Geisbert et al. 2010a; Richardson et al. 2013) (Tables 2.1, 2.2 and 2.3). Notably, a single i.m. injection with a rAd5-based vaccine expressing MARV-Angola GP resulted in complete protection with no signs of clinical illness in cynomolgus macaques after a high-dose (1,000 pfu) i.m. challenge with MARV-Angola 28 days later (Geisbert et al. 2010a). Additionally four NHPs who received the rAd5 MARV-Angola GP vaccine after three injections of MARV-Angola GP DNA in a prime-boost strategy were also completely protected; however, the failure of the DNA vaccine alone to protect against clinical illness suggests that rAd5 MARV was the key component (Geisbert et al. 2010a).

Sullivan and colleagues were the first to successfully protect NHPs from ZEBOV HF using a prime-boost strategy (Sullivan et al. 2000); cynomolgus monkeys were vaccinated three times with DNA expressing the GPs of ZEBOV, SEBOV, and ICEBOV and the NP of ZEBOV with a booster vaccination of a rAd5 vector expressing the ZEBOV GP 3 months later. All four vaccinated animals survived challenge after week 32 of the vaccination regimen when exposed to a low dose (6 pfu) of ZEBOV. The data for this study revealed that humoral immunity and T memory helper cells were strongly associated with protection; while cell-mediated immunity was important, it was not an absolute requirement for protection (Sullivan et al. 2000). Whether the DNA component of this regimen is absolutely needed is not clear, since there have been no reports on its efficacy when used alone, while the rAd5 component used alone is protective; as with MARV, a single injection of rAd5 expressing the ZEBOV GP resulted in complete protection from death and illness of cynomolgus macaques after a high-dose (1,000 pfu) i.m. challenge with homologous ZEBOV 28 days later (Sullivan et al. 2003). A DNA prime rAd5 strategy was also employed to demonstrate vaccine efficacy against the most recently discovered species of EBOV, BEBOV (Hensley et al. 2010). In brief, NHPs were initially vaccinated with DNA expressing ZEBOV GP and SEBOV GP and then boosted at weeks 4, 8, and 14. A little over a year later (week 53), animals were boosted with a rAd5-based vaccine expressing the ZEBOV GP. The animals were then challenged 7 weeks after the rAd5 boost with BEBOV. All four specifically vaccinated macaques survived the challenge, and only one animal showed evidence of clinical illness from the BEBOV exposure. While the study showed protection against BEBOV, the regimen requiring five injections over the period of nearly a year and half is not very practical in either natural or biodefense settings.

While filovirus transmission is not thought to be a major route of infection in nature, the inhalation route is among the most likely portals of entry in the setting of a bioterrorist event. Studies have shown that cynomolgus monkeys vaccinated once with a rAd5 vector expressing ZEBOV NP, ZEBOV GP, and SEBOV GP were completely protected against an aerosol ZEBOV challenge, while there was only partial protection against an aerosol SEBOV challenge (Pratt et al. 2010). However,

increasing the vaccination regimen to two injections over 99 days completely protected against a SEBOV aerosol challenge (Pratt et al. 2010).

Additionally, a two-injection filovirus vaccine platform was described that is based on a rAd5 vector expressing multiple antigens from five different filoviruses (ZEBOV NP, ZEBOV GP, SEBOV GP, MARV-Ci67 GP, MARV-Ravn GP, MARV-Musoke NP, MARV-Musoke GP) (Swenson et al. 2008a). In this study, two groups of cynomolgus monkeys were given an initial i.m. injection of this vaccine and then revaccinated 63 days later. The first group was challenged with MARV-Musoke 42 days after the second vaccination and was protected from lethal disease; this group was then subsequently back-challenged 72 days later with SEBOV. The second group was initially challenged with ZEBOV 43 days after the second vaccination with each animal surviving challenge and then back-challenged 69 days later with MARV-Ci67. All animals in these studies survived the back challenges as well. This platform using the same vaccination strategy has also showed protection against an initial SEBOV challenge (Pratt et al. 2010).

Based on the success of the rAd5 filovirus vaccine platform in NHPs, a phase I clinical trial was conducted using a rAd5 vaccine encoding the ZEBOV and SEBOV GPs. The study consisting of 31 volunteers showed that the vaccine was safe and that subjects developed antigen-specific cellular and humoral immune responses (Ledgerwood et al. 2010). While this study is encouraging, the high prevalence of preexisting immunity to adenoviruses in the human population may substantially limit the immunogenicity and clinical utility of the rAd5-based vaccines. The prevalence of anti-adenovirus antibody is up to 60 % in the general human population and up to 85 % in Africa, where filovirus vaccines are most needed (Schulick et al. 1997; Piedra et al. 1998). Additionally, Merck discontinued its HIV vaccine program based on rAd5 as it was reported that the vaccine appeared to increase the rate of HIV infection in individuals with prior immunity against the adenovirus vector used in the vaccine (Cohen 2007; Sekaly 2008).

Initial attempts to improve adenovirus-based vaccines against filoviruses focused on employing different adenoviruses with lower seroprevalence. However, these initial studies were not very successful (Geisbert et al. 2011). Vaccination of cynomolgus monkeys with recombinant adenovirus serotype 35 (rAd35) expressing the ZEBOV GP failed to completely protect animals against a lethal ZEBOV challenge. Similarly, vaccination of cynomolgus macaques with either recombinant adenovirus serotype 26 (rAd26) or a modified adenovirus in which only the seven short hexon hypervariable regions of Ad5 were exchanged from human adenovirus serotype 48 (each expressing the ZEBOV GP) failed to protect animals against a lethal ZEBOV challenge. A strategy to prime with rAd26 vectors expressing ZEBOV GP and boost with rAd35 vectors expressing ZEBOV GP was able to protect NHPs against a lethal ZEBOV challenge. However, to date, rAd5 is the only adenovirus serotype capable of inducing a protective response against EBOV as a single-injection vaccine. While the data associated with the filovirus rAd5 vaccine platform is impressive, a study revealed that when macaques are pre-immunized against Ad5 and then vaccinated with the rAd5 vaccine expressing the ZEBOV GP,

the NHPs are not protected against disease or death after challenge with ZEBOV (Geisbert et al. 2011) (Table 2.3).

The most recent studies to improve rAd5-based filovirus vaccines have utilized an additional boost with an adenovirus vector expressing interferon (IFN)-α as well as changing the vaccination route (Richardson et al. 2013) (Table 2.3). While it appears that incorporation of a boost vaccination with the adenovirus vector expressing IFN-α did not have any benefit regarding overcoming preexisting immunity, the administration of the rAd5-based ZEBOV GP vaccine by a combined intranasal and intratracheal route did improve survival of NHPs against homologous ZEBOV challenge when compared with vaccination by i.m. injection.

2.3.4 Rhabdovirus-Based Vaccines

2.3.4.1 Vesicular Stomatitis Virus

Over the last decade, Rose and colleagues have pioneered the use of VSV, the prototypic member of the *Rhabdoviridae* family, as an expression and vaccine vector (Roberts et al. 1999, 2000, 2001). VSV is very suitable as a vaccine expression vector, as it grows to high titer ($>10^9$ pfu/ml) in vitro, can be propagated in almost all mammalian cells, can induce strong humoral as well as cellular responses in vivo, and has the capacity to elicit both mucosal and systemic immunity (Rose et al. 2001; Zinkernagel et al. 1978a, b; Fehr et al. 1996). Furthermore, preexisting immunity to VSV is rare, and infection is not typically associated with serious disease, although VSV-associated encephalitis has been reported (Reif et al. 1987; Gaidamovich et al. 1966; Quiroz et al. 1988).

A recombinant VSV (rVSV)-based system has proven to be among the most successful vaccine platforms for MARV to date and has been proven equally effective against EBOV (Tables 2.1 and 2.4). A single i.m. vaccination of cynomolgus monkeys with a rVSV-MARV-Musoke GP vector elicited complete protection against a high-dose (1,000 pfu) i.m. challenge of homologous MARV given 28 days later (Jones et al. 2005). These animals were also protected when rechallenged 113 days later. Additionally, the MARV-Musoke vaccine proved protective against the most genetically disparate MARV strain, Ravn, and what appears to be the most virulent strain, Angola, suggesting that it may confer cross-protection against all the diverse strains of MARV (Daddario-DiCaprio et al. 2006a). Studies have also shown that a single vaccination of cynomolgus monkeys with rVSV-MARV-Musoke GP completely protected animals against a homologous aerosol challenge of MARV given 28 days later (Geisbert et al. 2008a).

For EBOV, a single i.m. vaccination of cynomolgus monkeys with a rVSV vector expressing only ZEBOV GP also elicited complete protection against a high-dose (1,000 pfu) i.m. challenge of homologous ZEBOV given 28 days later (Jones et al. 2005). However, cross-protection against another species of EBOV,

SEBOV, was not achieved as SEBOV challenge of the survivors resulted in fatal disease (Jones et al. 2005). A single i.m. vaccination of cynomolgus monkeys with rVSV-ZEBOV-GP was also able to completely protect animals against a homologous aerosol challenge of ZEBOV given 28 days later (Geisbert et al. 2008a). Importantly, protection can be conferred by these vaccines via various routes. Immunization of NHPs with the rVSV-ZEBOV-GP vector by either the oral or intranasal route resulted in complete protection of all animals against a high-dose (1,000 pfu) i.m. homologous ZEBOV challenge (Qiu et al. 2009). Recently, the mechanism of rVSV-ZEBOV-GP protection from lethal challenge with ZEBOV was evaluated, and results suggested that antibodies are necessary and correlate with protection of cynomolgus macaques (Marzi et al. 2013).

The ideal filovirus vaccine should be a single-injection vaccine that can protect primates against the various species and/or strains of EBOV and MARV. This is important because endemic areas of filoviruses overlap and since the specific strain or species of filovirus may not be immediately known in the case of a biological weapon attack. With this goal in mind, a study was conducted where cynomolgus monkeys were vaccinated with a multivalent vaccine consisting of equal parts of the rVSV-filovirus-GP vaccine vector for MARV, EBOV, and SEBOV (Geisbert et al. 2009). After 28 days the groups of the animals were challenged with either MARV, ZEBOV, SEBOV, or ICEBOV. Importantly, none of the vaccinated macaques succumbed to a filovirus challenge, showing the utility this platform could have as a single-injection multivalent vaccine.

The BEBOV outbreak in 2007 offered a new challenge to develop a strategy to protect against an emerging species of EBOV using existing vaccines that were available at the time of the outbreak. This strategy was tested in cynomolgus macaques against heterologous challenge with BEBOV. The NHPs in this study were vaccinated with rVSV-ZEBOV-GP or rVSV-ICEBOV-GP separately and challenged with BEBOV 28 days after vaccination. While the rVSV-ICEBOV-GP vector did not provide any additional protection when compared to mock-vaccinated control NHPs in the study (33 % survival), the rVSV-ZEBOV-GP vaccine protected 75 % of animals against lethal infection (Falzarano et al. 2011). Recently, the utility of combining rVSV-SEBOV-GP and rVSV-ZEBOV-GP vectors using either a single-injection blended vaccination approach or in a prime-boost regimen against heterologous BEBOV challenge in cynomolgus macaques was evaluated (Mire et al. 2013). Furthermore, the ability of a single injection of a newly developed homologous rVSV-BEBOV-GP vaccine vector to provide protection against homologous BEBOV challenge was assessed in this study. The rVSV-BEBOV-GP vector protected against homologous challenge with BEBOV. The prime-boost strategy with the rVSV-SEBOV-GP (prime) and rVSV-ZEBOV-GP (boost 14 days post rVSV-SEBOV-GP vaccination) vectors, which were available at the time BEBOV emerged, was capable of providing cross-protection against BEBOV challenge 35 days after prime vaccination. These results were promising and showed that a condensed, prime-boost vaccine regimen of available heterologous rVSV-filovirus-GP vaccines could be considered as a paradigm for controlling newly emerging EBOV species.

In addition to its efficacy as a preventive vaccine, the rVSV vaccine platform has also been used as a postexposure treatment for filovirus infections (Table 2.6). Remarkably, treatment of rhesus macaques with rVSV-MARV-GP shortly after a homologous high-dose MARV challenge resulted in complete protection of all animals from clinical illness and death (Daddario-DiCaprio et al. 2006b). Subsequent studies demonstrated that the rVSV-filovirus-GP vectors for ZEBOV and SEBOV protected 50 and 100 % of rhesus macaques, respectively, when administered as postexposure prophylaxis after high-dose homologous virus challenge (Feldmann et al. 2007; Geisbert et al. 2008b). The rVSV-filovirus-GP vectors were administered 20–30 min after filovirus challenge in these studies. A major question is how long after virus exposure can the rVSV-filovirus-GP vectors be effective? To address this question, rhesus macaques were treated with rVSV-MARV-GP 24 h post-homologous MARV challenge which resulted in protection of five of six monkeys while, remarkably, two of six animals were protected when the vaccine was administered 48 h after infection (Geisbert et al. 2010b).

Replication-competent vaccines, including the rVSV platform, are not without concern when it comes to their safety, especially in immunocompromised persons. However, initial results of various rVSV vectors in NHPs have been promising as no toxicity was seen in rhesus macaques following intranasal inoculation with wild-type VSV, rVSV wild-type, and two rVSV-HIV vectors, although neurovirulence was noted in one of four animals after direct intrathalamic inoculation of rVSV (Johnson et al. 2007). To date, no toxicity has been seen in over 100 NHPs given rVSV-MARV or rVSV-EBOV (Jones et al. 2005; Daddario-DiCaprio et al. 2006a; Geisbert et al. 2008a, 2009; Qiu et al. 2009; Falzarano et al. 2011; Marzi et al. 2013; Mire et al. 2013). Furthermore, there has been no significant vaccine vector shedding detected in these experiments despite immunization doses of up to 10^7 pfu (Jones et al. 2005; Daddario-DiCaprio et al. 2006a; Geisbert et al. 2008a, 2009, 2008c; Qiu et al. 2009; Falzarano et al. 2011; Marzi et al. 2013; Mire et al. 2013) which suggests, along with the natural low transmissibility of VSV (Tesh et al. 1975; Hanson 1952), that spread to persons outside the vaccine target population is unlikely.

To specifically address its safety, the rVSV-ZEBOV-GP vaccine was evaluated in two animal models for the immunocompromised state, NOD-SCID mice (Jones et al. 2007) and SHIV-infected rhesus monkeys (Geisbert et al. 2008c), along with neurovirulence testing of the rVSV-ZEBOV-GP and rVSV-MARV-GP vaccines in cynomolgus macaques by intrathalamic injection (Mire et al. 2012). No evidence of overt illness or neurovirulence was noted in any of the animals. In addition, the rVSV-ZEBOV-GP vector was recently used to treat a laboratory worker after a recent laboratory accident (Gunther et al. 2011). The vector was administered around 40 h after potential ZEBOV exposure. The patient developed fever, headache, and myalgia 12 h after injection which were readily controlled with antipyretics and analgesics. No other adverse effects were reported. Because it is not certain that infection actually occurred, efficacy of the vaccine in this case could not be evaluated. Regarding possible vaccine virus mutation to more virulent variants, some comfort can be taken from noting the case of the live recombinant vaccinia

vaccine for rabies that has been under field investigation in wild animals in the United States, Canada, and Europe since the 1980s and 1990s (Slate et al. 2005) with no evidence of evolution to more pathogenic forms.

2.3.4.2 Rabies Virus

Recently, a RABV bivalent filovirus vaccine platform (RABV/ZEBOV) was tested in NHPs (Blaney et al. 2013) (Table 2.5). Four groups of rhesus macaques were used in this study. Group 1 consisted of three control animals receiving a single injection of the RABV vaccine (BNSP333); group 2 consisted of four animals that received a single injection of the bivalent RABV/ZEBOV vaccine vector (BNSP333-GP); group 3 had four animals that received a single injection of the replication-restricted RABV vector expressing the ZEBOV GP (BNSP ΔG-GP); and group 4 consisted of four animals that received two doses (a prime and boost 28 days later) with a beta-propiolactone inactivated version of the BNSP333-GP vector. The vaccinated NHPs were challenged 70 days after vaccination and followed for up to 28 days post challenge.

The study showed that all animals in groups 2 and 3 were able to generate a humoral immune response to the ZEBOV GP immunogen with the humoral immune response being the major response when compared to the cellular response during the vaccination phase of the study. ZEBOV viremia was detected in all groups to some extent with all animals in group 1, one of four animal in groups 2 and 3, and three of four animals in group 4 all having viremia at day 6 post challenge. Each control in group 1 succumbed to ZEBOV challenge with 50 % succumbing for groups 3 and 4, whereas all the animals in group 2 survived the lethal challenge. These results were interesting considering all animals in groups 2–4 had generated IgG antibodies against the ZEBOV GP with no differences in the avidity of the antibodies between the groups. When the IgG response was further analyzed, it was found that the IgG1 response was important for protection using this vaccine vector suggesting that there is an antibody-dependent cellular cytotoxicity and complement activation mechanism for protection in the NHPs. While these results are promising, this system has yet to be tested for safety in NHPs although neurovirulence studies in mice were encouraging (Papaneri et al. 2012). In addition, this study utilized rhesus macaques for testing in a preventive vaccination setting, and further studies in the more robust cynomolgus model will be needed for better comparison with other filovirus vaccines.

2.3.5 Human Parainfluenza Virus Type 3

Human parainfluenza virus type 3 (HPIV3), a member of the family *Paramyxoviridae*, is a common pediatric respiratory pathogen. Live-attenuated vectors based on HPIV3 are actively being investigated as vaccines for HIPV3

and other pediatric pathogens (Durbin et al. 2000; Karron et al. 2003). Bukreyev and colleagues recently developed HPIV3 a vector expressing the ZEBOV GP and a vector expressing two antigens the ZEBOV GP and NP (Bukreyev et al. 2007) (Table 2.5). Combining an intranasal and intratracheal vaccination in cynomolgus macaques with the ZEBOV GP afforded the best protection against a high-dose (1,000 pfu) intraperitoneal (i.p.) challenge of homologous ZEBOV 28 days post vaccination (Geisbert et al. 2008a). In this study, six of seven HPIV3 ZEBOV GP-vaccinated animals survived challenge, and four of seven animals were protected against clinical illness. When the vaccine regimen using the HPIV3 ZEBOV GP vaccine was expanded to two doses over a 67-day period, the efficacy was improved, with three of three cynomolgus monkeys protected against clinical illness and death.

The HPIV3 system is replication competent which brings up similar safety concerns as the rVSV platform. Additionally, a majority of all adult humans have preexisting immunity to this common childhood pathogen, which presents a potential challenge using this vaccine vector analogous to rAd5. This concern was recently addressed as it was demonstrated that reinfection of NHPs with HPIV3 expressing ZEBOV GP is possible and results in an immune response to ZEBOV GP, indicating that vaccination might be feasible despite preexisting immunity (Bukreyev et al. 2010). Unfortunately, this study did not provide data on protective efficacy which as seen with the RABV/ZEBOV vector antibody response does not always correlate with protection (Blaney et al. 2013).

2.4 Conclusions and Future Directions

While initial progress on developing vaccines against filoviruses was slow, tremendous progress has been made over the last decade. At least four viral-vectored vaccines have shown the ability to protect NHPs against lethal MARV and EBOV challenges, and one of these, rVSV, was even shown to be effective as a postexposure prophylaxis. This progress was so encouraging that efforts were made to move candidate filovirus vaccines into clinical trials. However, there have been four recent events that have caused concern and have dampened enthusiasm for rapid movement toward vaccines suitable for human use. These include: (1) the recent awareness that the Angola strain of MARV is more pathogenic in primates than strains that most vaccines had been tested against; (2) the discovery of the new BEBOV species of EBOV in 2007; (3) studies suggesting that aerosol exposure may be more difficult to protect against than intramuscular injection; and (4) concerns regarding most vaccine studies in NHPs being tested against an attenuated cell culture variant of ZEBOV.

Studies have shown that the Angola strain of MARV causes a higher case fatality rate in humans and more rapid disease course in NHPs than other MARV strains. Early vaccine efficacy studies focused on the Musoke strain of MARV which was derived from a nonfatal human case and causes a more protracted disease in NHPs.

While rAd5- and rVSV-based vaccines have recently been shown to confer protection against this MARV strain, other vaccines have yet to be assessed. A greater concern than MARV-Angola is the newest species of EBOV, BEBOV. Three studies have evaluated vaccines in NHPs against BEBOV. One study using a rVSV-based BEBOV GP single-injection vaccine demonstrated complete protection of NHPs against disease from a homologous BEBOV challenge. However, multivalent vaccines comprised of ZEBOV and/or SEBOV antigens while protecting animals against death were unable to protect all animals from clinical illness. Moreover, no single-injection vaccine containing ZEBOV and/or SEBOV antigens has been able to completely protect NHPs from lethal BEBOV challenge. This complicates vaccine development as single-injection protection may require incorporation of BEBOV antigens further adding to the number of components in a filovirus vaccine.

An ideal filovirus vaccine would protect in a natural setting which includes contact or parenteral routes of exposure and in a biodefense scenario which would likely involve aerosol exposure. While one study using rVSV-based vaccines demonstrated protection of NHPs from homologous ZEBOV or MARV challenge, another study suggests that protection against aerosol exposure may present additional challenges. Specifically, it was recently shown that a VEEV replicon-based vaccine was unable to protect any NHPs against homologous SEBOV infection when administered as a single-injection vaccine. A prime-boost strategy with the same vaccine was able to protect all animals against homologous SEBOV challenge; however, all animals experienced more than mild clinical disease.

Perhaps the most significant hurdle that has recently been encountered in advancing filovirus vaccines for human use involves the recent discovery that ZEBOV seed stocks have acquired mutations in the GP gene by cell culture passage (Volchkova et al. 2011; Kugelman et al. 2012) and these viruses appear to be less pathogenic in NHPs (Geisbert 2013). Specifically, for EBOVs, the GP gene expresses thee different products (the spike GP1,2, the soluble (s)GP, and small soluble (ss)GP) by alternating the use of three overlapping reading frames (ORFs). The ratio of expression of these three proteins is controlled by a stretch of seven (7) consecutive template uridines (7U) known as the RNA editing site. sGP is the primary expression product of the GP gene. However, it has recently been shown that cell culture passage has a tendency to cause the insertion of an additional U residue in the viral genome at the GP editing site (8U) (Volchkova et al. 2011; Kugelman et al. 2012). One of these studies also showed that the majority of ZEBOV seed stocks used to evaluate candidate filovirus vaccines contain higher proportions of the 8U mutation than the wild-type 7U sequence (Kugelman et al. 2012). This mutation may have a very significant impact on virulence as it has a direct effect on the amount of sGP produced by infected cells. For wild-type EBOVs with the 7U phenotype, 80 % of the product of the GP gene is sGP. Cell culture-adapted EBOVs with higher 8U content and lower 7U content will thus produce proportionally lower amounts of sGP. While the function of sGP is unknown, it has speculated to act as a decoy and plays a role in subverting the host immune response (Volchkov et al. 1998; Ito et al. 2001; Mohan et al. 2012).

Interestingly, recent studies have shown that ZEBOV seed stocks with high proportions of 7U at the GP editing site cause a more rapid disease course in cynomolgus macaques than ZEBOV stocks with high proportions of 8U at the GP editing site (C.E. Mire, T.W. Geisbert, unpublished observations). More importantly, recent testing of several candidate vaccines that previously were shown to provide complete protection of NHPs against 8U ZEBOV infection indicated that only one of these vaccines, rVSV, was able to provide complete protection of macaques against a predominantly 7U ZEBOV seed stock (Geisbert 2013). Clearly, more work needs to be conducted to better define the impact of the 8U mutation on virulence and the ability of candidate vaccines to protect against the wild-type 7U EBOV seed stocks.

A thorough understanding of the pathogenesis of filoviruses in relevant animal models is essential not only for further evaluation of the efficacy of existing vaccine candidates but also in light of the "animal rule" enacted by the US FDA in 2002 [reviewed in Roberts and McCune (2008)], which established requirements for the evidence needed to demonstrate effectiveness of new drugs and biological products when human efficacy studies are not ethical or feasible. This rule would most likely be enacted for filoviral HF drugs and vaccines. This rule states that a product can be licensed based on evidence of effectiveness derived from studies in well-characterized animal models and the usual demonstration of biological activity and safety in humans. Thus, the validation of NHPs as accurate and reliable models of human filoviral HF has been and will be critical to the final evaluation and testing of candidate vaccines. Ultimately, no vaccine against filovirus infection will be approved for human use until it can protect NHPs from viremia and clinical illness.

The extent to which laboratory animal models corroborate with findings in humans is very important in the characterization of filovirus pathogenesis. To do this, more effort will need to be directed toward the application of modern immunological and molecular techniques to the study of human filovirus infection during the sporadic outbreaks in Central Africa (Bausch et al. 2008). In particular, a deeper understanding of the correlates of immunity in both humans and animal models of filoviral HF will be paramount to achieving this goal.

Lastly, if FDA approval of a filovirus vaccine should occur, there are still questions about economic incentives for pharmaceutical companies to produce a MARV or EBOV countermeasure. The areas most affected by outbreaks of filoviruses are generally in poor countries, so economic incentives will have to come from other nations or private foundations. However, the concerns of industrialized countries when it comes to protecting the military and others considered susceptible to use of filoviruses as bioweapons are the most likely driving force. The responsibility will then become the international community's to ensure that these vaccines are available to the persons in the most need in endemic areas where pathogenic filoviruses are found in nature.

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Chapter 3
Alphavirus Replicon Vectors for Prophylactic Applications and Cancer Intervention

Peter Pushko and Irina Tretyakova

Abstract Alphavirus replicons represent self-replicating RNA molecules resembling alphaviral genomic RNA, except replicons encode antigen(s) of interest in place of an alphaviral structural polyprotein. Because viral structural genes are missing, replicon RNA cannot initiate replication of an alphavirus. However, due to the presence of intrinsic RNA-dependent RNA polymerase activity, replicons are capable of self-amplification in vitro and in vivo resulting in high levels of expression of antigen of interest. For vaccination or therapeutic purposes, replicons can be delivered in vivo by replicon particles. The latter represent viruslike particle vectors (VLPVs) that encapsidate replicon RNA and deliver it into target cells for antigen expression. The viruslike nature and self-replicating RNA features ensure efficient priming of innate immunity and adjuvant effect, while high-level expression provides antigen for induction of cell-mediated and humoral immune responses. Replicon vectors have been developed from several alphaviruses including Venezuelan equine encephalitis virus (VEEV), Semliki Forest virus (SFV), and Sindbis virus (SINV). Applications of replicon particles included prophylactic and therapeutic vaccines for infectious diseases and cancer, as well as adjuvants for enhancement of immune responses. In several preclinical models including nonhuman primates, alphavirus replicons have shown exceptional promise as safe and effective vaccines and adjuvants. Experimental replicon vaccines included vaccines against influenza, Ebola, Marburg, and Lassa viruses. Bivalent vaccines protecting from both Ebola and Lassa viruses have been also described. Protective effects have been reported for cancer indications after therapeutic vaccination with replicon vaccines expressing tumor-associated antigens. Clinical trials involving alphavirus replicons are underway. In this review, an attempt is made to summarize the state of the art of the alphavirus replicon-based technology for prophylactic and therapeutic applications. The advantages and challenges of the replicon technologies are presented, and the future of this promising platform is discussed.

P. Pushko (✉) • I. Tretyakova
Medigen Inc., Riverside Technology Park, 8420 Gas House Pike, Suite S, Frederick, MD 21701, USA
e-mail: ppushko@medigen-usa.com

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3.1 Introduction

During recent years, several alphavirus-based vectors have been developed as experimental vaccines for infectious diseases and cancer, as well as novel adjuvants. Alphavirus replicons represent self-replicating RNA molecules resembling alphaviral genomic RNA, except replicons encode antigen(s) of interest in place of an alphaviral structural polyprotein. Replicon vectors have been generated from several alphaviruses. Historically, the first alphaviral replicon systems have been derived from Sindbis virus (SINV), Semliki Forest virus (SFV), and Venezuelan equine encephalitis virus (VEEV). Replicon vectors derived from SINV, SFV, and VEEV have been configured as vaccines for human and/or veterinary applications. Search of PubMed database conducted on May 1, 2014 by using two keywords (alphavirus replicons) resulted in retrieving 377 published articles. Search by using three keywords (alphavirus replicon vaccine) resulted in 214 articles. Finally, four keywords search (alphavirus replicon clinical trials) retrieved 16 articles from PubMed database. Thus, alphavirus replicons seem to be actively developed for vaccine applications. Alphavirus replicon vectors have shown exceptional promise in preclinical trials including nonhuman primates (Geisbert et al. 2002; Hevey et al. 1998). Human clinical trials were also conducted (Slovin et al. 2013; Wecker et al. 2012; Bernstein et al. 2009). This review represents an attempt to summarize available information on alphavirus replicon vectors for the development of prophylactic and therapeutic vaccines for infectious diseases and cancer.

In this review, the term "replicon" will be used to indicate alphaviral genome-derived RNA molecule that encodes RNA replicase and is therefore capable of self-amplification (replication). Replicons are generally engineered to express foreign gene of interest in eukaryotic cell lines. Chemical instability of replicon RNA prevents the broad use of "naked" RNA replicons for in vitro and in vivo applications. Therefore, for vaccination or therapeutic purposes, replicons can be encapsidated into viruslike particle vectors (VLPVs), or "replicon particles," by using alphavirus structural proteins supplied in trans. The genetic constructs for expression of alphavirus structural proteins and encapsidation of replicons are named "helpers." Encapsidated replicons are often termed replicon particles, for example, VEEV replicon particles (VRPs) (Schafer et al. 2009; Kamrud et al. 2008). Obviously, the term "VRP" is not applicable to replicon particles that are made from SINV or SFV. Since replicon particles essentially represent VLPs encapsulating replicon RNA, a broader functional term of "VLP vectors" (VLPVs) may be suitable. The VLPVs are capable of delivering replicon RNA into target cells in vitro and in vivo for antigen expression. In this review, we will use terms "replicon particles," VRPs, and "VLPVs" interchangeably to indicate alphavirus-like particles that encapsulate replicon RNA and are capable of delivering replicon RNA to target cells. Replicon particles, or VLPVs, cannot replicate beyond the initially infected target cells because replicons do not encode alphaviral structural proteins. The concept of VLP vectors has been also used in other viruses, such as polyomavirus (Eriksson et al. 2011; Tegerstedt et al. 2005; Chang

et al. 2011). For example, prior research suggested that murine polyomavirus VLPs can be useful vectors for gene therapy and immune therapy and for vaccine applications (Tegerstedt et al. 2005).

3.2 Biology of Alphaviruses

As mentioned earlier, replicons have been derived from several representatives of the *Alphavirus* genus. *Alphavirus* is one of two genera in the viral family Togaviridae. The other Togaviridae genus is *Rubivirus* with rubella virus (Strauss and Strauss 1994). Alphaviruses are classified according to antigenic characteristics, as they have antigenic sites on the capsid and on the envelope glycoproteins. Viruses can be differentiated by serological tests, particularly neutralization assays. Many alphaviruses can cause disease in people and can also be identified by clinical manifestations. Therefore, it is critically important that live alphavirus does not regenerate during preparation of VLPVs, or replicon particles (Pushko et al. 1997). There are approximately 30 alphavirus species capable of infecting invertebrates (mosquito) as well as vertebrates such as humans, rodents, fish, birds, and larger mammals including horses (Strauss and Strauss 1994). Transmission between species and individuals occurs mainly via mosquitoes, which places the alphaviruses into the group of arboviruses—or arthropod-borne viruses. Viruses are maintained in nature by mosquito-vertebrate-mosquito cycles. The mosquito borne pathogenic arboviruses include Venezuelan (VEEV), western (WEEV), and eastern equine encephalitis (EEEV) viruses, chikungunya virus (CHIKV), and others (Strauss and Strauss 1994; Schwartz and Albert 2010). Restricted interactions between viruses, invertebrate vector species, and vertebrate hosts tend to confine the geographic spread of alphaviruses. Occasionally, a virus may escape its usual ecological niche and cause widespread epizootics (VEEV) or urban epidemics (CHIKV). Human infections are seasonal and are usually acquired in endemic areas. For example, recently, a 17-year-old female traveled to Central America and developed clinical symptoms including fever, headaches, and myalgias. Laboratory tests revealed infection with VEEV (Muniz 2012).

Alphaviruses often are medically relevant as they can cause human disease. Clinical disease occurs in either of two general forms, depending upon the virus: one is typified by fever, malaise, headache, and/or symptoms of encephalitis (e.g., EEEV, WEEV, or VEEV viruses), while the other by fever, rash, and arthralgia (e.g., CHIKV, Ross River, Mayaro, and SINV). For example, VEEV, which is endemic in South and Central America, can cause severe neurological disease. In contrast, CHIKV, an etiological agent of chikungunya fever, an emerging global infectious disease, is characterized by severe arthralgia. Infection is transmitted via infected mosquitoes. Various mosquito species are known to be potential vectors for different arboviruses. For example, *Culex* spp. mosquitoes are considered a possible vector for SINV or Ockelbo virus (Werblow et al. 2013). CHIKV is transmitted by mosquitoes of *Aedes* spp. (Chen and Wilson 2012). In the vertebrate

host including humans, transient viremia and dissemination occur as virus is released from cells during lytic infection cycle. Infection with seroconversion in the absence of clinical disease is common, but severe disease can also occur and can be either incapacitating or, in cases of encephalitis, occasionally fatal. Virus is eliminated by the immune system; however, central nervous system pathology or arthritis may persist for weeks. Initial resistance is conferred by unspecific defense mechanisms including interferon. Antibodies play a critical role in recovery and resistance, and T-cell responses are also important (Strauss and Strauss 1994). Long-lasting protection is mostly restricted to the same alphavirus and is dependent on the presence of neutralizing antibodies. Diagnosis is suggested by clinical manifestations and by known risk of exposure to virus. Confirmation is typically by virus isolation and identification, or by a specific rise in IgG antibody, or the presence of IgM antibody. Disease surveillance and virus activity in natural hosts are used to determine if any control measures are needed to reduce populations of vector mosquitoes or to vaccinate hosts, especially horses. There are no FDA-approved human vaccines for alphaviruses (Strauss and Strauss 1994; Tretyakova et al. 2013). However, experimental live attenuated vaccines TC-83 and 181/25 for VEEV and CHIKV, respectively, do exist (Tretyakova et al. 2013). These vaccines are used under the Investigational New Drug (IND) Protocols in individuals at particularly high risk of exposure, such as laboratory or medical workers. Generally, the use of live attenuated strains such as TC-83 for preparation of alphavirus replicon and helper systems can significantly alleviate biosafety concern that pathogenic alphavirus can regenerate during VLPV preparation.

3.3 Genome and Structure of Alphaviruses

For preparation of alphavirus replicon and helper systems, understanding of the alphavirus life cycle (Fig. 3.1), genome, and virion structure is critically important. Alphavirus genome inside the virion is a positive-sense, monopartite, single-stranded RNA genome, approximately 12 kb in length. Genomic RNA is capped and polyadenylated. After introduction into permissive eukaryotic cell, alphaviral genomic RNA serves as mRNA for translation of nonstructural proteins (NSPs) that function as RNA-dependent RNA polymerase, or replicase (Strauss and Strauss 1994), as depicted in Fig. 3.1. The NSPs are encoded in the 5′ two-thirds of the genome (Fig. 3.2a). After NSPs are made in the cells, they direct synthesis of a complementary antisense (−) RNA by using genomic RNA as a template. In turn, the newly synthesized (−) strand RNA serves as a template for the synthesis of progeny genomic (+) strand RNA (Fig. 3.1). This process is mediated by the viral NSP proteins (replicase complex). In addition, the (−) strand RNA serves as a template for synthesis of subgenomic (+) strand RNA, which functions as mRNA for synthesis of structural proteins. The subgenomic RNA covers approximately the 3′ one-third of the genome. In the alphaviruses, subgenomic RNA encodes the structural proteins that interact with genomic RNA and form progeny virions

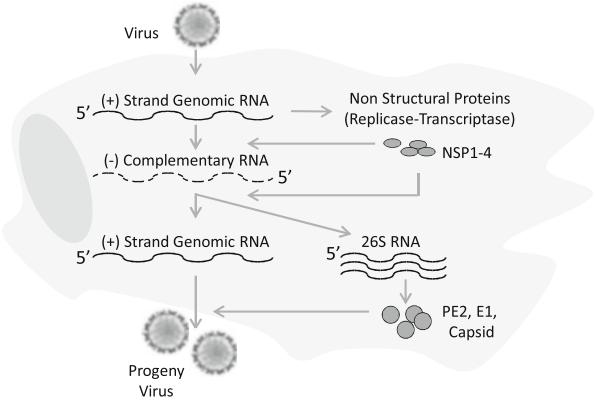

Fig. 3.1 Alphavirus life cycle. Indicated are (+) and (−) polarities of RNA during replication, as well as subgenomic RNA for expression of structural proteins PE2 and E1

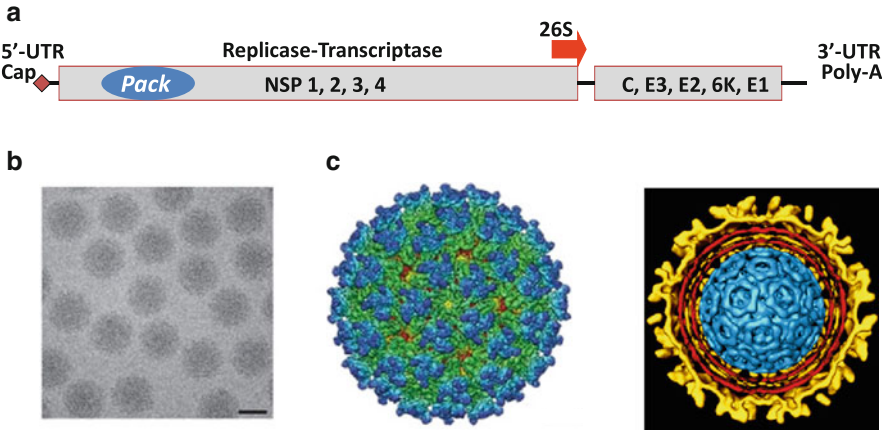

Fig. 3.2 Alphavirus genome and virion particle structure. (**a**) Alphavirus RNA genome. Indicated are nonstructural proteins (NSP), structural proteins (C, E3, E2, 6K, E1), 26S subgenomic promoter, as well as 5′ and 3′ untranslated regions (UTR) and genome packaging signal. (**b**) Alphavirus particles, by high-resolution cryoelectron microscopy. VEEV TC-83 strain embedded in vitreous ice is shown (Zhang et al. 2011). (**c**) Image reconstruction of alphavirus virion particle (Paredes et al. 1993; Zhang et al. 2011)

(Fig. 3.1). In contrast, in engineered RNA replicons, subgenomic RNA encodes the gene of interest in place of alphavirus structural proteins, as described below. Accordingly, in order to encapsidate replicons into VLPVs, or replicon particles, structural proteins are provided in trans from the helper constructs.

From the structural perspective, alphavirus virions are spherical, enveloped particles 60–70 nm in diameter and icosahedral symmetry (Fig. 3.2b, c). The lipid-containing envelope generally contains two surface glycoproteins E1 and E2 that mediate virus attachment, fusion, and penetration. The E1 and E2

glycoproteins are arranged into trimers of E1/E2 heterodimers (Strauss and Strauss 1994). The icosohedral nucleocapsid contains capsid protein and genomic RNA. Alphavirus virions mature by budding through the plasma membrane. Within the recent years, considerable knowledge was generated regarding the structure and functional organization of alphavirus particles (Vaney et al. 2013; Paredes et al. 1993). This includes the crystal structures of the envelope glycoprotein complexes at neutral and at acid pH, as well as image reconstructions of intact virions at neutral pH to resolutions of 4–7 Å. For example, structure of live attenuated vaccine strain TC-83 of VEEV was determined by using cryo-electron microscopy (cryo-EM) at 4.4 Å resolution. Density map clearly resolved regions (including E1 and E2 transmembrane helices and cytoplasmic tails) that were missing in the crystal structures of domains of alphaviruses. Interestingly enough, E3 protein was observed on mature TC-83 virions (Zhang et al. 2011). The new data provided unprecedented detail in the understanding of the alphavirus virion structure as well as improved understanding of the biology of the virus and the process of the alphavirus particle assembly during maturation and disassembly during cell entry.

Generally, the alphavirus infection cycle begins with E2 protein binding to host receptors on the surface of a host cell. This is followed by internalization of the virus and its transport into the acidic intracellular vesicles. The low pH induces a rearrangement of the E2/E1 dimer, thus activating fusion activity of E1 (Wahlberg and Garoff 1992). E1 inserts its hydrophobic fusion loop into the membrane of the host cell vesicle, forms E1 trimers, and refolds to pull the host cell and viral membranes together, thus causing membrane fusion and virus infection (Gibbons et al. 2004). Alphavirus genome delivery occurs directly at the plasma membrane in a time- and temperature-dependent process. Upon attaching to the cell surface, intact RNA-containing viruses become empty shells, which could be identified by antibody labeling. The rate at which full particles were converted to empty particles increased with time and temperature (Vancini et al. 2013).

In addition to binding the host cell receptor, E2 protein participates in other processes throughout the virus life cycle. During viral replication, this protein is synthesized as a precursor, PE2, and acts to facilitate the folding of its E1 protein. Furthermore, similarly to the vesicular entry pathway, the exit pathway also involves transport through cellular compartments that have an acidic pH. Interestingly enough, the PE2/E1 pair is more acid resistant than the E2/E1 dimer, and this feature likely protects E1 from random fusion during transport through the exit pathway (Wahlberg et al. 1989). Late in transport, the cellular enzyme furin cleaves PE2 to produce the mature E2 protein plus a small peripheral protein, E3 (Zhang et al. 2003). Finally, the trimers of E1/E2 heterodimers are transported to the plasma membrane, where they interact with the nascent nucleocapsid cores in the cytoplasm to form the intact progeny viruses that bud out of the host cell for the next round of infection. The virus then exits by budding from the cell surface, with some alphavirus species retaining E3 and others releasing it.

Thus, the alphavirus gains entry into cells by a process of receptor-mediated endocytosis followed by membrane fusion in the acid environment of the endosome. Cryoelectron micrograph analyses and 3D reconstructions showed that SINV virus retains its overall icosahedral structure at mildly acidic pH, except in the

membrane-binding region, where monomeric E1 associates with the target membrane and the E2 glycoprotein retains its original trimeric organization (Cao and Zhang 2013). CHIKV infection of susceptible cells is also mediated by E1 and E2. Glycoprotein E2, derived from furin cleavage of the PE2 precursor into E3 and E2, is similarly responsible for receptor binding, while E1 is responsible for membrane fusion. Glycoprotein organization of CHIKV particles was revealed by X-ray crystallography (Voss et al. 2010). The structures of SINV and CHIKV alphaviruses show that the mature E2 protein is an elongated molecule containing three domains with immunoglobulin-like folds: the amino-terminal domain A, located at the center; domain B at the tip; and the carboxy-terminal domain C, located close to the viral membrane. The structures of the PE2/E1 and E2/E1 pairs suggest specific residues that may control their dissociation at low pH and explain how PE2 and E2 regulate virus fusion (Kielian 2010).

3.4 Preparation of Replicon RNA

Alphaviruses, including attenuated strains of VEEV, have been configured as vaccine vectors (Strauss and Strauss 1994; Frolov et al. 1996). The availability of the full-length infectious clone technology greatly facilitated the development of vectors. Using infectious clone technology, alphavirus RNA can be easily made by using transcription in vitro and then transfected into permissive cells to initiate replication (Strauss and Strauss 1994; Davis et al. 1989, 1994). Generally, two types of VEEV vectors were prepared and evaluated: (1) double-promoter and (2) replicon vectors (Fig. 3.3). In the double-promoter vectors, a gene of interest is introduced into the full-length viral RNA downstream from a duplicated 26S

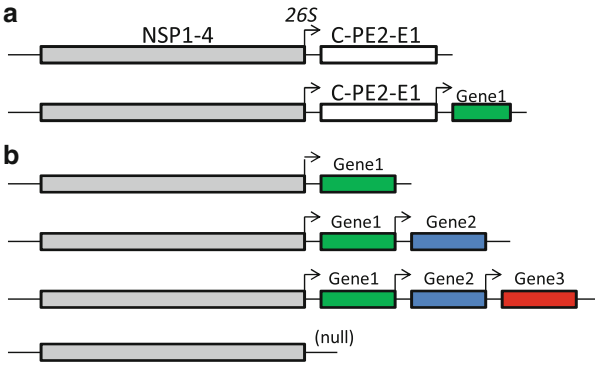

Fig. 3.3 Alphavirus genomic RNA and alphavirus vectors. (**a**) Alphavirus genomic RNA (*top*) and double-promoter vector (*bottom*). Indicated are locations of nonstructural proteins (NSP), 26S promoter, and structural proteins (C-PE2-E1), and location of foreign gene of interest. (**b**) Alphavirus replicon vectors. Locations of foreign gene(s) are indicated. 26S promoter is indicated with an *arrow*. Null replicon does not encode any foreign gene

promoter, resulting in independent transcription of two subgenomic mRNAs for both the viral structural proteins and the gene of interest (Hahn et al. 1992; Davis et al. 1996). This is depicted in Fig. 3.3a, along with parental genomic alphaviral RNA. These double-promoter vectors assemble into infectious, replication-competent viruses that are capable of propagating and expressing gene of interest through multiple rounds of replication in vitro and in vivo. However, during multiple rounds of replication in vitro or in vivo, the foreign gene of interest within the double-promoter vector tends to be deleted, which limits applications of this type of vector.

In the other type of alphavirus vectors, replicon vectors, the structural genes were deleted, and a gene of interest was introduced in place of the structural protein genes downstream from a single viral 26S promoter (Pushko et al. 1997; Xiong et al. 1989; Liljestrom and Garoff 1991; Frolov et al. 1997; Zhou et al. 1995; Mossman et al. 1996; Seregin et al. 2010). As with the double-promoter vectors, RNA replicons retained the nonstructural protein (NSP) genes and cis-acting elements (Fig. 3.3b) and were capable of RNA replication, transcription, and high-level expression of heterologous genes in the cell cytoplasm. However, when introduced into cells, replicons are restricted to a single cycle of replication as the lack of the structural proteins precludes alphavirus particle assembly and spread to uninfected cells. The lack of expression of structural protein genes also minimizes anti-vector immune responses in vivo (Pushko et al. 1997). It has been shown that VEEV- and EEEV-based replicons appear to be less cytopathic than Sindbis virus-based constructs and they can readily establish persistent replication in BHK-21 cells (Petrakova et al. 2005).

Double-promoter or bicistronic replicons have also been described (Carrion et al. 2012; Pushko et al. 2001). In these vectors, one heterologous gene of interest was placed downstream from the first 26S promoter, while the second heterologous gene was placed downstream from the second 26S promoter (Pushko et al. 2001). Such replicons expressed both genes in vitro and induced immune response in vivo to both expressed heterologous proteins (Pushko et al. 2001). Potentially, three or more genes can be expressed from independent 26S promoters (Fig. 3.3b). However, the number of genes can be limited by the size of the genes, as well as by other factors, such as vector capacity, genetic stability of the vector expressing multiple genes, the effects of expressed proteins on host cell metabolism, and others.

In recent years, replicons that contain no foreign GOI (null replicons, Fig. 3.3b) have been made as a novel type of adjuvant (Thompson et al. 2006). For example, co-inoculation of null replicon particles (not expressing any transgene) with inactivated influenza virions, or ovalbumin, resulted in a significant increase in antigen-specific systemic IgG antibodies, compared with antigen alone. Pretreatment of replicon particles with UV light largely abrogated adjuvant effect. These results demonstrated that VEEV alphavirus replicon particles possess intrinsic adjuvant activity and suggest that vector RNA replication may be responsible for this activity (Thompson et al. 2006).

3.5 Replicon Vector Delivery In Vivo

For vaccination, alphavirus replicons (Fig. 3.3b) can be delivered in permissive cells in vivo by using several methods. For example, replicons can be delivered in vivo as naked RNA (Johanning et al. 1995) (Fig. 3.4) or by plasmids directing the synthesis of replicons in vivo (Dubensky et al. 1996; Berglund et al. 1998). For example, intradermal electroporation of SFV naked replicon RNA elicited strong immune responses in mice (Johansson et al. 2012). Unfortunately, instability of naked RNA limits its use for in vivo applications. Improved T-cell responses to conserved HIV-1 regions were observed by electroporating SFV replicon DNA compared to that with conventional plasmid DNA vaccine (Knudsen et al. 2012). Tetravalent replicon vaccines against botulinum neurotoxins were prepared by using DNA-based SFV replicon vectors (Yu et al. 2013). However, there are technical and regulatory difficulties associated with plasmid DNA transfections in vitro and in vivo. Recombinant viruses were also used to prepare replicon vaccines (Vasilakis et al. 2003; Sun et al. 2013). Adenovirus-vectored SFV replicon construct expressing the E2 glycoprotein from classical swine fever virus (CSFV), rAdV-SFV-E2, induced immunity against a lethal CSFV challenge (Sun et al. 2013). Furthermore, transfection-independent system for packaging alphavirus replicon vectors was generated by using modified vaccinia virus Ankara (MVA) vectors to express all of the RNA components necessary for the production of VEEV replicon particles. Infection of mammalian cells with these recombinant MVA vectors resulted in robust expression of VEEV structural genes, replication of the alphavirus replicon vector, and high titers of replicon particles. Interestingly

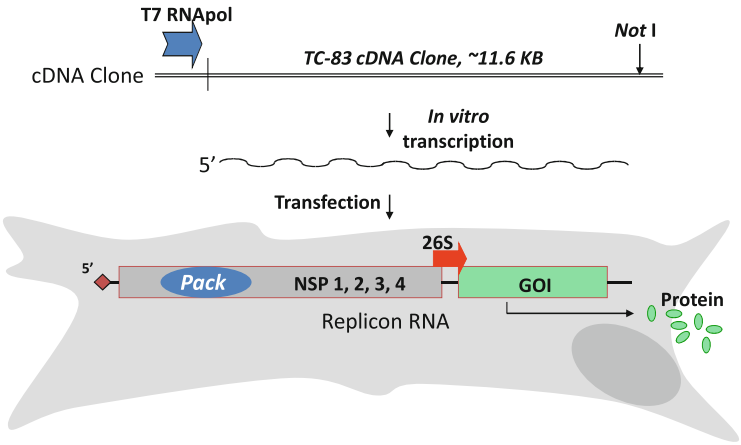

Fig. 3.4 Preparation of replicon RNA vector from the cDNA clone and expression of foreign gene of interest (GOI) in eukaryotic cell. The cDNA clone of TC-83 replicon is shown on *top* including T7 RNA polymerase promoter and NotI restriction site, which are used for runoff in vitro transcription. Replicon RNA that is made in vitro is transfected into permissive eukaryotic cell by electroporation or other method

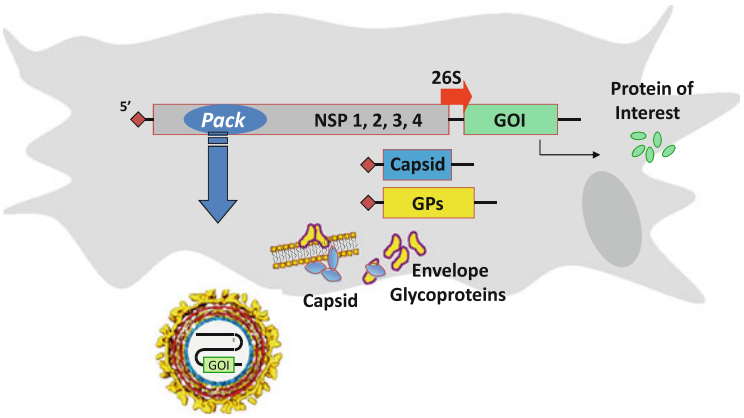

Fig. 3.5 Encapsidation of alphavirus RNA replicons into replicon particles in eukaryotic cells. Replicon particles represent viruslike particle vectors (VLPVs) that encapsulate replicon RNA. VLPVs are made in eukaryotic cells that are cotransfected with replicon RNA, capsid helper RNA, and GP helper RNA. Capsid and GP helpers express alphavirus capsid and glycoproteins, which recognize packing signal within the replicon RNA and form replicon particles

enough, replicon packaging was achieved in a cell type (fetal rhesus lung) that has been approved for vaccine manufacturing for human use (Vasilakis et al. 2003). There are challenges for virus-based replicon encapsidation process, including the need for live virus clearance from replicon particle preparations.

In spite of the fact that various vaccination strategies exist that utilize alphavirus replicons for vaccination purposes, preparation of alphavirus-like VLPVs, or replicon particles by using packaging RNA helpers (Fig. 3.5), remains the workhorse of alphavirus vector technologies (Pushko et al. 1997; Zhou et al. 1995; Mossman et al. 1996; Carrion et al. 2012). The process of preparation of replicon particles by cotransfection of replicon and helper RNAs is described below in more detail.

3.6 Preparation of Replicon Particles by Using RNA Helper Systems

One of the first replicon-helper vaccine vector systems was developed from an attenuated strain of VEEV (Pushko et al. 1997). The replicon RNA consisted of the cis-acting 5′ and 3′ ends of the VEEV genome, the complete NSP gene region, and the subgenomic 26S promoter. The genes encoding the VEEV structural proteins were replaced with heterologous gene of interest (GOI), such as the influenza virus hemagglutinin or the Lassa virus nucleocapsid gene (Pushko et al. 1997). After transfection into eukaryotic cells by electroporation, replicon RNAs directed the efficient, high-level synthesis of the influenza or Lassa virus protein of interest (Fig. 3.5). For packaging of replicon RNAs into vector particles, the VEEV capsid and glycoproteins are supplied in trans by expression of VEEV nucleocapsid and

glycoproteins from helper RNA(s) co-electroporated with the replicon (Fig. 3.5). A number of different helper constructs, expressing the VEEV structural proteins from a single or two separate helper RNAs, were derived from various attenuated VEEV strains (Pushko et al. 1997; Seregin et al. 2010). Regeneration of infectious virus was not detected when replicons were packaged using a bipartite helper system (Fig. 3.5) encoding the VEE capsid protein and glycoproteins on two separate RNAs (Pushko et al. 1997).

3.7 Applications of Alphavirus VLPVs (Replicon Particles)

Applications of alphavirus VLPVs, or replicon vector particles, involve development of human and veterinary vaccines against infectious diseases and immunotherapies for cancer, as well as adjuvants. The representative examples of these applications are described below and summarized in Table 3.1.

Table 3.1 Representative experimental alphavirus replicon vaccines for infectious diseases

Alphavirus replicon	Indication	Protective antigen	Testing in vivo	Notes	References
VEEV	Influenza	HA	Rodent		Pushko et al. (1997)
	Influenza	HA	Swine	Veterinary	Bosworth et al. (2010)
	BVDV	E2	Calves	Veterinary	Loy et al. (2013)
	Lassa	N, GPC	Rodent		Pushko et al. (1997, 2001)
	Machupo	GPC	Rodent		Carrion et al. (2012)
	Junin	GPC	Rodent		Seregin et al. (2010), Carrion et al. (2012)
	Ebola	NP, GP	Rodent, NHP		Geisbert et al. (2002), Pushko et al. (2000, 2001), Herbert et al. (2013)
	Marburg	NP, GP	Rodent, NHP		Hevey et al. (1998)
	Dengue	prME, E	NHP		White et al. (2013)
	HIV	gag	Rodent, human	Phase I clinical	Wecker et al. (2012), Carroll et al. (2011)
	CMV	gB, pp65/IE1	Human	Phase I clinical	Bernstein et al. (2009)
SINV	HCV	E2E1	Rodent		Zhu et al. (2013)
	CPV	VP2	Dog	Veterinary	Dahiya et al. (2011)
SFV	HIV	Gp160	NHP		Berglund et al. (1997)
	Influenza	NP, HA	Rodents		Berglund et al. (1999)
SAV[a]	ISAV	HE	Salmon	Veterinary	Wolf et al. (2013)

[a]*SAV* salmonid alphavirus, *ISAV* infectious salmon anemia virus

3.8 Alphavirus Replicons as Vaccines for Infectious Diseases

In the first study of VEEV replicon systems, subcutaneous immunization of BALB/c mice with VRP expressing either influenza HA or Lassa virus N gene (HA-VRP or N-VRP, respectively) induced antibody responses to each expressed protein. After two inoculations of HA-VRP, complete protection against intranasal challenge with influenza was observed (Pushko et al. 1997). Similarly, immunization of mice with SFV vectors encoding the influenza virus HA and nucleoprotein (NP) resulted in immune responses that were protective against challenge infection with influenza virus (Berglund et al. 1999).

Some of the applications of VEEV replicon vaccines involved vaccine development for viral hemorrhagic fevers. For example, one of the first VEEV replicon vaccines has been developed for Marburg filovirus (MBGV), for which there are no vaccines or treatments (Hevey et al. 1998). MGBV causes an acute hemorrhagic fever with a high mortality rate in people. VEEV RNA replicon was used to express genes for MBGV glycoprotein (GP), nucleoprotein (NP), VP40, VP35, VP30, or VP24. Guinea pigs were vaccinated with recombinant VEEV replicons packaged into VEEV-like particles and then experimentally infected with MBGV in a BSL4 laboratory. Survival and viremia in animals were evaluated. Results indicated that either GP or NP was protective antigen for MBGV, while VP35 afforded incomplete protection. As a more definitive test of replicon vaccine efficacy, nonhuman primates (cynomolgus macaques) were vaccinated with VEEV replicons expressing MBGV GP and/or NP. Three monkeys received packaged control replicons; these died 9 or 10 days after challenge, with typical MBGV disease. MBGV NP afforded incomplete protection, sufficient to prevent death but not disease in two of three macaques. However, three monkeys vaccinated with replicons which expressed MBGV GP, and three others vaccinated with both replicons that expressed GP or NP, remained aviremic and were completely protected from disease (Hevey et al. 1998).

RNA replicons derived from an attenuated strain of VEEV alphavirus have also been configured as candidate vaccines for another filovirus, Ebola hemorrhagic fever (Pushko et al. 2000). Similarly to other alphavirus replicons (Fig. 3.3), the Ebola virus (EBOV) nucleoprotein (NP) or glycoprotein (GP) gene was introduced into the VEEV RNA downstream from the 26S promoter in place of the VEEV structural protein genes. The resulting recombinant replicons expressing the NP or GP gene were packaged into VEEV replicon particles (NP-VRP and GP-VRP, respectively) using a bipartite helper system that provided the VEEV structural proteins in trans and prevented the regeneration of replication-competent VEEV during packaging (Fig. 3.5) (Pushko et al. 1997). The immunogenicity of NP-VRP and GP-VRP and their ability to protect against lethal Ebola infection were evaluated in BALB/c mice and in two strains of guinea pigs. The GP-VRP alone, or in combination with NP-VRP, protected both strains of guinea pigs and BALB/c mice, while immunization with NP-VRP alone protected BALB/c mice, but neither strain

of guinea pig (Pushko et al. 2000). Protection of nonhuman primates against EBOV challenge proved more challenging (Geisbert et al. 2002). Nevertheless, VEEV replicon particles were successfully evaluated as experimental vaccines against Sudan (SUDV) and EBOV filoviruses in nonhuman primates (Herbert et al. 2013). VRP vaccines were prepared that expressed the GP of either SUDV or EBOV. A single intramuscular vaccination of cynomolgus macaques with high dose of VRP expressing SUDV GP provided complete protection against intramuscular challenge with SUDV. Vaccination against SUDV and subsequent survival of SUDV challenge did not fully protect cynomolgus macaques against intramuscular EBOV back-challenge. However, a single simultaneous intramuscular vaccination with VRP expressing SUDV GP combined with VRP expressing EBOV GP did provide complete protection against intramuscular challenge with either SUDV or EBOV in cynomolgus macaques. Finally, intramuscular vaccination with VRP expressing SUDV GP completely protected cynomolgus macaques when challenged with aerosolized SUDV, although complete protection against aerosol challenge required two vaccinations with this vaccine (Herbert et al. 2013).

Vaccine development against dengue has also been reported by using VEEV replicons. VRP replicon particles expressing dengue virus E antigens as subviral particles [prME] and soluble E dimers [E85] successfully immunized and protected macaques against dengue virus (White et al. 2013). Anti-vector antibodies did not interfere with a booster immunization. Interestingly enough, compared to prME-expressing vectors, the E85 vectors induced neutralizing antibodies faster, to higher titers, and with improved protective efficacy. This study also mapped antigenic domains targeted by vaccination versus natural infection, revealing that, unlike prME-VRP and live virus, E85-VRP induced only serotype-specific antibodies, which predominantly targeted EDIII, suggesting a protective mechanism different from that induced by live virus and possibly live attenuated vaccines. A tetravalent E85-VRP dengue vaccine induced a simultaneous and protective response to all four serotypes after two doses given 6 weeks apart. Balanced responses and protection in macaques provided further support for exploring the immunogenicity and safety of this vaccine candidate in humans (White et al. 2013).

Other vaccines include vaccines against arenaviruses, such as Lassa, Junin, and other arenaviral hemorrhagic fevers. Arenaviruses cause severe disease in people. For example, Junin virus (JUNV) is the etiological agent of the potentially lethal, reemerging human disease, Argentine hemorrhagic fever. The replicon system was engineered from live investigational VEEV vaccine TC-83 that expressed glycoproteins (GPC) of JUNV (Seregin et al. 2010). Preclinical studies testing the immunogenicity and efficacy of TC83/JUNV GPC were performed in guinea pigs. A single dose of the TC-83 alphavirus-based vaccine expressing only GPC was immunogenic and provided partial protection, while a double dose of the same vaccine provided a complete protection against JUNV (Seregin et al. 2010).

A SINV virus vector was used to induce humoral and cellular responses against hepatitis C virus (HCV). The recombinant vector, pVaXJ-E1E2, expressing HCV glycoproteins E2 and E1, was constructed by inserting the E1E2 gene into the replicon pVaXJ, a DNA vector derived from Sindbis-like virus XJ-160 (Zhu

et al. 2013). The replication-defective replicon particles were produced by transfecting BHK-21 packaging cell line with pVaXJ-E1E2. Mice were vaccinated using a prime-boost regimen with SINV replicon particles combined with Freund's incomplete adjuvant via intramuscular injection, and HCV-specific IgG antibody levels and cellular immune responses were detected by IFA and IFN-γ ELISPOT, respectively (Zhu et al. 2013).

SFV replicon RNA vectors expressing the envelope protein gp160 of HIV-1IIIB were evaluated in cynomolgus macaques. Monkeys were immunized four times with recombinant SFV particles. Whereas two out of four monkeys showed T-cell-proliferative responses, only one monkey had demonstrable levels of antibodies to HIV-1 gp41 and gp120 as shown by enzyme-linked immunosorbent assay (ELISA) and Western blot. The vaccinated monkeys and four control animals were challenged with 10,000 MID100 (100 % minimum infectious doses) of cell-free monkey cell-grown SHIV-4 virus. Three out of four vaccinated monkeys had no demonstrable viral antigenemia and low viral load as opposed to one of the four naive control animals (Berglund et al. 1997).

Several experimental veterinary vaccines have also been developed from alphavirus replicons including replicons derived from VEEV and other alphaviruses. For example, replicon particles that expressed bovine viral diarrhea virus sub-genotype 1b E2 glycoprotein were generated (Loy et al. 2013). Expression was confirmed in vitro by using antibodies specific to E2 glycoprotein. Experimental replicon particle vaccine was generated in Vero cell culture and administered to BVDV-free calves in a prime-boost regimen at two dosage levels. Vaccination resulted in neutralizing antibody titers that cross-neutralized both type 1 and type 2 BVD genotypes after booster vaccination. Additionally, high-dose vaccine administration demonstrated protection from clinical disease and significantly reduced the degree of leukopenia caused by viral infection (Loy et al. 2013). Other experimental veterinary vaccines have also been developed from alphaviruses. An alphavirus-derived replicon particle vaccine expressing the H3N2 swine influenza virus hemagglutinin gene induced protective immunity against homologous influenza virus challenge (Bosworth et al. 2010). A replicon expression system based on the salmonid alphavirus (SAV) that encodes the infectious salmon anemia virus (ISAV) hemagglutinin-esterase (HE) was found to be an efficacious against infectious salmon anemia (Wolf et al. 2013). Following a single intramuscular immunization, Atlantic salmon (*Salmo salar*) were effectively protected against subsequent ISAV challenge. These results have shown that the alphavirus replicon approach may represent a novel immunization technology for the aquaculture industry (Wolf et al. 2013).

A SINV replicon-based DNA vaccine containing VP2 gene of canine parvovirus (CPV) was delivered by *E. coli* to elicit immune responses (Dahiya et al. 2011). The orally immunized dogs developed CPV-specific serum IgG and virus neutralizing antibody responses after vaccine administration. The cellular immune responses were analyzed using lymphocyte proliferation test and flow cytometry and indicated successful CPV-specific sensitization of both CD3+CD4+ and CD3+CD8+ lymphocytes. This research demonstrated that SINV replicon-based DNA vaccine

delivered by *E. coli* can be considered as a promising approach for vaccination of dogs against CPV (Dahiya et al. 2011).

3.9 Sequential Immunization with VLPVs

One of the advantages of alphavirus VLPVs, or replicon particle vectors, is that they do not induce significant self-immunity (Pushko et al. 1997). Even when anti-vector response was detected, this did not prevent booster vaccinations (White et al. 2013). This allowed efficient boosts with the same vectors, and even sequential immunizations with vectors expressing distinct immunogens. As described above, subcutaneous immunization of BALB/c mice with VEEV replicon vectors expressing either influenza HA or Lassa virus N gene (HA-VRP or N-VRP, respectively) induced antibody responses to each expressed protein. After two inoculations of HA-VRP vectors, complete protection against intranasal challenge with influenza was observed. Furthermore, sequential immunization of BALB/c mice with two inoculations of N-VRP prior to two inoculations of HA-VRP induced an immune response to both HA and N, which was essentially equivalent to immunization with either VRP construct alone. Protection against influenza challenge was not affected by previous N-VRP immunization (Pushko et al. 1997).

3.10 Multivalent and Bicistronic Replicon Vaccines

Vaccination against multiple pathogens can be achieved by blending several species of replicon particles, each species protecting against a single pathogen of interest. Alternatively, protection against multiple pathogens can be achieved by vaccination with replicon particles, which encapsidate multi-cistronic replicons co-expressing multiple protective antigens in a tandem fashion (Fig. 3.3b). An example of blended, tetravalent dengue vaccine derived from VEEV replicons has been described (White et al. 2013). Preparation of bivalent and bicistronic replicons and encapsidation of bicistronic replicons into VLPVs has resulted in bivalent replicon vaccines capable of protecting against two pathogens, Lassa and Ebola viruses. Experimental individual vaccines for Lassa virus and bivalent and bicistronic vaccines for Lassa and Ebola viruses were developed from an RNA replicon of attenuated VEEV. Recombinant replicons were incorporated into viruslike replicon particles. Expression of antigens was confirmed by immunofluorescence assay (Fig. 3.6). Guinea pigs vaccinated with particles expressing Lassa virus nucleoprotein or glycoprotein genes were protected from lethal challenge with Lassa virus (Pushko et al. 2001). Vaccination with particles expressing Ebola virus glycoprotein gene also protected the animals from lethal challenge with Ebola virus. In order to evaluate a single vaccine protecting against both Lassa and Ebola viruses, blended and bicistronic particles were prepared that expressed

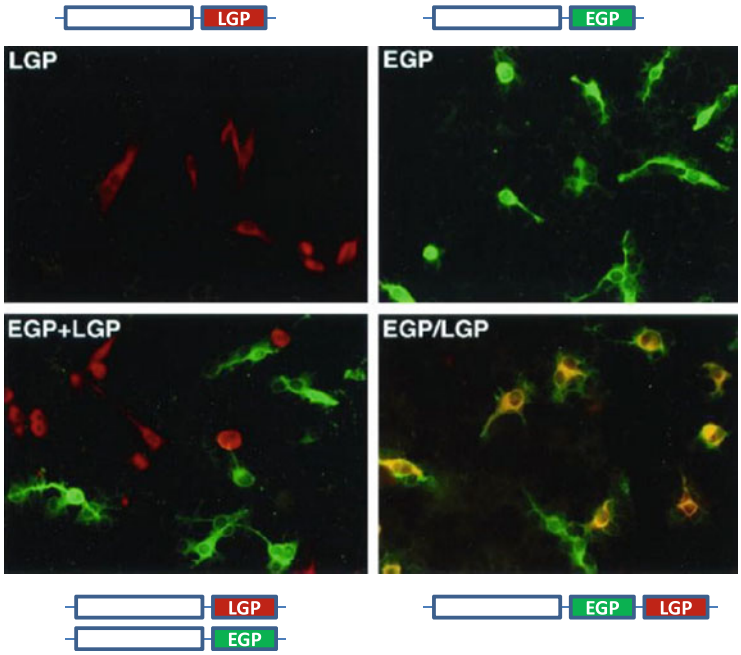

Fig. 3.6 Expression of Lassa and Ebola antigens from VEEV replicons in BHK-21 cells, by immunofluorescence (Pushko et al. 2001). *Top panel* depicts expression of individual Lassa (*left*) and Ebola (*right*) genes. *Bottom panel* shows expression of both Lassa and Ebola antigens from blended vaccine formulation (*left*) and from bicistronic replicon expressing both Lassa and Ebola antigens from the same replicon vector (*right*). *Yellow color* indicates co-localization of both antigens in the same cell

glycoprotein genes of both Ebola and Lassa viruses. Vaccination of guinea pigs with either bicistronic replicon particles or with a blended mixture of particles expressing Ebola and Lassa virus glycoprotein genes protected the animals against challenges with Ebola and Lassa viruses. The results showed that immune responses can be induced against multiple vaccine antigens co-expressed from an alphavirus replicon and suggested the possibility of engineering multivalent vaccines based upon alphavirus vectors for arenaviruses, filoviruses, and possibly other emerging pathogens (Pushko et al. 2001). Recently, bicistronic VEEV VLPVs were described that co-expressed Junin and Machupo arenavirus GPC genes (Carrion et al. 2012).

3.11 Clinical Trials

Clinical trials involving alphavirus replicon vaccines are underway. In order to develop cytomegalovirus (CMV) vaccine, a two-component alphavirus replicon particles expressing CMV gB or a pp65/IE1 fusion protein, previously shown to

induce robust antibody and cellular immune responses in mice, were evaluated in a randomized, double-blind phase 1 clinical trial in CMV seronegative individuals. Forty subjects received a low dose or high dose of vaccine or placebo by intramuscular or subcutaneous injection at weeks 0, 8, and 24 (Bernstein et al. 2009). The vaccine was well tolerated, with mild to moderate local reactogenicity, minimal systemic reactogenicity, and no clinically important changes in laboratory parameters. All vaccine recipients developed ex vivo, direct IFN-γ ELISPOT responses to CMV antigens and neutralizing antibodies. Polyfunctional CD4(+) and CD8(+) T-cell responses were detected by polychromatic flow cytometry. This alphavirus replicon particle vaccine was safe and induced neutralizing antibody and multifunctional T-cell responses against three CMV antigens that are important targets for protective immunity (Bernstein et al. 2009).

Another clinical trial was conducted to evaluate experimental HIV-1 vaccine. On the basis of promising preclinical data (Williamson et al. 2003), safety and immunogenicity of an alphavirus replicon HIV-1 subtype C *gag* vaccine, expressing a nonmyristoylated form of *gag*, were evaluated in two double-blind, randomized, placebo-controlled clinical trials in healthy HIV-1-uninfected adults (Wecker et al. 2012). Escalating doses of vaccine or placebo were administered subcutaneously to participants in the USA and Southern Africa. Although both trials were stopped prematurely due to various reasons, safety and immunogenicity were evaluated through assessments of reactogenicity, reports of adverse events, and assessment of replication-competent VEEV viremia. Immunogenicity was measured using the enzyme-linked immunosorbent assay (ELISA), chromium 51-release cytotoxic T lymphocyte (CTL), gamma interferon (IFN-γ) ELISPOT, and other assays. Vaccine was well tolerated and exhibited only modest local reactogenicity. There were five serious adverse events reported during the trials; however, none were considered related to the study vaccine. In contrast to the preclinical data (Williamson et al. 2003), immune responses in humans were limited. Only low levels of binding antibodies and T-cell responses were seen at the highest doses. This trial also highlighted the difficulties in the developing of HIV vaccine (Wecker et al. 2012).

3.12 Alphavirus Replicons as Vaccines Against Cancer

Cellular immunotherapy based on autologous dendritic cells (DCs) targeting antigens expressed by metastatic cancer has previously demonstrated clinical efficacy. However, the logistical and technical challenges in generating such individualized cell products require the development of alternatives to autologous DC-based cancer vaccines. Particularly attractive alternatives include delivery of antigen and/or activation signals to resident antigen-presenting cells, which can be achieved by alphaviral vectors expressing the antigen of interest and capable of infecting DCs.

The rationale for developing immunotherapeutic vectors and opportunities to enhance their effectiveness has been reviewed elsewhere including the use of alphaviruses (Osada et al. 2012). Alphavirus replicon vectors have been used in multiple studies to develop new immunotherapies against tumor-associated antigens (TAAs). Efforts to evaluate and discover TAAs as diagnostic and therapeutic markers for cancer have succeeded in identification of several TAAs. Many TAAs represent "self"-antigens and, as such, are subject to the constraints of immunologic tolerance. There are significant immunological barriers to eliciting antitumor immune responses to self-antigens. VEEV-derived alphavirus replicon vector system that has shown in vivo tropism to dendritic cells has been used to develop vaccines using expression of TAAs. For example, VEEV vectors have been shown to overcome the intrinsic tolerance to the "self"-TAA rat neu and elicited an effective antitumor immune response using VEEV replicon vector and a rationally designed target antigen in a rigorous rat mammary tumor model (Nelson et al. 2003). The VEEV vectored immunotherapy has shown the capacity to generate 50 % protection in tumor challenge experiments ($p = 0.004$). The establishment of immunologic memory was confirmed by both second tumor challenge and Winn assay ($p = 0.009$). Minor antibody responses were identified and supported the establishment of T helper type 1 (Th1) antitumor immune responses by isotype. Animals surviving in excess of 300 days with established effective antitumor immunity showed no signs of autoimmune phenomena. These experiments supported the establishment of T-lymphocyte-dependent, Th1-biased antitumor immune responses to a non-mutated "self"-TAA in an aggressive tumor model. Importantly, this tumor model is subject to the constraints of immunologic tolerance present in animals with normal developmental, temporal, and anatomical expression of a non-mutated TAA. These data supported the development and potential clinical application of VEEV replicon vectors along with the appropriately designed target antigens for antitumor immunotherapy (Nelson et al. 2003).

Prostate-specific membrane antigen (PSMA) is a transmembrane protein expressed in all types of prostatic tissue. PSMA may represent a promising diagnostic and possibly therapeutic TAA target (Slovin et al. 2013). PSMA-VRP was made and evaluated in phase I clinical trial for patients with castration-resistant metastatic prostate cancer (CRPC). Two cohorts of three patients with CRPC metastatic to bone were treated with up to five doses of either $0.9 \times 10(7)$ IU or $0.36 \times 10(8)$ IU of PSMA-VRP at weeks 1, 4, 7, 10, and 18, followed by an expansion cohort of six patients treated with $0.36 \times 10(8)$ IU of PSMA-VRP at weeks 1, 4, 7, 10, and 18 (Slovin et al. 2013). No toxicities were observed. In the first-dose cohort, no PSMA-specific cellular immune responses were seen but weak PSMA-specific signals were observed by ELISA. The remaining nine patients, which included the higher cohort and the extension cohort, had no PSMA-specific cellular responses. PSMA-VRP was well tolerated at both doses. While there did not appear to be clinical benefit nor robust immune signals at the two doses studied, neutralizing antibodies were produced by both cohorts suggesting that the vaccine dosing was not optimal (Slovin et al. 2013).

Another promising strategy of the use of alphavirus vectors for cancer therapy can be targeting and potentially eliminating of tumors by cytopathic effect exhibited by alphavirus vectors. For example, distribution of recombinant SFV particles (recSFV) and SFV naked viral RNA replicon was studied in tumor-free and 4T1 mammary tumor-bearing mice (Vasilevska et al. 2012). The predominant tumor targeting by recSFV was observed at a reduced dose, whereas the dose increase led to a broader virus distribution in mice (Vasilevska et al. 2012).

3.13 Alphavirus Replicons as Adjuvants

One of the most recent applications of alphavirus replicon vectors includes their use as novel adjuvants. VEEV replicon particles (VRP) have been used to augment humoral, cellular, and mucosal immune responses in mice. For the adjuvant purpose, replicons do not even need to express any transgene. For example, co-inoculation of VRP with no transgene (null VRPs) along with inactivated influenza virions resulted in a significant increase in antigen-specific systemic IgG and IgA antibodies, compared to antigen alone. Pretreatment of VRP with UV light diminished this adjuvant effect. These results demonstrate that alphavirus replicon particles possess intrinsic systemic and mucosal adjuvant activity and suggest that VRP RNA replication is the trigger for adjuvant activity (Thompson et al. 2006).

It was also demonstrated that VRP adjuvant induced an increased and balanced serum IgG subtype response to co-delivered antigen, with simultaneous induction of antigen-specific IgG1 and IgG2a antibodies (Thompson et al. 2008). VRP adjuvant also increased both systemic and mucosal antigen-specific CD8+ T-cell responses, as measured by an IFN-γ ELISPOT assay. Additionally, VRP further increased antigen-specific T-cell immunity in an additive fashion following co-delivery with the TLR ligand, CpG DNA (Thompson et al. 2008). VRP infection led to recruitment of CD8+ T cells into the mucosal compartment, possibly utilizing the mucosal homing receptor, as this integrin was upregulated on CD8+ T cells in the draining lymph node of VRP-infected animals, where VRP-infected dendritic cells reside. This newly recognized ability of VRP to mediate increased T-cell response towards co-delivered antigen provides the potential to both define the molecular basis of alphavirus-induced immunity and improve alphavirus-based vaccines (Thompson et al. 2008).

To assess the adjuvant activity of null VRP in the context of a licensed inactivated influenza virus vaccine, rhesus monkeys were immunized with either influenza Fluzone vaccine alone or with Fluzone vaccine mixed with null VRP and then challenged with a human seasonal influenza virus, A/Memphis/7/2001 (H1N1) (Carroll et al. 2011). Compared to Fluzone alone, Fluzone with null VRP-immunized animals had stronger influenza-specific CD4(+) T-cell responses (4.4-fold) with significantly higher levels of virus-specific IFN-γ (7.6-fold) and IL-2 (5.3-fold) producing CD4+ T cells. Fluzone/null VRP-immunized animals

also had significantly higher plasma anti-influenza IgG ($p < 0.0001$, 1.3 log) and IgA ($p < 0.05$, 1.2 log) levels. In fact, the mean plasma anti-influenza IgG titers after one Fluzone/null VRP immunization was 1.2 log greater ($p < 0.04$) than after two immunizations with Fluzone vaccine alone. After virus challenge, only Fluzone/null VRP-immunized monkeys had a significantly lower level of viral replication ($p < 0.001$) relative to the unimmunized control animals. Although little anti-influenza antibody was detected in the respiratory secretions after immunization, strong anamnestic anti-influenza IgG and IgA responses were present in secretions of the Fluzone/null VRP-immunized monkeys immediately after challenge. There were significant inverse correlations between influenza RNA levels in tracheal lavages and plasma anti-influenza HI and IgG anti-influenza antibody titers prior to challenge. These results demonstrate that null VRP dramatically improves both the immunogenicity and protection elicited by a licensed inactivated influenza vaccine (Carroll et al. 2011).

3.14 Advantages and Challenges for Alphavirus Replicon Vectors

Alphavirus replicon vectors are characterized by high-level expression of heterologous genes in cultured cells, little or no regeneration of plaque-forming virus particles, and the capability for sequential immunization to multiple pathogens in the same host. Induction of protective immunity against many pathogens and cancer-related indications has been demonstrated by using VEEV, SINV, and SFV replicon vectors. The vaccine products derived from attenuated VEEV viruses incorporate multiple redundant safety features (Pushko et al. 1997). Another feature of VEEV replicon vectors is the ability to target dendritic cells (DCs). Dendritic cells consist of heterogeneous phenotypic populations and have diverse immunostimulatory functions dependent on both lineage and functional phenotype. As exceptionally potent antigen-presenting cells, DCs represent excellent targets for generating effective antigen-specific immune responses. VRP replicon particle vectors derived from VEEV have been reported to transduce murine and human DC. The receptive DC subsets, the degree of restriction for this tropism, and the extent of conservation between rodents and humans have been extensively studied and characterized. By using fresh peripheral blood DCs, mononuclear cells, monocyte-derived macrophages, and monocyte-derived DCs, it has been demonstrated that VEEV vector has similar tropism for DCs between humans and rodents. It has been also observed that the VEEV target population represents a subset of immature myeloid DCs and that VRP-transduced immature DCs retain intact functional capacity, for example, the ability to resist the cytopathic effects of VRP transduction and the capacity to acquire the mature phenotype. These studies supported the demonstration of selective VRP tropism for human DCs and provide further insight into the biology of the VRP vector, its parent virus, and human DCs (Nishimoto et al. 2007).

Regarding the development of veterinary vaccines by using alphavirus vectors, one of the attributes of a good veterinary vaccine is the capability to permit differentiation of vaccinated vs. infected animals (DIVA). The DIVA concept relies on the principle that a vaccinated animal will have a different immune response than an animal that is infected with the wild-type pathogen and that this immune response is readily detectable by some immunoassay. Alphavirus vectors can be readily configured to allow DIVA, thus allowing the development of next-generation veterinary vaccines (Vander Veen et al. 2012).

3.15 Future Directions

The alphavirus replicon technology offers great potential for the next generation of human and veterinary vaccines (Pushko et al. 1997; Vander Veen et al. 2012). Both replicon particles and replicon DNA vaccines have demonstrated robust and balanced immune responses with subsequent protection against a variety of diseases that have implications for both human and animal health. Further improvements in both safety and the replicon vector design may significantly advance the field of replicon-based vaccines. Improvements of replicon packaging technologies in order to achieve better packaging efficacies and preventing recombination of replicons and helpers will be an important goal for further development of alphavirus vector development. In addition, replicons derived from other viruses can also be useful for many applications in vitro and in vivo, such as replicon derived from Kunjin flavivirus (Pijlman et al. 2006). The optimal vaccine regimens for prophylactic and therapeutic interventions may include a combination of distinct alphavirus vectors or combinations of alphavirus vectors with other vaccine-relevant immunogens.

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Chapter 4
Current Status and Future of Polio Vaccines and Vaccination

Konstantin Chumakov

Abstract The history of polio vaccines and their use illustrates the concept of evolution of vaccines driven by changing epidemiological and socioeconomic conditions. The development of two vaccines against poliomyelitis—inactivated Salk vaccine (IPV) and live oral Sabin vaccine (OPV)—is among the most consequential achievements of prophylactic medicine of the past century. Each with their own strengths and weaknesses, they were used over the past 50 years in different settings and different regimens and combinations. This resulted in virtual elimination of the disease in almost the entire world with the exception of a few countries. Continuation of the eradication campaign coordinated by WHO may soon result in complete cessation of wild poliovirus transmission, and poliovirus may join smallpox virus in the club of extinct pathogens. However, unlike smallpox vaccination that was stopped after the interruption of virus circulation, vaccination against poliomyelitis will have to continue into the foreseeable future, due to significant differences in the nature and epidemiology of the viruses. This chapter reviews the reasons for the need to maintain high population immunity against polioviruses, makes the case for developing a new generation of polio vaccines, and discusses their desirable properties as well as new vaccine technologies that could be used to create polio vaccines for the post-eradication environment.

4.1 Introduction

Vaccines occupy a unique place among medical biotechnology products. Among the oldest of such products, some vaccines were developed and are still manufactured using centuries-old methods. Increasing demands for safety, efficacy, and manufacturing efficiency create strong pressures to use modern technologies for vaccine manufacture requiring introduction of innovative approaches. Vaccines against poliomyelitis are among the most widely used and successful vaccines ever,

K. Chumakov (✉)
Office of Vaccines Research and Review, Center for Biologics Evaluation and Research, Food and Drug Administration, 10903 New Hampshire Avenue, Silver Spring, MD 20993, USA
e-mail: Konstantin.Chumakov@fda.hhs.gov

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and thus they represent a paradigm for other vaccines. Their introduction almost 60 years ago resulted in virtual elimination of the dreadful disease from the face of the Earth. This dramatic change in the epidemiology of poliomyelitis and shifts in societal perception of the risk-benefit balance triggered several important changes in polio immunization policies. Potential complete eradication of the disease in the foreseeable future may require replacement of the currently used vaccines with products having a new target profile more suitable for post-eradication environments. This process represents a clear illustration of the evolution of vaccines in response to epidemiological and socioeconomic changes and the need to continuously work on updating vaccine manufacturing technology. This chapter will review the history of polio vaccines and discuss the reasons for developing new products. It will also review some innovative approaches that are now being explored for polio vaccines and could be also used for development of other products.

4.2 Natural History of Poliomyelitis and Milestones in Discovery of Polio Vaccines

Poliomyelitis is a neurological disease that manifests itself by flaccid paralysis that follows a few days of febrile illness and in many cases lasts for the rest of the life of its victims. In the most severe bulbar cases, death ensues due to paralysis of respiratory muscles (Baker 1949). The disease was first described in the eighteenth century by a British doctor Michael Underwood (Underwood 1789), but it was known for many centuries before that, as evidenced by ancient images found in Egypt depicting typical victims of poliomyelitis. However, for most human history poliomyelitis occurred as a sporadic disease that occasionally afflicted children and young adults, giving it its other name "infantile paralysis" (Badham 1834–35; Heine 1840; Cornil 1863; Jacobi 1874–75). At the turn of the twentieth century, the nature of the disease changed, and it gradually became an epidemic disease with a global reach (Putnam and Taylor 1893; Flexner and Clark 1912–13; Frost 1913).

The reasons for this transformation were changes in socioeconomic conditions that led to improved hygiene. In the past most children were infected with poliovirus in infancy and early childhood while they were still protected by maternal antibodies and were less susceptible to the virus. Because of a very low attack rate (one out of several hundred infected individuals), this early encounter with the virus led to a relatively small number of clinical cases but left the rest of those who were exposed to the virus with a life-long immunity. Therefore, wild polioviruses were vaccinating the human population against themselves and thus restricted their own spread. With improved sanitation and hygiene, the first encounter with poliovirus occurred later when children were no longer protected by maternal antibodies, and as a result the number of paralytic cases increased. Lower population immunity created the possibility for virus to spread rapidly and cause outbreaks of increasing size and severity.

The first isolation of poliovirus was reported in 1909 by Austrian scientists Karl Landsteiner and Erwin Popper (Landsteiner and Popper 1909). At the same time Flexner and Lewis demonstrated that monkeys can be infected with the virus (Flexner and Lewis 1909) and that they can be made resistant to the virus by either passive transfer of antibodies from immune animals or active immunization (Flexner and Lewis 1910). Subsequent studies revealed that there are three distinct serotypes of poliovirus (Burnet and Macnamara 1931; Bodian et al. 1949; Kessel and Pait 1949) that belong to the human *Enterovirus* genus (Pallansch et al. 2013) within the Picornaviridae family (Racaniello 2013). These small RNA viruses contain a single molecule of positive-strand RNA of about 7,440 nucleotides inside an icosahedral protein capsid composed of 60 copies of each of four structural proteins. The virus attaches to a protein receptor called CD155 expressed on the surface of susceptible cells, penetrates the cells through endocytosis, and releases its genomic RNA into the cytoplasm to direct synthesis of all viral proteins. All poliovirus proteins are synthesized as a single precursor polypeptide chain of about 2,200 amino acids, which is then autocatalytically cleaved to generate a variety of proteins with different functions needed to synthesize viral progeny and subvert host metabolism and defense systems. Poliovirus infection is highly productive yielding thousands of infectious particles from each infected cell, which then dies and lyses; however, in some rare cases the virus may establish chronic infection. The mechanisms of chronic infection and its role in viral pathogenesis are not fully understood. This aspect will be briefly touched upon later in this chapter.

The increasingly severe nature of polio outbreaks in the twentieth century attracted the attention of both the general public and scientists who sought to develop measures against the disease. A boost to public awareness was the fact that US President Franklin D. Roosevelt had contracted poliomyelitis at the age of 39 leaving him partially paralyzed for life. Together with his friend Basil O'Connor, he helped to establish the National Foundation for Infantile Paralysis that would later become known as the March of Dimes. This charitable organization raised money to help polio victims and also to fund research leading to the prevention of the disease.

Many leading scientists became involved in the work on poliomyelitis that enabled the development of anti-polio vaccines. Demonstration that serum from convalescents can protect from poliomyelitis (Kramer et al. 1932) and that monkeys can be immunized by inactivated virus (Brodie 1934) led to the attempt to actively immunize humans (Brodie and Park 1935). These early trials were unsuccessful and several recipients of this vaccine developed paralytic disease (Leake 1935).

In 1949 a significant breakthrough was achieved by John F. Enders, Thomas H. Weller, and Fred C. Robbins who developed in vitro cell cultures and demonstrated that they could support growth of poliovirus in the laboratory (Enders et al. 1949). For this discovery that opened a route to laboratory research on poliovirus, including development of vaccines, they were awarded the 1954 Nobel Prize in physiology and medicine.

Other key studies were pursued by William Hammon and others who explored the use of serum from people immune to poliomyelitis to protect against the

disease. A large clinical study showed that gamma globulin from these sera completely protected against paralysis (Hammon et al. 1952). This provided decisive proof that humoral immunity is sufficient for protection, and therefore that creation of a vaccine that induced such an immune response might be possible.

The work on vaccines progressed in two directions. The first was led by Dr. Jonas Salk and his associates who developed a protocol for formalin inactivation of poliovirus grown in cell cultures. Under carefully controlled conditions, virus lost infectivity while retaining immunogenicity. The vaccine was administered as an intramuscular injection. The results of clinical trials of this vaccine were publicly revealed in April of 1955. They demonstrated very high protective efficacy of the vaccine, which was subsequently confirmed by its mass use that immediately followed this announcement.

Other groups were pursuing creation of live attenuated vaccines. They aimed to select strains of polioviruses that would replicate in vaccine recipients but would not be able to infect the central nervous system. Enders, Weller, and Robins demonstrated that passaging of virus in cultured cells led to reduction in its neurovirulence (Enders et al. 1952). Hilary Koprowski was developing a live vaccine based on mouse-adapted strains (Koprowski et al. 1952; Koprowski 1958). The most successful strains were developed by Albert Sabin (Sabin 1954a, b, 1955a, b). Live vaccine was administered orally by putting a drop of vaccine directly into a child's mouth or in small sugar cubes. Use of this oral polio vaccine (OPV) made from these strains was hampered by the existence of Salk's inactivated polio vaccine (IPV) and by lingering doubts about the safety of vaccine made from live virus. However, large-scale clinical studies conducted in the former Soviet Union and some other countries in Eastern Europe demonstrated its safety and high efficacy as well as low production costs and ease of administration (Chumakov 1960; Sabin 1961a). The next section of this chapter will compare properties of OPV and IPV in detail. Here we will just mention that these properties determined the ultimate overwhelming dominance of OPV in public health systems worldwide for the next 50 years. Another factor leading to increasing acceptance of OPV despite availability and high efficacy of IPV was the so-called Cutter incident (Nathanson and Langmuir 1963a, b, c; Offit 2005). Just 2 weeks after IPV licensure, it was found that some batches of the vaccine produced by Cutter Laboratories contained residual live virus that had escaped inactivation, leading to several paralytic and even lethal cases caused by vaccination. The Cutter incident had a profound and long-lasting effect on regulation of vaccines and led to creation of a legal framework for compensation of victims of vaccine-related injuries. More importantly, this tragic episode had a silver lining by opening the door to OPV that became the instrument for not only disease control but possibly for its complete eradication.

4.3 OPV vs. IPV

IPV was licensed on April 12, 1955, 10 years to the day after the passing of polio's most famous victim—Franklin D. Roosevelt. Its introduction in the USA and European countries led to a spectacular decline of the incidence of paralytic poliomyelitis. However, immunization with IPV does not induce sterilizing immunity, meaning that while being completely protected against paralysis, vaccine recipients can be successfully infected with poliovirus and pass on the virus to their contacts. In other words, IPV is not very effective in preventing spread of the virus and breaking chains of its transmission. On the other hand, immunization with OPV makes the intestinal tract of vaccine recipients refractory to subsequent infection, virus replication, and shedding of the virus in stool. Another attractive property of OPV is its ability to cause a "herd effect" by spreading the vaccine virus from a primary vaccine recipient to his/her contacts—siblings, playmates, etc.—and thus immunizing them against the disease. These are perhaps the biggest advantages of live vaccine over inactivated. Combined with some other benefits of OPV such as lower cost and easier administration, these advantages led, after licensure of OPV in the early 1960s, to a dramatic shift from the use of IPV to almost exclusive use of OPV. With the exception of three countries in Scandinavia that by then had eliminated poliomyelitis and therefore had no incentive to switch to another vaccine, all other countries replaced IPV in their immunization schedules with OPV. The additional advantages of OPV include a significantly lower production cost and ease of administration. While IPV is given through intramuscular injections and therefore requires qualified medical personnel, OPV is given orally by depositing a drop of the vaccine into the mouth of a child. Removing the need for trained medical personnel to administer vaccine is a major advantage especially in resource-limited countries. The shift from IPV to OPV was also facilitated by no-cost licensure by Sabin of his attenuated strains to any manufacturer who would agree to follow his advice on the manufacturing process. In 1972 he donated his strains and granted control of their use to the World Health Organization.

Despite several obvious advantages of OPV, its mass worldwide use revealed some troubling weaknesses. The first was discovered relatively early after reports of rare cases of paralytic poliomyelitis following administration of OPV (Chang et al. 1966; Feigin et al. 1971; Wright et al. 1977). The link between these cases of vaccine-associated paralytic polio and OPV was long suspected but hard to prove until the introduction of molecular genetic methods and nucleotide sequencing (Nottay et al. 1981). These tests unambiguously proved that vaccine-associated paralytic polio (VAPP) is caused by a mutated form of the vaccine virus that regained neurovirulent properties (reversion to virulence). The incidence of VAPP varied in different countries, but one of the most representative studies conducted in the USA showed that paralysis occurred once per roughly 600,000 first doses of the vaccine (Alexander et al. 2004). Therefore, in the USA there were 5–10 cases of VAPP per year. As long as the morbidity caused by wild polioviruses was significantly higher, this level of adverse reactions did not attract broad attention. However, at some point VAPP became the leading cause of poliomyelitis

in the country and made the continued use of OPV ethically tenuous. This point was reached in the 1990s, when a new generation of IPV became available, and made it possible for some countries to switch from OPV to sequential use of IPV and OPV and then to the exclusive use of inactivated vaccine.

Another sobering discovery that was made relatively late in the use of OPV was the realization that reverted poliovirus can not only cause paralysis in vaccine recipients and their immediate contacts but it can also establish chains of transmission in populations and cause outbreaks of paralytic polio. The first discovery of the so-called circulating vaccine-derived polioviruses (cVDPV) was made in Hispaniola in the year 2000 (Kew et al. 2002), but other earlier outbreaks caused by cVDPV were revealed retrospectively by comparing nucleotide sequences of the virus isolates (Centers for Disease Control and Prevention 2001). Since 2000, dozens of outbreaks caused by cVDPV of all three serotypes were identified (Kew et al. 2004; Centers for Disease Control and Prevention 2012). Most often, though, such outbreaks are caused by derivatives of the Sabin type 2 strain (Fig. 4.1). The largest cVDPV outbreak started in Nigeria in 2006 and is still not over at the time of this writing (Wassilak et al. 2011) and was triggered by viruses that emerged independently from multiple sources (Burns et al. 2013). The events that triggered this outbreak were first suspension of all vaccination activities for several months, followed by resumption of vaccination campaigns. A significant cohort of nonimmune children that emerged during the pause in polio immunizations may have provided a fertile ground for emergence and spread of cVDPV. Similar events took place on a much smaller scale in the former Soviet Union in the 1960s (Korotkova et al. 2003).

Yet another observation that increased doubts about continued use of OPV was the discovery of another type of vaccine-derived polioviruses, namely, those that

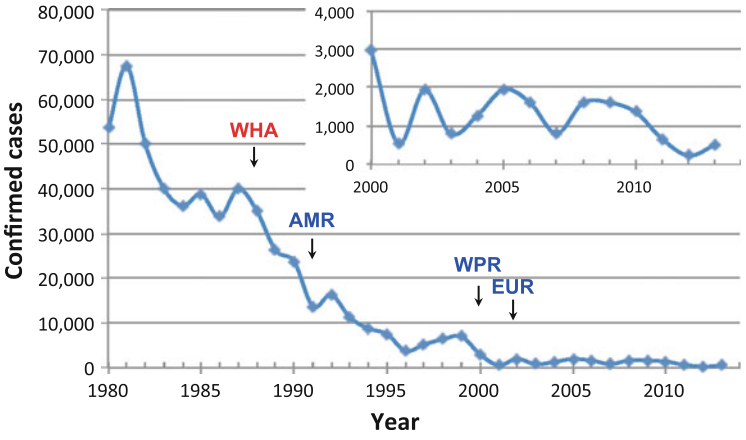

Fig. 4.1 Worldwide number of confirmed paralytic cases of poliomyelitis caused by wild polioviruses based on official WHO reports. Insert shows the incidence in the past 15 years. *Arrows* indicate the timing of the WHA decision to launch global polio eradication campaign and regional certifications in the America, Western Pacific, and European regions

were found in persons chronically infected with poliovirus (Feigin et al. 1971; Lopez et al. 1974; Davis et al. 1977; Minor 2001). Patients with some types of primary immunodeficiencies characterized by failure to produce antibodies (agammaglobulinemia) can become persistently infected with vaccine poliovirus during immunization and proceed to chronically excrete poliovirus for a prolonged period of time, often for years. Prolonged excretion of poliovirus was also observed in otherwise healthy people (Martín et al. 2004). These immunodeficiency-associated vaccine-derived polioviruses (iVDPV) may also regain virulence and in some cases were found to cause paralysis of their carriers (Hidalgo et al. 2003). Obviously, besides a threat to the patients, iVDPV are capable of reseeding the environment with virulent polioviruses and potentially restart virus circulation in regions where it has already been stopped (Minor 2009). Finally, one more type of vaccine-derived polioviruses, called ambiguous (aVDPVs), has been isolated from environmental samples (sewage, water, etc.) (Blomqvist et al. 2004; Cernáková et al. 2005; Centers for Disease Control and Prevention 2009; Roivainen et al. 2010). The origin of these viruses is unknown. However, since there is no natural reservoir for poliovirus except for humans, it is believed that aVDPVs are excreted by either unknown immunodeficient carrier(s) or are a result of cryptic circulation of cVDPV that continues undetected because of the absence of paralytic disease. In either case this phenomenon represents a significant concern, and discovery of the three types of VDPV has put to rest the previous dogma that Sabin viruses can revert only partially. It is now universally recognized that VDPVs can be as virulent as wild strains. The inevitability with which they emerge in countries using OPV has become a compelling justification for stopping OPV use in countries that eliminated transmission of wild poliovirus strains and replacing it with IPV.

The development that made the switch from OPV to IPV possible was the production in the 1980s by the Dutch National Institute for Public Health and the Environment (RIVM) of the enhanced potency IPV (eIPV) (van Wezel et al. 1984). Unlike the classical Salk vaccine that was made by formaldehyde inactivation of virus contained in harvests from cell cultures infected with poliovirus, eIPV was prepared by a more sophisticated process. First, instead of conventional monolayer cell cultures, cells were grown on a suspension of microcarrier beads in bioreactors. This resulted in a much higher cell density and increased virus yields. Second, the virus was purified from the harvest by a combination of size-exclusion and ion-exchange chromatographies and was largely free from most cellular components. As a result each dose of IPV could contain a greater amount of antigen leading to its higher potency. This new technology that was developed by a government public health institution was then quickly adopted by a number of large vaccine manufacturers and is now the basis for all IPV produced in the world. This technological breakthrough resulting from the successful interplay of public and private sectors was described in detail in an excellent review by Blume (2005).

The process of gradual replacement of OPV with eIPV is continuing as circulation of wild polioviruses is stopped in more countries and as they improve their economical circumstances making a more expensive IPV option a viable alternative to OPV. Replacement of OPV with IPV was facilitated by the introduction of

combination vaccines in which IPV is added to other antigens, including vaccines against diphtheria, tetanus, acellular pertussis vaccine, as well as hepatitis B or *Haemophilus influenzae*. This allowed IPV to be introduced without adding more injections to the existing immunization schedules. Describing further development of polio vaccines and the reasons behind it requires us to cover one of the most important public health endeavors of the past 25 years, namely, the worldwide polio eradication campaign.

4.4 Polio Eradication

The introduction of IPV in 1955 triggered a significant decline in polio incidence, and the switch to OPV in most countries in the early 1960s continued this trend so that by the next decade, polio was no longer a significant problem in developed parts of the world. However, it continued to actively spread in resource-limited countries mostly because of the inadequate vaccine coverage. The idea of polio eradication was proposed by Albert Sabin based on the absence of an animal reservoir for the virus (Sabin 1961b, c, 1965; Hampton 2009). His strategy envisioned the use of OPV in mass campaigns conducted during a short time, often just 1 day when all children in the target age group (usually between 0 and 4 or 5 years old) would receive vaccine simultaneously regardless of their prior immunization status. These campaigns, which were later called National Immunization Days (NID), were aimed at stopping circulation of wild polioviruses.

The first organized polio eradication campaign was proposed by the Pan American Health Organization in 1985, which resolved to completely eliminate polio from the Americas (de Quadros 1992, 1997). The initiative was strongly supported by the Rotary International organization that continuously remained one of the key players in the worldwide campaign, US Agency for International Development (USAID), UNICEF, Inter-American Development Bank, and other donors. In addition to the NIDs, the campaign relied on extensive epidemiological monitoring based on acute flaccid paralysis (AFP) surveillance (Andrus, de Quadros et al. 1992). AFP is the primary clinical manifestation of poliomyelitis but can also result from other infectious and noninfectious causes. Its incidence throughout the world is rather uniform (1–2 cases a year per 100,000 of population). This creates a possibility to evaluate the quality of local surveillance systems: reported rate below this level indicates the need for improved surveillance. Each case of AFP is followed up, including virological examination to confirm or reject the diagnosis of poliomyelitis. Further differentiation between wild and vaccine polioviruses and among serotypes is performed by immunological tests and nucleotide sequencing that also enables to determination of the phylogenetic relatedness of the isolates. This powerful molecular epidemiology approach helps to trace virus transmission and identify the source of virus that caused each paralytic case (Kew et al. 1990).

The campaign in the Americas was highly successful and resulted in complete elimination of polio in 1991—just 6 years after the start of the program. This prompted the World Health Assembly, the governing body of the WHO, to resolve

in 1988 that polio should be eradicated worldwide by the year 2000. The strategy was similar to that used in the Americas (Chumakov and Kew 2010). The world was divided into six regions that coordinated immunization campaigns, tracked their progress, and reported it to the WHO headquarters. Stopping wild polio circulation in each region followed by a period of extensive surveillance leads to regional certification. After all regions are certified free from circulation of wild viruses, poliovirus would be declared eradicated worldwide after 2 years with no paralytic cases or isolation of wild poliovirus from patients or the environment. During the 12 years during which global eradication was expected to be completed, there was a dramatic decline in the incidence of disease (Fig. 4.2). The number of endemic countries declined from 125 in 1988 to 20 in 2000 and to just 3 at the time of this writing. The transmission of wild type 2 polioviruses was completely interrupted in 1999, and type 3 appears to have been eliminated in 2012. The number of independent genetic lineages has significantly decreased. All these indicators suggested that the program was moving in the right direction, but progress was stalled at the turn of the century because of a variety of factors that will be discussed below. As a result 25 years after the inception of the eradication campaign, there are still three countries in which transmission has never been interrupted (Pakistan, Afghanistan, and Nigeria), and progress in some regions is compensated by unexpected outbreaks of the disease in others. In May of 2014 this prompted WHO to declare poliovirus spread a public health emergency of International concern.

The new global strategy adopted in 2013 envisions that wild polioviruses circulation will be interrupted in 2015 and that the final certification could be achieved in 2018 (WHO 2013). These optimistic projections are based on the recent progress, but since many similar predictions in the past have turned out to be incorrect, we must remain cautious.

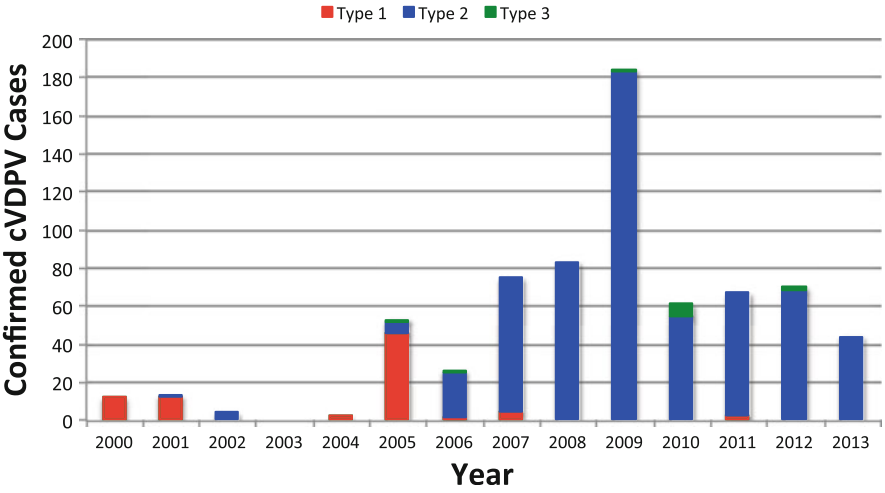

Fig. 4.2 Worldwide number of confirmed paralytic cases of poliomyelitis caused by circulating vaccine-derived polioviruses. Data from http://www.polioeradication.org

The reasons for the failure to eradicate poliovirus during the originally projected timeframe include previously unknown aspects of poliovirus biology, as well as complex social, economical, and religious factors, and the deteriorating security situation in many regions of the world. Vaccination of children, and especially conducting NIDs, is complicated if not impossible in the areas with active military conflicts. Prejudice against vaccination that exists and even promoted in some societies requires significant efforts on the part of the campaign to overcome active resistance to the immunization activities. Protracted campaigns also contribute to the fatigue of local public health workers that gradually lose faith its ultimate success. All these factors are beyond medicine or science and are difficult to overcome. In this chapter we will only review the new scientific knowledge that was derived from developments of the past 15 years that are relevant to the future strategy of dealing with polio, including creation of new vaccines.

One important factor contributing to the slowdown of progress of polio eradication was an unexpectedly low efficacy of OPV in some regions. For instance, in some states in northern India the per-dose seroconversion rate was found to be less than 10 %, requiring multiple repeated vaccinations to reach the population immunity level of 85–90 % needed to stop virus transmission (Patriarca et al. 1991; Grassly et al. 2006, 2007; O'Reilly et al. 2012). At this low level of immunogenicity, more than 15 doses of vaccine were needed to immunize most children, which takes up to 2 years. In some states in India with the most resilient circulation of wild polioviruses, every child under 5 years of age was immunized 10 times a year, bringing the total number of doses to 50 (!). Combined with extremely high birth rates, a significant susceptible population of children remained despite extraordinary immunization efforts. The only solution to this problem could be to increase the immunogenicity of OPV. Part of the reason for the low efficacy was interference among the three serotypes of vaccine virus after administration of trivalent OPV. To minimize the interference, monovalent vaccines against serotypes 1 and 3 were used (Nasr El-Sayed et al. 2008; John et al. 2011) supplementary to routine vaccination with trivalent OPV. The rationale behind this change was that type 2 poliovirus is the most robust of the three Sabin strains and strongly competes with the other two. In addition, wild type 2 poliovirus was eradicated in 1999, and therefore maintaining high immunity against type 2 poliovirus was a lower priority than stopping transmission of types 1 and 3. Introduction of monovalent OPV1 and OPV3 and then bivalent OPV1 + 3 vaccine succeeded, and the circulation of wild polioviruses in India was interrupted in 2011 (John and Vashishtha 2012; Kaura and Biswas 2012; O'Reilly et al. 2012).

The next unexpected revelation about the biology of poliovirus was the discovery in year 2000 of circulating vaccine-derived polioviruses that was already discussed above. There is a consensus among scientists that VDPVs are as virulent as wild strains of the virus, and must be looked at similarly (Agol 2006; Dowdle and Kew 2006; Minor 2009). Therefore, eradication of poliovirus must include not only wild strains but also VDPVs. Furthermore, since the only way to avoid emergence of VDPV is to stop the use of OPV, eradication can only be possible when the use of vaccine that led to eradication is terminated as well. The original solution to this

central paradox of polio eradication was to stop OPV use synchronously after global certification (Dowdle et al. 2003). The safety of this approach, however, is untestable and is fraught with danger. Serious doubts about the prudence of this approach were strengthened by the discovery of the so-called orphan polioviruses isolated in regions that were believed to be free from polio circulation for several years and that were genetically linked to the old local strains by using a "molecular clock" method (Jorba et al. 2008). This could be a result of either breaches in surveillance or a cryptic circulation of poliovirus in communities without overt clinical manifestations or a combination of both. Regardless of the reasons, the phenomenon of orphan polioviruses cannot be discounted in discussions of the strategy of OPV withdrawal because it is very hard to reach absolute certainty that polio is no longer present in a given community. Therefore, the current strategy envisions that availability and universal introduction of IPV is a prerequisite to withdrawal of OPV (WHO 2013). Since wild type 2 polioviruses were eliminated in 1999, the only source of type 2 paralytic polio is attributed to VDPV. Therefore, replacement of trivalent OPV by bivalent OPV1 + 3 for routine immunization could eliminate these cases and also be a test case for eventual withdrawal of all OPV vaccinations. Since wide use of bivalent OPV to stop transmission in endemic countries may have been linked to increased incidence of type 2 cVDPVs as a result of the diminished population immunity to this serotype (Arita and Francis 2011; Arya and Agarwal 2011), the switch from tOPV to bOPV must occur only in the context of maintaining high population immunity by switching to IPV.

There is no consensus at this time about whether the replacement of OPV with IPV must be done on an interim basis until there is more certainty that all wild viruses and VDPVs are removed from circulation and all stocks of poliovirus (including OPV) are securely contained or destroyed, or the use of IPV must continue indefinitely (Agol et al. 2005; Chumakov et al. 2007; Ehrenfeld et al. 2008). The arguments in favor of the first solution are based on saving of public health resources, which was the primary justification of the entire eradication campaign. On the other hand withdrawal of all protection against poliovirus will create an unprecedented epidemiological situation with the entire population born after OPV cessation being susceptible to the disease. This would create a significant vulnerability to accidental or intentional release of poliovirus back into circulation that could trigger a new pandemic of unpredictable proportions. This scenario becomes even scarier considering that poliomyelitis acquired by nonimmune adults is clinically more severe than the disease in infants and children. Therefore, after passage of some time, the entire population would become susceptible to a highly contagious and deadly/crippling disease, and poliovirus could become an ideal bioterrorism weapon. Containment of poliovirus and even complete destruction of all its stocks can diminish these concerns, but cannot resolve them completely. First, it is very difficult if not impossible to verify containment and destruction, but more importantly, modern technology allows live poliovirus to be synthesized from chemicals within a short time and at a very low cost (Cello et al. 2002). Thus, to many experts in the field, it appears increasingly likely that immunization against poliovirus must continue indefinitely.

It may be appropriate at this point to draw some parallels with the eradication of smallpox. While being a more deadly and contagious disease, eradication of smallpox was by far a more straightforward endeavor. The main distinction is that the diagnosis of smallpox is much easier, can be based on a quick examination, and does not require sophisticated laboratory procedures including nucleotide sequencing as is the case for poliovirus. The second difference is the very high disease to infection rate for smallpox: most susceptible individuals who were infected with variola virus developed the disease with its characteristic symptoms. In contrast, only one of a few hundred children infected with poliovirus proceed to develop any symptoms, making it very hard to quickly identify outbreaks of the disease. A good example is the first outbreak of cVDPV in Haiti and Dominican Republic that went undetected for the first 1½ years (Kew et al. 2002). These differences, combined with frequent and often severe adverse reactions to smallpox vaccinations, were a compelling reason to stop immunization against smallpox. However, decades later concerns about bioterrorism led to the development and stockpiling of a new generation of smallpox vaccines with an improved safety profile that is now ready to be used in case of emergency. Theoretically, a similar approach could be used for poliomyelitis, but difficulties in timely diagnosis will make such emergency response ineffective and will likely result in a new pandemic of poliomyelitis unless a sufficient level of population immunity is maintained universally.

These considerations take us to the next question of what is the ultimate objective of any eradication campaign and what is the strict definition of the term. Dealing with any infectious disease can go through three phases (Dowdle and Birmingham 1997; Dowdle 1998). First is control, i.e., application of preventive measures (e.g., vaccination) that lead to reduction of the disease burden to a socially acceptable level, which is maintained by continuous prophylaxis. The next phase is elimination, which is similar to control but reduces the morbidity to zero. Elimination is sustained by continuous vaccination to maintain high immunity levels that prevents the spread of the pathogen. Finally, eradication also means the complete absence of morbidity, but unlike elimination, it no longer requires preventive measures and vaccination. From the considerations presented above, it is clear that complete stopping of all polio vaccination is not prudent in the foreseeable future, and therefore in the strict sense of the word, the campaign should rather be called elimination but is referred to as eradication mostly for historical reasons.

4.5 New Generation of Polio Vaccines

As discussed above, continued use of OPV has become unacceptable because of safety and ethical considerations. However, its replacement with IPV involves significant challenges. The most important of these include its higher cost and the need for intramuscular injections delivered by qualified medical personnel. Another problem is that the lower ability of IPV to induce mucosal immunity precludes the

ability to break chains of transmission of the virus (Anis et al. 2013). Finally, current IPV is manufactured from highly virulent virus, which poses production biosecurity risks. Therefore, a new generation of polio vaccines is being explored for use after eradication, with properties that include lower cost, increased ability to induce mucosal immune responses, and addressing the biosecurity concerns (Ehrenfeld et al. 2009). For a live vaccine, a more genetically stable virus that would not revert to virulence would be essential. The current research and development efforts described below include both new OPV and IPV vaccines.

Elucidation in the 1980s and 1990s of the molecular mechanisms of poliovirus attenuation and reversion to virulence led to several efforts to create attenuated strains with higher genetic stability. Most of these efforts were aimed at restricting the emergence and accumulation of point mutations responsible for reversion. Since most VDPV strains are recombinants between Sabin strains and other non-polio enteroviruses, it is believed that recombination may also play a role in reversion to virulence. Evaluation of genetic stability is performed in vitro (in cultured cells) and in vivo (in animal experiments), but ultimately vaccine safety must be confirmed in humans. While several studies reported increased stability as measured in vitro, proving it in clinical studies represents a major challenge. Given the relatively low frequency of vaccine-induced complications (1 in about 600,000 first doses), to achieve the statistical power needed for definitive conclusions about the superiority of a new strain would require a clinical study of unprecedented size. Another consideration that complicates the development of a more stable attenuated strain is the absence of reliable in vitro or animal biomarkers of poliovirus safety. For this reason there have not been many studies in this direction until the creation of a consortium of several laboratories funded by the Bill and Melinda Gates Foundation that was tasked to develop a more genetically stable strain of type 2 OPV. At the time of this writing, the work is still ongoing. Therefore, we can only describe the general principles employed in this work.

One of the determinants of virulence and attenuation are mutations in a stem-loop domain (designated the F-domain of stem-loop VI) of the 5′-untranslated region. This domain is part of an internal ribosome entry site (IRES) and is believed to be involved in the interactions between translation initiation factors and the ribosome and the viral RNA molecule (Guest et al. 2004; Kauder and Racaniello 2004). It was reported that some of these factors are tissue-specific, and thus mutations in this region may affect tissue tropism and restrict virus replication in neuronal cells. Recombinants in which this region of poliovirus was replaced with the homologous element from human rhinoviruses were found to be strongly attenuated (Gromeier et al. 1996; Chumakov et al. 2001). These rhinovirus-poliovirus chimeras are now studied for their use as oncolytic agents against gliomas (Dobrikova et al. 2012). Such chimeric viruses could potentially be used as vaccines with improved stability.

Another approach aimed at the same attenuation determinant takes advantage of the observation that structural stabilization of this stem-loop structure leads to increased virulence, while its destabilization leads to attenuation. For instance, attenuation of type 3 poliovirus was achieved by mutating a stable G:C pair to a

weak G:U pair that destabilized the entire hairpin structure. During reversion, this G:U pair is replaced by the original G:C pair. Stability of A:U pairs is intermediate between G:C and G:U, so if the RNA hairpin is reengineered by replacing G:C and G:U pairs with A:U, the overall stability of the structure will remain roughly unchanged, and the virulence of such virus will also stay the same. This change, however, will result in higher genetic stability because it takes two mutations to convert an A:U pair to a more stable G:C pair, and the intermediates in this process (either G:U or A:C pairs) have a lower structural stability and hence lower fitness. A number of constructs created based on this principle were shown to have superior genetic stability and are now being considered as candidates for a more genetically stable vaccine virus (Macadam et al. 2001, 2006; Rowe et al. 2001).

Another way to impair the function of the IRES element is to delete or insert additional nucleotides, which leads to distortion of its overall conformation. Such manipulations, however, are not stable because virus can easily restore fitness by excising the inserts or filling the deletions with an unrelated piece of RNA of similar size from other sources. A way to overcome this instability was proposed by Wimmer and his colleagues, who took advantage of a cis-acting replicative element (*cre*) in viral RNA. Normally located in the center of the RNA molecule, it is critically important for initiation of RNA replication. Transplantation of the *cre* element from its normal position to the IRES region in the 5′-UTR strongly attenuated the virus (Toyoda et al. 2007). Since *cre* plays a critical role in RNA replication, the virus cannot excise this element, and thus the resulting attenuated constructs are genetically stable.

Viral RNA replicases are notoriously error prone, generating a lot of mutations and being one of the reasons for the genetic instability of viral RNA genomes. Despite the obvious problems created by high mutation rates, the ability to rapidly generate mutations gives viruses some advantages by allowing them to rapidly adapt to growth in new or changing environments. Therefore, the fidelity of viral replicases is optimized not to be very high or very low. This was demonstrated by the discovery of mutations in the polymerase gene that result in mutant replicase with increased fidelity (Pfeiffer and Kirkegaard 2003), which had an impaired ability to infect animals (Vignuzzi et al. 2006, 2008). This observation suggested the use of high-fidelity polymerase mutants to (1) decrease the rate of reversion and (2) provide an additional mechanism of attenuation.

All organisms including polioviruses have a certain bias in the use of synonymous codons. This is widely used in biotechnology when a foreign protein is expressed in a heterologous system. To maximize the yield of its product, the gene coding for the target protein is recoded by using codons most frequently used in the expression system. This process is called codon optimization. In experiments with poliovirus it was found that the reverse process—codon deoptimization (i.e., engineering viral genomes to use codons that are normally avoided in the poliovirus genome)—reduces viral fitness and decreases the yield of infectious virus (Burns et al. 2006). The resulting crippled virus cannot easily revert to restore its fitness because the change was a result of multiple mutations in different parts of the genome.

The mechanism by which codon deoptimization reduces viral fitness may be more complex than simply using rare codons. Besides codon usage bias, most organisms also manifest a codon pair bias (Gutman and Hatfield 1989). It means that there is a preference in the way codons coding for neighboring amino acids are selected: some codon pairs are used more frequently than others. If this order is changed by swapping different synonymous codons in the sequence, the result is similar to codon deoptimization, even though the overall codon usage remains unchanged (Coleman et al. 2008). The reason behind codon pair bias is yet to be established. To complicate the situation even further, it was found that in the poliovirus genome the frequency of G following C (the presence of dinucleotide CpG) and A following U (UpA) is lower than would be expected in a random sequence. If poliovirus RNA is recoded into a sequence with a higher number of CpG and UpA dinucleotides, the size of its plaques decreases proportionally to the number of changes introduced (Burns et al. 2009). For viruses generated by all these "genome scrambling" approaches, the yield of infectious virus decreases significantly, while the yield of physical particles is affected to a smaller degree. The biological mechanisms behind these phenomena are still unknown, as well as it is unclear whether all these observations represent the same phenomenon or have distinct reasons behind them. Nevertheless genome scrambling may have important applications in the development of attenuated and inactivated vaccines (Mueller et al. 2010).

So far we have described novel rational ways to attenuate virus in a more stable way and to restrict reversion by preventing point mutations. Another aspect of the search for a more genetically stable poliovirus is to try to restrict its ability to recombine with other viruses. Poliovirus and enteroviruses in general are highly promiscuous and recombine with high frequency (Cooper 1977; Furione et al. 1993; Agol 1997; Combelas et al. 2011). This property is highly advantageous because it allows them to evolve rapidly and to mitigate the damage caused by point mutations by replacing defective parts of their genome with functional pieces hijacked from other viruses. It appears likely that recombination helps vaccine viruses to replace parts of their genome that were crippled by attenuation and as a result to regain some fitness. Therefore, restricted recombination frequency may be a desirable property for an improved vaccine strain.

The work in this direction is complicated by our limited knowledge about the mechanisms of recombination. It is believed that homologous recombination plays an important role for poliovirus. Therefore, recoding relevant portions of the vaccine poliovirus genome to minimize homology with other viruses may reduce recombination frequency. Finding polymerase mutations with lower intrinsic recombination frequency could also be helpful in limiting the ability of viruses to exchange parts of their genome (Runckel et al. 2013). However, the ultimate utility of these approaches is unknown. It is still unclear whether recombination events themselves or selection based on fitness are the rate-limiting step that determines the frequency of the emergence of recombinant viruses. Work in these directions is ongoing and as a minimum promises to produce new knowledge about this fascinating aspect of poliovirus biology.

The list of shortcomings of the current IPV includes its relatively high cost, the need for intramuscular injections, and the lower mucosal immune response. In the post-eradication environment, it will also be joined by biosecurity concerns since it is manufactured from highly virulent strains that must be grown in large quantities. Despite all best efforts to contain the virus, there will always be a small chance of accidental or intentional release of live virus into the environment, the consequences of which could be catastrophic. Therefore, it has been proposed that IPV manufacture should be based on attenuated strains with a better biosafety profile.

This work is being pursued in several directions. One obvious solution would be to make inactivated vaccine from the attenuated Sabin strains to produce what is now known as Sabin IPV (sIPV). An additional advantage of this solution would be to maintain a "warm base" for OPV manufacture, in case there should be a need to restart its production in the future. This work started in the early 1990s (Doi et al. 2001) and demonstrated that while the immunogenicity of type 1 Sabin IPV was at least as good as the immunogenicity of conventional IPV (cIPV) made from the wild Mahoney strain, the immunogenicity of IPV made from the two other serotypes of Sabin viruses, especially of type 2, was inferior to wild-type IPV (Dragunsky et al. 2004, 2006; Tano et al. 2007). Further development revealed that the amount of type 1 sIPV antigen needed to induce an immune response comparable to that of cIPV prepared from the Mahoney strain was significantly lower. The reverse was true for type 2 viruses (Westdijk et al. 2011). As a result the optimal composition of trivalent sIPV was different from that in the cIPV. As of this writing, sIPV was licensed in Japan (Shimizu 2012) and phase 3 clinical evaluation was completed in China. In Japan it is produced by Kaketsuken and Biken in the form of combination vaccines with diphtheria, tetanus, and pertussis (DTP) antigens for subcutaneous administration. Since there is no poliomyelitis in China or Japan, clinical studies of sIPV were performed using a seroconversion endpoint that demonstrated that with appropriate formulation its efficacy is comparable to conventional IPV. The Institute for Translational Vaccinology in the Netherlands (formerly a part of RIVM and NVI) supported by the World Health Organization has developed an sIPV production process (Verdijk et al. 2011) and licensed it to a number of manufacturers in developing countries. Therefore, the first of the new generation IPV is Sabin IPV, manufacture of which is believed to carry lower biosecurity risks.

There are still important questions about sIPV that need to be resolved. Some of them are related to standardization of this new class of IPV, selection of appropriate potency testing methods, and reference reagents. Other issues that need further studies are related to quantification of biosecurity risks associated with its production and the types of safety tests that should be a part of its manufacture. While intuitively it appears that using attenuated virus for making inactivated vaccine is safer than using wild strains, this risk is not easily quantifiable, because if released into circulation, Sabin viruses can easily regain their virulence (see discussion about VDPV). In addition, according to the current Global Action Plan adopted by the WHO (World Health Organization 2004), after wild virus circulation is stopped and OPV use is terminated, Sabin strains must be contained under the same

strict conditions as wild strains. Therefore, sIPV manufacturing facilities will have to be upgraded to BSL3/polio containment level, defeating a significant part of the reason behind its development and introduction. Therefore, while being a step in the right direction, sIPV may not be the ultimate solution for the future.

A number of research groups are also working on development of even safer alternative strains that could be used for IPV production. The main requirement for such strains is that they must be completely apathogenic and that this attenuated phenotype be stable in vitro and in vivo, so that they could not revert to virulence and restart circulation even if they were released into the environment. The approaches used for generating such stably attenuated viruses are similar to those that were discussed above in the section describing development of new OPV2. They include replacement of reversion-prone IRES elements of Sabin polioviruses with homologous regions from non-neurotropic viruses such as human rhinoviruses (Gromeier et al. 1996; Chumakov et al. 2001; Dobrikova et al. 2012), stabilization of attenuating domains in the IRES by reengineering the F-domain stem-loop using A:U pairs (Macadam et al. 2006), moving the *cre* element to the 5′-UTR (Toyoda et al. 2007), introduction of high-fidelity mutations in the polymerase gene (Vignuzzi et al. 2008), and scrambling coding sequences to alter codon usage bias, codon pair bias (Toyoda et al. 2007), or the number of CpG and UpA dinucleotides (Burns et al. 2009). Proof of principle studies performed for all these approaches in vitro showed that the resulting virus may have a higher genetic stability. However, whether they could be used for manufacture of a sufficient quantity of poliovirus antigen needed for IPV production and whether they will be more stable in vivo (and thus more acceptable from the biosecurity standpoint) are yet to be established. Obtaining reliable information about the latter aspect is quite challenging because there is no adequate preclinical (animal) model of poliovirus transmissibility and genetic stability in vivo.

The ideal solution to biosecurity concerns would be a manufacturing process that does not require any infectious virus. While antigens for many other vaccines can be successfully produced in various expression systems such as baculovirus, yeasts, etc. the difficulty for using this approach for poliovirus vaccine is that most if not all of its protective epitopes are formed by secondary or even tertiary interactions between stretches of amino acids from different polypeptide chains. Their activity is highly sensitive to conformational changes, and therefore only native virus particles can elicit protective immune response. There is no effective in vitro system of poliovirus assembly that could be used to produce amounts of poliovirus particles needed for vaccine manufacture. The assembly process of poliovirus capsids is quite complex and is not fully understood. However, it is known that it involves auto-proteolytic cleavage of one of the protein precursors that takes place only after RNA is encapsidated inside these particles and “locks” the entire structure in a proper conformation. Empty particles containing no poliovirus RNA that are produced during virus replication or during expression of poliovirus proteins are quite unstable. Stabilization by protein engineering may potentially solve this problem (Porta et al. 2013). This could open a way to producing immunogenic empty capsids to be used as vaccines in a process that would require no live poliovirus.

Another avenue of research and development of new inactivated poliovirus vaccines aims to lower the cost and/or improve their immunogenicity (thereby reducing the dose of antigen needed for inducing a protective immune response). Cost reduction could be achieved by increasing the yields of virus by introducing new manufacturing processes and cell substrates. It has been reported that use of suspension cultures of PerC6 cells in serum-free medium allows cells to grow to a much higher densities and results in a higher virus yields (Sanders et al. 2013). Another way to reduce vaccine cost is to use alternative routes of delivery that would increase immunogenicity and allow dose-sparing. Adding adjuvants is a well-known solution to increase immunogenicity and dose-sparing, and there are a number of groups actively exploring the use of various conventional and novel adjuvants in combination with poliovirus vaccines. Among conventional adjuvants, alum was shown to increase immunogenicity of IPV (Verdijk et al. 2013; Westdijk et al. 2013). Novel adjuvants such as oil-in-water adjuvants (Baldwin et al. 2011) and agonists of toll-like receptors and other components of the innate immune system are also under investigation. Some adjuvants were shown to also increase the mucosal immune response after intramuscular administration, and this aspect is also is under study (Ivanov et al. 2006).

The skin is the first line of defense against many pathogens and therefore contains many immunologically active cells, including dendritic cells and macrophages that scout for invading pathogens. Therefore, intradermal administration of antigens is believed to be more effective compared to intramuscular administration. Clinical trials with intradermal delivery of a fractional dose of IPV demonstrated that this is indeed the case, but the dose-sparing effect fell short of the target 1/5 of the intramuscular dose (Resik et al. 2010; Cadorna-Carlos et al. 2012; Nelson et al. 2012; Soonawala et al. 2013). Effective priming immunization after one intradermal dose of IPV was demonstrated by an anamnestic response to a booster dose of the vaccine (Resik et al. 2013). Therefore, intradermal delivery is a viable option that can also eliminate the need for injections if needle-less devices are used. Another possibility for intradermal delivery is the use of "microneedle patches" (Hiraishi et al. 2011; del Pilar Martin et al. 2012; Kim et al. 2012; Edens et al. 2013). These small arrays of dissolvable plastic microneedles coated with antigen can be painlessly applied to the skin similar to a Band-Aid to deliver IPV intradermally. The utility and efficiency of this approach are now under investigation.

All these new developments relate to stand-alone IPV that may play a role in the endgame of polio eradication and help to transition from OPV to IPV. However, in the long-term perspective, IPV will be used in combination with other antigens in the form of tetravalent (DTaP-IPV), pentavalent (DTaP-IPV-HiB or DTaP-IPV-HepB), or hexavalent vaccines combining all these antigens. Combination vaccines provide the maximum public health benefit while minimizing cost and the number of injections needed for vaccine delivery. Such vaccines are already used in developed countries, and affordable versions of combination products needed for the rest of the world may use some of the approaches described above.

Closely related to the development of novel poliovirus vaccines are attempts to create new tools that could mitigate their adverse effects. As discussed above, OPV can induce chronic infection in immunodeficient patients. At present there is no cure that would help these patients clear infection and stop shedding of virulent iVDPVs. Development of antiviral drugs is underway that promise to not only be useful for this purpose but that could potentially help in an emergency response to protect people if an outbreak occurs after eradication is complete or to treat people accidentally exposed to poliovirus (Collett et al. 2008). Passive immunotherapy could also be used for these purposes either alone or in combination with anti-poliovirus drug(s). Its efficacy was well-demonstrated in the pre-vaccine era (Hammon et al. 1952), but it was not used because of the difficulty of producing intravenous immunoglobulin. Monoclonal antibody technology has made it possible to create human antibodies highly effective against poliovirus (Chen et al. 2011, 2013), and their utility is being studied along with antiviral drugs.

4.6 Conclusions

The history of poliovirus vaccines represents a fascinating story of an evolving relationship between two highly effective vaccines, each having their advantages and disadvantages (Fig. 4.3). Being the first of two, IPV triumphantly demonstrated that polio can be successfully prevented but opened the Pandora's box of vaccine-

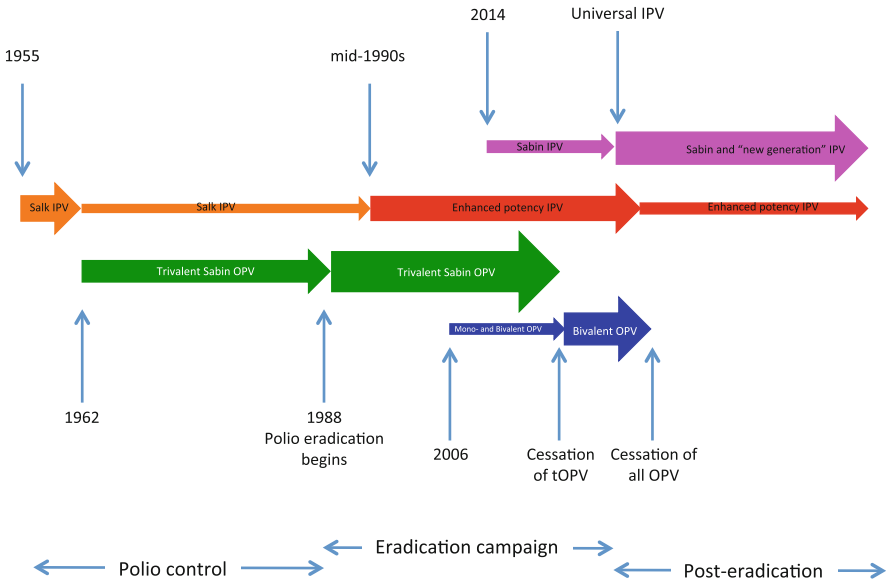

Fig. 4.3 Timeline illustrating evolution of polio vaccines

induced injuries that in turn led to the emergence of the modern regulatory and legal framework for vaccine development and use. This also opened the door for OPV that for many years was the vaccine of choice and led to remarkable progress in the control of poliomyelitis. This success of OPV will inevitably lead to its own demise and the need to be replaced by a safer inactivated vaccine. However, the new IPV is likely to be different from the IPV that we know now. Thus, the ever-changing epidemiological and socioeconomic landscape determines the need to continuously update the existing vaccines and to introduce innovative products that meet new challenges.

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Chapter 5
Current Status of Hantavirus Vaccines Development

Evgeniy A. Tkachenko, Tamara K. Dzagurova, and Peter E. Tkachenko

Abstract Hantaviruses are associated with two human diseases: hemorrhagic fever with renal syndrome (HFRS) in Eurasia and hantavirus pulmonary syndrome (HPS) in the Americas. These viruses are carried by persistently infected rodents and are transmitted to humans by aerosolized rodent excreta. The number of reported cases of hantavirus infection is growing in many countries. New hantavirus strains have been increasingly isolated worldwide raising public-health concerns. There is still no effective antiviral treatment against hantavirus infections. Prevention can be partially achieved by rodent avoidance, but it is not realistic in many endemic areas. The realistic preventive program has to be based on safe and effective multivalent vaccines specific for local epidemiological environment. This chapter summarizes the current status of hantavirus epidemiology and development of preventive strategy to control hantavirus infections. The current and novel hantavirus vaccines are discussed in terms of the demand, population at risk, and the potential market size for specific endemic areas.

5.1 Introduction

Hantaviruses (family *Bunyaviridae*, genus *Hantavirus*) are enveloped, single-stranded, negative-sense RNA viruses, carried primarily by rodents or insectivores of specific host species (Krüger et al. 2011). In humans hantaviruses cause two diseases, hemorrhagic fever with renal syndrome (HFRS) in Eurasia (Yanagihara and Gajdusek 1988) and hantavirus pulmonary syndrome (HPS) in the New World

E.A. Tkachenko (✉) • T.K. Dzagurova
Russian Academy of Medical Sciences, Moscow, Russia

Chumakov Institute of Poliomyelitis and Viral Encephalitides, Moscow, Russia
e-mail: evgeniytkach@mail.ru

P.E. Tkachenko
First Moscow Medical State University, Moscow, Russia

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(Nichol et al. 1993; Lopez et al. 1996). Four HFRS human pathogens are *Hantaan* (HTNV) and *Seoul* (SEOV) viruses in Asia (where approximately 90 % of worldwide incidences occur) and *Puumala* (PUUV) and *Dobrava*/*Belgrade* (DOBV) viruses in Europe. Two hantaviruses, *Sin Nombre* (SNV) and *Andes* (ANDV), cause most HPS cases in North and South America, respectively.

The clinical features of HFRS were first described in 1930s in north-central Sweden (Myhrman 1934; Zetterholm 1934) and in Russia Far East (Targanskaia 1935; Smorodintsev et al. 1959; Sirotin and Keiser 2001). Approximately at the same time, a similar disease was described in Manchuria, China (Ishii et al. 1942; Johnson 2001). The Swedes called the disease as epidemic nephropathy, while the Russians and Japanese as Far Eastern nephrosonephritis and Songo fever, respectively. During the Korean War (1951–1953), a disease, known as Korean hemorrhagic fever, appeared among several thousand United Nations personnel (Johnson 2001), leading to a quarter century of efforts to identify the causative agent (Schmaljohn 2009). In 1976 HTNV was finally isolated from the lungs of Korean field mice (Lee et al. 1978) and in 1981 the virus was cultivated in cell culture (French et al. 1981). Several diseases that were clinically similar were soon shown to be caused by viruses related to HTNV. In 1983 the term "HFRS" was adopted by the World Health Organization to consolidate the nomenclature of the diseases (Bull WHO 1983). In 1994, the clinical features of HPS were first described in the southwestern part of the United States (Duchin et al. 1994).

Each year approximately 60,000–100,000 HFRS cases are reported worldwide, mostly in China and Russia (Zhang et al. 2010; Tkachenko et al. 2013). The most severe forms of HFRS are caused by DOBV and HTNV, with 5–12 % mortality. PUUV and SEOV cause less severe infections with mortality rate less than 1 % (Vapalahti et al. 2003). Although HPS is much smaller in numbers with about 3,000 cases throughout North and South America during the 1993–2012 period, SNV, ANDV, and related viruses can cause HPS in the Americas with much higher fatality rate, ~35 % (Macneil et al. 2011). Humans get mainly infected from aerosolized rodent excreta, but HPS may be also transmitted from person to person (Enria et al. 1996).

There is still no effective antiviral treatment against hantavirus infections. The main treatment of severe HPS or HFRS cases is purely supportive, often in intensive care unit surroundings. This means mechanical ventilation or even extracorporeal membrane oxygenation for HPS and all forms of extracorporeal blood purification (mostly hemodialysis) for HFRS (Maes et al. 2009). Ribavirin is not widely available and should only be given intravenously at early stage of the disease. In practical terms, the drug is applicable only during outbreaks caused by highly pathogenic Hantaan virus in Korea. In China encouraging results have been obtained only when ribavirin was given during the first 5 days after onset (Huggins et al. 1991). In a limited field study of HPS in the United States, no convincing beneficial effect could be demonstrated with ribavirin (Mertz et al. 2004).

The reported cases of hantavirus infections are increasing in many countries, and new hantavirus strains have been increasingly identified worldwide, which constitutes a public-health problem of increasing global concern. Hantavirus infection

might be underestimated even in countries were the disease is known due to its clinically asymptomatic and nonspecific mild manifestations. The lack of simple and validated diagnostics complicated diagnosis in hospitals (Bi et al. 2008). In addition, the increasing domestic and international travel exacerbates the risk of infection. Nevertheless, hantavirus-induced diseases are easily preventable as far as safe and efficacious vaccines are available.

5.2 Epidemiology of Hantavirus Infections and Rational for Vaccine Development

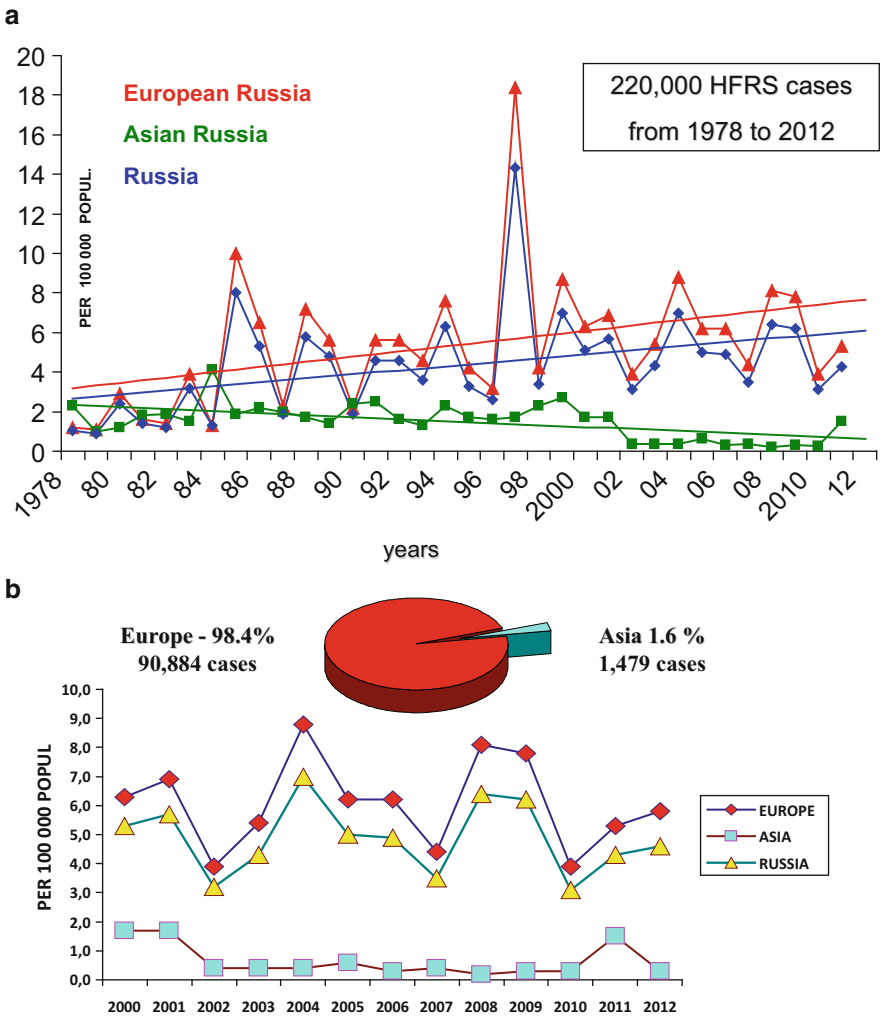
5.2.1 Hemorrhagic Fever with Renal Syndrome in Russia

The clinical features of the first HFRS cases in Russian Far East were described by Targanskaia in 1935 (Targanskaia 1935). The disease was called "hemorrhagic nephrosonephritis" (Churilov 1941). It was long believed that the area of distribution of this infection was limited to Far Eastern part (Amur River basin) of Russian. Therefore, retrospectively so-called Tula fever, known since 1930, can be considered as the first discovery of HFRS in Russia. The disease attracted attention of physicians in 1930 in Tula region, 120 km from Moscow, where during 5 years (1930–1934) 95 cases of "Tula fever," including 5 fatal cases, were reported in 1936 (Terskikh 1936). For a long time, "Tula fever" was considered, without sufficient evidence, as a peculiar leptospirosis and then as a rickettsiosis. In 1958–1959, during a large outbreak (850 cases), a quite conclusive clinical and pathoanatomical evidence of the identity of "Tula fever" with Far Eastern "hemorrhagic nephrosonephritis" was obtained.

The perception exists that in the 1950s–1960s, the disease was considered as a major medical problem in the European Russia and the end of the 1960s the disease was registered in 18 administrative regions under different names (Tula, Yaroslavl, Ural fevers, etc.). In 1954 M. Chumakov proposed the name "hemorrhagic fever with renal syndrome." In 1983 this name was recommended by the WHO Working Group to unify a nomenclature of very similar clinical diseases in Europe and Asia (Chumakov 1963; Bull WHO 1983).

Since 1978 (when HFRS has been included in the official reporting system of the Russian Ministry of Public Health) to 2012, a total of 220,177 cases had been registered in 57 from 83 administrative regions of Russia with annual average morbidity rate ~6.5 per 100,000 population. Among these cases, 214,744 cases were reported from 46 out of 58 administrative regions of the European Russia (97.5 % of total HFRS cases) and 5,433 cases from 11 out of 25 regions of the Asian Russia (2.5 %). Human epidemics have had cycles with a frequency of 3–4 years (Fig. 5.1).

The analysis of the dynamics of morbidity due to HFRS in the twenty-first century has not allowed to reveal the tendency in reduction of HFRS morbidity in



YEARS	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	TOTAL
EUROPE	7,246	8,203	4,480	6,133	10,126	7,152	7,107	5,005	9,306	8,954	4,484	5,982	6,706	90,884
ASIA	129	134	123	111	111	196	90	122	69	109	88	109	88	1,479
RUSSIA	7,375	8,337	4,603	6,244	10,237	7,348	7,197	5,127	9,375	9,063	4,572	6,091	6,794	92,363

Fig. 5.1 HFRS morbidity in Russia. (**a**) 1978–2012, (**b**) 2000–2012

Russia (annual average morbidity of more than 7,000 cases) (Fig. 5.1). The distribution of HFRS in Russia was found to be scattered throughout the country. However, different geographical regions are distinguished by the morbidity rates due to HFRS that vary considerably. In the Asian Russia, 93 % HFRS cases were

registered in four Far Eastern regions (Primorsk, Khabarovsk, Amur, and Jewish regions) and significantly less in the Western Siberia with the lack of reported cases in Eastern Siberia (Tkachenko et al. 2013). In the European Russia, most high rates of annual HFRS incidence occur in the eastern area. Here there are 11 administrative regions with high HFRS morbidity (20 per 100,000 population) including Bashkiria region with the highest morbidity in Europe. Practically 40 % of all HFRS cases in Russia are registered on the territory of Bashkiria with annual average morbidity rates of more than 50 per 100,000 (Tkachenko et al. 1999, 2013). During the last years, in addition to the primary factor, massive reproduction of bank voles infected by PUUV, very extensive construction activity of people coming from cities to rural endemic areas to build country houses resulted in significant increase of human contacts with infected rodents and in increase of HFRS morbidity in European Russia.

In general, in Russia morbidity is higher in rural areas as compared to urban. However, in Eastern European endemic area morbidity rate in large cities is approximately three times higher than those in rural areas. Most HFRS cases in the European Russia occurred during the summer and fall, while cases in the Far Eastern regions of Asian Russia occurred in fall and winter. About 70 % of the total HFRS patients were in the 20–49-year age group; children under the age of 14 years represented approximately 5 % of the cases. Males outnumbered females by a ratio of 4:1. The analysis of risk factors showed that the major risk was associated with occasional activities in the forest, gardening, and farming activities (Tkachenko et al. 1999, 2013).

Results of serological prospective studies of convalescents who were diagnosed with HFRS more than 25 years ago showed a long-term persistence of hantavirus-specific antibodies (Myasnikov et al. 1986). In Russian endemic areas hantavirus antibody prevalence rate was found to be different. The highest seroprevalence was observed in highly endemic HFRS areas with the highest rates of natural infection (up to 30 % in Bashkiria). Among the random population without clinical manifestations of HFRS, the seropositive men-women ratio is 2:1. However, among the HFRS patients, this ratio is 4:1. The difference may be explained primarily by the fact that HFRS in women is frequently diagnosed as pyelonephritis and other diseases with mild and even asymptomatic manifestations. More frequent antibody findings in subjects of older ages may be explained by increasing number of human contacts with sources of infection later in life. The fact of detection of hantavirus antibody in healthy individuals may be explained by milder, even asymptomatic nature of the infection, as well as by misdiagnosis.

Evidence for the mode of transmission of hantavirus to humans derives principally from epidemiological observations. Experimental evidence of hantavirus transmission within rodent population provided additional view on the way how the virus is transmitted to humans. The natural hantavirus infection in rodents indicates that the virus persists in rodent reservoir and causes chronic, apparently asymptomatic infection and shedding over a long period of time with urine, feces, and respiratory secretions (Gavrilovskaya et al. 1990). Aerosolized droplets containing the virus are sufficient to transmit hantavirus horizontally among

rodents. Evidence for respiratory route of hantavirus infection was demonstrated during two laboratory outbreaks involving 126 HFRS cases (Kulagin et al. 1962; Tkachenko et al. 1999). The source of unforeseen airborne infected dust was identified in large shipment cages containing forest mouselike rodents brought to the research institute's animal facilities from natural foci of infection and kept in large cages for 1–3 months. Bank voles (*M. glareolus*) were predominant among the trapped forest rodents. In a number of cases, the airborne transmission could be the only possible way of human infection.

Thus, numerous epidemiological studies of infections acquired in natural conditions suggest that close human contact with rodents should be a risk factor for hantavirus infection. The victims are primarily persons who are working permanently in accordance with their occupational duties in active natural HFRS foci or those who only visit endemic areas periodically but frequently enough to be infected by virus from wild animals. There is no evidence of human-to-human, secondary transmission or nosocomial HFRS outbreaks in Russia.

Analysis of results of hantavirus antigen detection in lung tissues of about 70 species of small mammals showed that practically each landscape zone has natural foci of the infection with different levels of virus circulation. Hantaviruses were hosted by different rodent species in all analyzed areas as it was shown by antigen detection in mammals belonging to different species (Tkachenko et al. 1987; Slonova et al. 1985; Gavrilovskaya et al. 1983a; Ivanov et al. 1989). Hantavirus antigen was also detected in tissues of 13 species of birds, trapped in the Russia Far East (Tkachenko and Lee 1991).

The first hantavirus strains were isolated in Russia at the end of the 1970s by using bank vole laboratory colonies (Gavrilovskaya et al. 1983b) and since 1983 in Vero-E6 cell cultures (Tkachenko et al. 1984). Using tissue cultures, more than 100 hantavirus strains were isolated from HFRS patients and necropsy materials, rodent lung tissues from 8 different species, and from 1 species of birds (Tkachenko et al. 1984, 2005a; Ivanidze et al. 1989; Slonova et al. 1992, 1996; Dzagurova et al. 1995; Klempa et al. 2008). Immunological studies and genotyping of hantavirus strains revealed at least eight hantavirus species circulated in Russia: HTNV, PUUV, SEOV, DOB/BELV, TULV, KHBV, TOPV, and HTNV-like (Amur/Soochong virus) (Slonova et al. 1990; Niklasson et al. 1991; Plyusnin et al. 1994, 1996; Tkachenko, 1995; Horling et al. 1996; Dekonenko et al. 1996; Yashina et al. 2001). The vast majority of rodents and insectivore species as well as other mammal and bird orders harboring hantavirus are probably ancillary hosts. Currently the epidemiological significance of certain rodents is established in different regions of Russia. In Russia Far East, HFRS cases are etiologically associated mainly with HTNV, with HTNV-like (Amur/Soochong virus), and, in the less extent, with SEOV. The principal hosts of these viruses are *A. agrarius*, *A. peninsulae,* and *R. norvegicus*. HFRS cases registered in European regions are caused mainly by PUUV associated with bank vole, *M. glareolus,* and less by DOB/BELV associated with two species, *A. agrarius* (central European regions) and *A. ponticus* (southern regions). The principal hosts of TULV, KHBV, and TOPV are *Microtus arvalis*, *Microtus fortis,* and *Lemmus sibiricus,* respectively.

Recently, novel hantaviruses have been discovered in the Black Sea coast area of European Russia, and major's pine vole, *Microtus majori*, was identified as a novel hantavirus host (Klempa et al. 2013a). The newly discovered hantavirus, provisionally called "Adler" virus (ADRV), is closely related to TULV. Amino acid differences with TULV (5.6–8.2 % for nucleocapsid protein and 9.4–9.5 % for glycoprotein precursor) are on the border line of the current ICTV species definition criteria (7 %). Sympatric occurrence of ADRV and TULV in the same region suggests that ADRV is not a geographical variant of TULV but a host-specific taxon. High intracluster sequence variability indicates the long-term presence of the virus in this region. The pathogenic potential of ADRV needs to be determined.

Until recently, HFRS cases in European Russia were associated with PUUV only. However, during the last years in Central European Russia, three large HFRS outbreaks caused by DOB/BELV were detected (more than 700 cases). A detailed investigation of outbreaks had revealed the striped field mouse (*Apodemus agrarius*) as a virus reservoir. In addition, the *A. agrarius*-borne DOB/BELV lineage (DOB-Aa) or genotype Kurkino (DOB/KURV) was identified as the causative infectious agent (Klempa et al. 2008, 2013b). The results of comparative analyses of epidemiological data of PUUV-HFRS and DOB/KURV-HFRS outbreaks indicate that 97 % of total DOB/KURV-HFRS cases were diagnosed in rural and only 3 % in urban areas (Tkachenko et al. 2005b; Mutnykh et al. 2011). At the same time, 30 % of PUUV-HFRS cases in Bashkiria were diagnosed in rural areas and 70 % of cases were found in urban areas. Most PUUV-HFRS cases were diagnosed during August–December with the HFRS peak in October, while DOB/KURV-HFRS cases were diagnosed during November–March peaking in December. However, clinical symptom differences between PUUV-HFRS and DOB/KURV-HFRS diseases were not identified. Analysis of risk factors showed that in PUUV-HFRS area, the major risk factors were linked with a short-time stay in the forest (55 %), gardening, and farming activities (36 %), while those in DOB/KURV-HFRS area were connected with hibernal cattle breeding (73 %) and other agricultural activities (25 %).

In 2000, DOB/BELV hantavirus was detected in the Sochi region, southern part of European Russia. At the same area HFRS cases were diagnosed among febrile patients (Tkachenko et al. 2005a). It suggests that the *A. ponticus*-born DOB/BELV lineage (DOB-Ap) or genotype Sochi (DOB/SOCV) hantavirus associated with the Black Sea field mouse, *Apodemus ponticus* (a novel host rodent), is the causative agent of the human HFRS. *A. ponticus* is naturally spread in the southern European Russia and in regions between the Black and the Caspian Sea. The Sochi virus was isolated in Vero-E6 cell cultures from *A. ponticus* and an HFRS patient with fatal outcome (Tkachenko et al. 2005b; Dzagurova et al. 2012).

In 2000–2011, 56 HFRS cases caused by Sochi virus were diagnosed in 7 administrative regions of Krasnodar province including 38 cases in Sochi metropolitan area (Tkachenko et al. 2013; Klempa et al. 2008; Dzagurova et al. 2009). To our current knowledge, Sochi virus seems to be the most pathogenic representative of DOB/BELV lineage of hantaviruses. The case fatality rate was determined to be as high as 14 %. Nearly 60 % of clinical cases were defined as severe (including fatal

cases) and nearly 40 % were classified as clinically moderate. Four times more males than females were affected. Notably, the age average among HFRS patients was around 30, and the proportion of young individuals (7–15 years old) was relatively high, 10 % (Dzagurova et al. 2008a).

The comparative analyses of clinical manifestations of patients with HFRS caused by five different hantaviruses showed that these viruses can cause mild, moderate, and severe forms of the disease. However, severe forms were more associated with DOB/SOCV (14 %) and HTNV (5–8 %) infections than with HFRS caused by PUUV, SEOV, and DOB/KURV (up to 1 % severe forms). In Russia, 97.7 % of the total number of HFRS were caused by PUUV associated with bank vole, *Myodes glareolus*. Only 2.3 % of HFRS cases were caused by other hantaviruses, HTNV, HTNV-like Amur/Soochong virus, and SEOV (1.5 % all together), and by DOB/BELV (0.8 %). Thus, PUUV virus plays the major role in the HFRS morbidity in Russia.

Periodical and massive reproduction of rodents, with the forming epizootics among them, is the main and determinative factor that influences HFRS epidemics in humans. The prevention of the HFRS disease mainly includes measures aimed at reducing exposure to live rodents and their excreta. However, rodent control measures are expensive and difficult to maintain over a long period of time. Preventive vaccination is the only effective measure to control hantavirus infection and reduce HFRS morbidity in endemic regions. The HFRS morbidity can be used to estimate the potential population at risk and the required HFRS vaccine doses. In Russia, vaccination campaign has to cover 20 European regions (where HFRS is caused mainly by PUUV and less by DOB/BELV) with a population of ~45 million as well as four Far Eastern regions (where HFRS is caused mainly by HTNV, HTNV-like, and Amur/Soochong virus and less by SEOV) with a population of ~5 million. Approximately 50 % of the population in these regions (25 million) potentially are at risk and are potential recipients of vaccine against HFRS.

Hemorrhagic Fever with Renal Syndrome in Europe For the period of 80 years since the first description of HFRS human cases in Sweden (Myhrman 1934; Zetterholm 1934), the list of European countries with HFRS incidence reached to date 35: Austria, Belgium, Bosnia and Herzegovina, Bulgaria, Croatia, Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Luxembourg, the Netherlands, Norway, Portugal, Romania, European Russia, Slovenia, Slovakia, Sweden, Switzerland (Vapalahti et al. 2003; Heyman et al. 2009), Albania (Eltari et al. 1987; Antoniadis et al. 1996), Belarus (Zhavoronok et al. 2008), Estonia (Vasilenko et al. 1987), Georgia (Kuchuloria et al. 2009), Latvia (Lundkvist et al. 2002), Lithuania (Moteyunas et al. 1990), Macedonia (Gligic et al. 2010), Moldova (Mikhaylichenko et al. 1994), Montenegro (Papa et al. 2006), Poland (Gut et al. 2013), Serbia (Gligic et al. 2010), Turkey (Ertek and Buzgan 2009), and Ukraine (Micevich et al. 1987). HFRS is a widespread infection in Europe with clear effects on public health. Unfortunately, hantavirus infection remains to be underestimated or not recognized by the medical and public-health authorities in many countries, mainly because of the lack of diagnostics. Practically, with

exception of Finland, Russia, Sweden, Belgium, Yugoslavia, and France, in all other countries, HFRS cases became recognizable and diagnosed only after 1990 (Heyman et al. 2009). Still many cases remained undiagnosed due to subclinical manifestations and nonspecific symptoms at the early stage of the disease.

Epidemiological analysis of HFRS morbidity in Europe is complicated due to the absence of statistical data in most European countries (with Russia exception). It seems reasonable to use the European Network for Diagnostics of Imported Viral Diseases (ENIVD) information, collected and published by Paul Heyman and other authors (Heyman et al. 2008, 2009, 2011), as well as case reports and description of HFRS outbreaks. Since 2012, approximately 300,000 HFRS cases have been reported in 35 European countries. The distribution of HFRS was found to be scattered throughout Europe. HFRS morbidity varies considerably in different countries with the highest rate in the European part of Russia, where HFRS cases make up ~70–80 % of total number of HFRS cases registered in Europe (see below). In addition to Russia, there are countries with high annual HFRS morbidity. These countries include Finland (997.5 cases), Germany (544.6 cases), Sweden (276.8 cases), and Belgium (98.1 cases). In 1999, 2002, and 2005, Finland had about 2,500 serologically confirmed HFRS cases; in 2008, a record year, 3,259 cases were diagnosed. Belgium had peak years in 2007 (298 cases) and in 2008 (336 cases). In 2007, Sweden had 2,195 cases (Vaheri et al. 2011; Heyman et al. 2009, 2011; Makary et al. 2010). In 2004–2005 local outbreaks of HFRS were reported in Germany. A large outbreak with 1,688 cases was reported in 2007 and 2,017 cases were reported in 2010 (Hofmann et al. 2008; Faber et al. 2010). Climate changes seem to be responsible for the increase in the number of sporadic HFRS cases without any traceable geographical or temporal trends (Heyman 2007). With Russia exception, Norway, Sweden, and Finland account for the most of hantavirus infections in Europe.

In Europe, HFRS is caused by three hantavirus species, PUUV (carried by *M. glareolus,* bank vole); DOB/KURV (carried by *A. agrarius*, the striped field mouse); DOB/DOBV, genotype Dobrava (carried by *A. flavicollis*, yellow-necked mouse) (Klempa et al. 2013a); and by DOB/SOCV, genotype Sochi, associated with *A. ponticus* (the Black Sea field mouse). PUUV is the major cause of HFRS in Western, Central, and Northern European countries (Finland, Sweden, Norway, Germany, Belgium, France, and European Russia). DOB/KURV has been found in Germany, Slovakia, Russia and Slovenia. This virus commonly infects humans and is associated with DOBV/KUR-HFRS. DOB/DOBV is the main cause of severe HFRS in Southern Europe, including Greece, Albania, Bulgaria Slovenia, Croatia, Bosnia, Serbia, Montenegro (Vapalahti et al. 2003; Antoniadis et al. 1996; Avsic-Zupanc et al. 1999; Markotic et al. 2002; Papa et al. 2006; Lundkvist et al. 1997). The disease in the Balkans is seen primarily among adults, especially woodcutters, shepherds, military personnel and others whose occupations occasionally require them to work or sleep outdoors. Unlike HFRS in other parts of Europe, cases in the Balkans peak during the warmer months of the year, with 80 % of them registered from June to September (Avsic-Zupanc et al. 1999). In the Balkans, DOB/DOBV-HFRS patients have more severe clinical manifestations then PUUV-HFRS

patients. The mortality rate can be up to 12 % (Papa et al. 2006; Markotic et al. 2002). By contrast, DOB/KURV infections in Baltic countries, in Central Europe, and in Central European part of Russia are mainly associated with mild-to-moderate forms HFRS with very little fatalities if any (Vapalahti et al. 2003; Klempa et al. 2008; Dzagurova et al. 2009).

In January–May, 2009, 12 HFRS cases caused by PUUV were first registered in Turkey (Ertek and Buzgan 2009); 2 more cases were additionally diagnosed in August (Kaya et al. 2010).

HFRS is endemic in Austria where approximately 30 cases of HFRS are annually diagnosed. The last epidemics were observed in 2004 and 2007 with 72 and 78 documented PUUV-HFRS cases, respectively. In 2011–2013, the first DOB/DOBV and DOB/KURV-HFRS cases have been detected. Hantaviruses DOB/DOBV and DOB/KURV were also found in *A. flavicollis* and *A. agrarius*, respectively, captured at the place of residence of HFRS patients (Aberle et al. 2013).

Risk factors for HFRS include professions such as forestry, farming and military, or activities such as camping and the use of summer cottages. Humans are thought to be infected from aerosolized rodent excreta when exposed to hay and crop during harvesting, cleaning cellars, sheds, stables or summer cottages in the fall and handling wood (especially inside the dusty woodsheds). Hantaviruses are reasonable stable and can be viable (infectious) for more than 10 days at room temperature (Hardestam et al. 2007; Kallio et al. 2006). Moreover, bank voles excrete PUUV for several months, especially in saliva (Hardestam et al. 2008). The male gender is a clear risk factor with a male/female ratio of, for example, 1.67 in Finland and 1.52 in Sweden (Makary et al. 2010; Hjertqvist et al. 2010). Risk factors also include the use of rodent traps instead of poison rodent control campaign. Additional risk has been attributed also to woodcutting and house warming with firewood and spending time and working in the forest. Increased incidence or occupational risk is also associated with military activity, farming, forestry, camping, and summer cottages (Winter et al. 2009).

In summary, the HFRS disease is endemic in many European countries and hantavirus infection is a growing public-health problem. No specific therapy or vaccine is currently available. There is a need to develop advanced vaccines which should include PUUV and DOB/BELV antigens.

Hemorrhagic Fever with Renal Syndrome in Asia In Asia, clinical HFRS cases caused by HTNV, HTNV-like viruses (Amur/Soochong virus), and SEOV have been registered mainly in China, South and North Korea, and the Far Eastern regions of Russia. China is the major HFRS-endemic country in Asia and in the world. During 1950–2007, a total of 1,557,622 HFRS cases and 46,427 deaths (3 %) were reported in China with the highest annual peak in 1986, with 115,985 cases. HFRS has been reported in 29 of 31 provinces in China with annual morbidity up to 40,000–60,000 cases (Zhang et al. 2010). In 2004 the National Disease Reporting System was established by China CDC. From 2006 to 2011, a total of 64,250 HFRS cases and 762 deaths were reported with the case fatality rate of 1.18 % (Li 2013).

HFRS morbidity is associated mainly with the northeastern, eastern, central, and southwestern parts of China (humid and semi humid zones). The disease rarely occurs in the northwestern part (arid zone) with top rate of 20.3, 18.9, 8.2, 7.7, 5.0, and 4.6 cases/100,000 population in the Heilongjiang, Shandong, Zhejiang, Hunan, Hebei, and Hubei provinces, respectively (Fang et al. 2007). Rural areas account for more than 70 % of all HFRS cases; mainly peasants were infected (Chen and Qiu 1993). Poor housing conditions and high rodent density in residential areas seem to be responsible for most HFRS epidemics. The increase in HFRS morbidity from the end of the 1970s coincided with the fast socioeconomic development started in 1978 in China. During the 1980s and 1990s, China underwent large changes such as agricultural development, irrigation engineering, urban construction, mining, and highway and railway construction. These activities increase human exposure and contact with rodents. Because rats are more mobile than other hantavirus hosts (Plyusnin and Morzunov 2001), fast socioeconomic development also causes wide expansion of rats infected with SEOV. This fact might subsequently lead to the high nationwide prevalence of SEOV infections. However, improved housing conditions, improved hygiene, and human migration from rural areas to cities might contribute to the decline of HFRS cases since 2000. In general, HFRS cases are registered throughout the year with increase in winter and spring with the peak in November (Chen and Qiu 1993; Chen et al. 1986). Early epidemiological investigations found that the winter peak resulted from HTNV carried by *A. agrarius* and that the larger spring epidemic was mainly caused by SEOV carried by *R. norvegicus* (Chen et al. 1986). HFRS affects patients of any age (from infancy to >65 years), but mostly adolescents and young adults got infected (Chen and Qiu 1993; Chen et al. 1986). The incidence in males were over three times higher than females (Li 2013). Because *A. agrarius* and *R. norvegicus* rodents are the predominant carriers and distributed nationwide, HTNV and SEOV are obviously the major threat for HFRS in China. Epidemiological studies in China suggest that camping or living in huts in fields, living in a house on the periphery of a village, and cat ownership are supposed to be risk factors (Rio et al. 1994). The gradual change in the disease structure (proportions of mild and severe disease) might have contributed to the decreased mortality rates as well. In recent decades, as rats followed human activities and migration from rural to urban areas during the fast socioeconomic development in China, the proportion of mild HFRS cases caused by SEOV steadily increased while the proportion of more severe cases associated with HTNV infection decreased (Chen and Qiu 1993).

HTNV was first isolated from striped field mice in 1981 (Yan et al. 1982). Consistent with the geographical distribution of *A. agrarius*, HTNV has been found in all Chinese provinces except Xinjiang (Yan et al. 2007). In addition to *A. agrarius*, HTNV has been also found in *Apodemus peninsulae* in northeastern China (Zhang et al. 2007). Genetic analysis of the small (S) and medium (M) genome segments suggested that at least nine distinct lineages of HTNV are circulating in China (Zou et al. 2008). In general, HTNV variants display geographical clustering. Recently, reassortment between HTNV and SEOV was detected in *R. norvegicus* (Zou et al. 2008), which indicates that genetic

reassortment occurs naturally between two hantavirus species. Because reassortment is a way for segmented viruses to achieve high infectivity and adapt to new animal hosts, further studies are warranted to evaluate susceptibility of *A. agrarius* and *R. norvegicus* rodents to these unique reassortant viruses and to determine whether these reassortants can infect humans.

HFRS cases caused by SEOV were first reported in Henan and Shanxi provinces along the Yellow River in China (Hang et al. 1982). Subsequently, SEOV (strain R22) was isolated from *R. norvegicus* in Henan (Song et al. 1984), and SEOV has been found in almost all provinces of China except Qinghai, Xinjiang, and Xizang (Zhang et al. 2009). SEOV-associated HFRS seems to have recently spread to areas where it had not been reported during previous epidemics (Zhang et al. 2009). Most known SEOV variants (from lineages 1–4 and 6), including those from China, Brazil, Japan, South Korea, North America, and the United Kingdom, are genetically homogeneous. Lineages 1–4 are widely distributed and do not follow a geographical clustering pattern. Thus, the variants from lineages 1–4 and 6 are closely related and may have a more recent common ancestor. Because *R. norvegicus* is distributed nationwide and found to be more mobile than other hantavirus hosts, SEOV has become the largest threat for public health in China. It may bring even more potential threats to humans as rat species become more widespread along with globalization of the economy. Natural HFRS cases caused by SEOV have been found almost exclusively in China and other Asian countries. The lack of HFRS in other countries may result from better living conditions, low rat densities, and low rates of SEOV carried by the rats.

Hantaviruses are thought to have coevolved with their respective hosts. Each serotype and/or genotype of hantavirus appears to be primarily associated with 1 (or a few closely related) specific rodent host species. As described above, more than 100 species of rodents and several dozens of insectivores are widely distributed in HFRS-endemic areas in China (Zhang et al. 1997). Hantavirus-specific antibodies and/or antigens have been identified in, at least, 38 rodent species. Therefore, in addition to already known HTNV, SEOV, Dabieshan virus, Hokkaido virus, Khabarovsk virus, Vladivostok virus, and Yuanjiang virus, yet-unknown hantavirus species may be circulating in China. In-depth studies on hantavirus distribution in different geographical regions and hosts in China as well as genetic characterization of hantaviruses and elucidation of the relationship between these viruses and other known hantaviruses should help prevent the diseases they cause.

A comprehensive preventive strategy has been implemented to control HFRS in China. It includes public-health education and promotion, rodent control, surveillance, and vaccination (Zhang et al. 2004). Since the 1950s, on mainland China, the rat population has been controlled by using poison bait or trapping around residential areas. During the 1980s and 1990s, deratization around residential areas effectively decreased both rodent density and incidence of HFRS, especially the disease caused by SEOV (Luo and Liu 1990).

Improving general awareness and knowledge of pathogen source, transmission routes (how to avoid contact with a pathogen), diagnostics, vaccination, and general hygiene appears to be one of the most cost-effective ways to prevent infectious

diseases. Since the 1970s, public education on HFRS and other infectious diseases has been conducted by all possible means in China, especially in rural areas. After implementation of comprehensive preventive measures, including vaccination, in the past decade in China, HFRS morbidity has decreased dramatically. Only 11,248 HFRS cases were reported in 2007 (Zhang et al. 2010). Mortality rates also declined from the highest level of 14.2 % in 1969 to 1 % during 1995–2007.

Nevertheless, despite intensive measures implemented last years, HFRS remains a major public-health problem in China (Zhang et al. 2004), and during the last years, there is a trend in the increasing number of HFRS cases (Li 2013).

HFRS is one of the important acute febrile infections and a major public-health problem in South and North Korea. It was recognized for the first time in Korea in 1951 among soldiers of the United Nations (Smadel 1953). The causative pathogen Hantaan virus was discovered by Lee et al. in 1976 (Lee et al. 1978) and named after the Hantaan River crossing the endemic areas near the demilitarized zone between North and South Korea.

In South Korea, a total of 14,309 HFRS patients were hospitalized from 1951 to 1986, with one third being soldiers (Lee 1989). Hundreds of HFRS cases were registered in the 1970s and 1980s, with a sharp increase in the number of cases in the early 1990s, up to 1,200 cases per year. From 2001 to 2008, 323–450 HFRS cases were registered annually in the South Korea (South Korean Centers for Disease Control and Prevention 2008). The number of hospitalized HFRS patients has declined to 100–300 per year in recent years in South Korea (Baek et al. 2006).

Both HTNV and SEOV are known as the etiologic agents of HFRS in Korea. These viruses establish chronic infections in certain species of rodents and are transmitted to individual primarily via aerosols or fomites from feces, urine, and saliva of infected mice (Tsai 1987). HTNV, carried by *A. agrarius* and *A. peninsulae*, causes a severe form of HFRS and is mostly distributed in rural areas, whereas SEOV, carried by *Rattus norvegicus* and *Rattus rattus*, causes urban-acquired cases and may cause a milder clinical syndrome.

There are two epidemic periods of HFRS each year, the major (October–December) and minor (May–July) epidemic periods. The majority of cases (more than 75 % of patients) occur during the major epidemic period. SEOV infection is less seasonal in occurrence. There are two high-risk groups of HFRS—residents, who are mostly farmers, and Korean soldiers stationed in the field (Lee 1989). More than 500 HFRS cases were serologically confirmed and hospitalized annually in the 1980s (Lee 1989). However, the number of the reported cases has gradually decreased to approximately 300–400 cases per year since it was legally designated as a communicable disease in 2000 (Korea Center for Disease Control and Prevention 2004). Nevertheless, the associated factors have not been defined. The inactivated hantavirus vaccine (Hantavax™, Korean Green Cross, Korea) has been commercially available since October 1990. Because of its adoption into the national immunization program in 1992, it has been widely distributed to public-health centers and the Korean army (Cho et al. 2002).

The sporadic HFRS cases have been reported in India, Indonesia, Singapore, Sri Lanka, Thailand, Hong Kong, and Taiwan (Clement et al. 2006; Chandy et al. 2009;

Groen et al. 2002; Plyusnina et al. 2004; Chan et al. 1996; Vitarana et al. 1988; Tai et al. 2005; Suputthamongkol et al. 2005). Serological investigation showed evidences of hantavirus infections in humans in Israel, Kuwait, Laos, Malaysia, Philippines, and Vietnam (George et al. 1998; Pacsa et al. 2002; Rollin et al. 1986; Lam et al. 2001; Quelapio et al. 2000).

Hantavirus Pulmonary Syndrome in the New World Hantavirus pulmonary syndrome (HPS), was discovered in the southwestern United States in 1993 (Duchin et al. 1994). The causative agent was determined to be an unidentified North American member of the *Hantavirus* genus (Nichol et al. 1993). The clinical syndrome caused by this agent, ultimately named Sin Nombre virus (SNV), came to be called hantavirus pulmonary syndrome, HPS. This designation distinguished it from previously described hantavirus illnesses, which were characterized as HFRS. At the early stage of the disease, cardiac and respiratory functions are markedly impaired by virus infection. For this reason, some authors have proposed name "hantavirus cardiopulmonary syndrome." In the United States, HPS was retrospectively traced back to as early as 1975 (Wilson et al. 1994).

Until now, about 3,000 cases of HPS have been identified in small clusters and individual cases throughout North and South America, with a total of 616 cases occurring in the United States (between 11 and 48 cases annually) (Jonsson et al. 2010; CDC 2012a, b, c). More than half of the North American hantavirus cases occur in the Four Corners area of the Southwest, but infections have been reported in 34 US states. In Canada, cases of HPS are rare, fewer than eight being reported per year, with the first Canadian case of HPS identified retrospectively back to 1989 (Weir 2005). A 26 % case fatality of HPS was reported in Northern Alberta, Canada (Verity et al. 2000). Although generally occurring in rural areas, up to 25 % of cases occur in urban and suburban areas (CDC 2012b). Although reporting of the disease appears relatively sparse, the actual incidence may be somewhat higher due to asymptomatic infections. In a study performed in Baltimore (an area with very few reported cases of HPS), 44 % of mice and 0.74 % (nine patients) were serologically positive for hantavirus despite being otherwise healthy and asymptomatic (Zaki et al. 1996).

Although there appears to be a significant spectrum of disease, the case fatality rate for symptomatic HPS patients in the United States was 38 % (Zaki et al. 1996). Most cases occur during the late spring and early summer months, which may allow clinicians to distinguish the disease from influenza, which has a similar presentation (CDC MMWR 1993). Cases almost exclusively occur in people who sleep or work in areas where they may be exposed to rodents. Transmission of the virus occurs predominately through inhalation of aerosolized rodent urine, feces, or saliva; exposure may also occur through food contaminated by rodent saliva and excreta and through rodent bites (Jonsson et al. 2010; Schmaljohn and Hjelle 1997). Although human-to-human transmission has not been observed in North America, there have been a few documented cases of such transmission in South America (Jonsson et al. 2010). The largest risk factor is entering closed buildings with rodent infestations (Armstrong et al. 1995).

In the United States, the principal virus causing HPS is SNV, which chronically infects the deer mouse, *Peromyscus maniculatus*. The deer mouse habitat occupies a huge swath of the North American continent, sparing only areas nearing the Arctic Circle, a few states in the southeastern United States, and southern Mexico. Approximately 10 % of tested deer mice in this range are infected with SNV (Lonner et al. 2008). Additionally, closely related hantaviruses are hosted by other *Sigmodontinae* rodents in areas where deer mice are sparse, including Black Creek Canal virus, hosted by the cotton rat *Sigmodon hispidus* in Florida, and the Bayou virus, hosted by the swamp rat *Oligoryzomys palustris* in Louisiana and Texas.

In Argentina, the first case of HPS was confirmed by virus detection in 1995 (Lopez et al. 1996). Three clusters involving 29 cases and a severe outbreak with 18 HPS cases were later reported in 1995 and 1996, respectively (Levis et al. 1998). By the end of 2006, a total of 841 cases were reported in Argentina (Capria et al. 2007). In 1996, an outbreak of HPS was detected in the Neuquen region of southern Patagonia, and the source was traced to *Sigmodontinae* rodent, long-tailed rice rat, *Oligoryzomys longicaudatus*. The hantavirus detected in both patients and rats was named the Andes virus (ANDV) (Lopez et al. 1996). In 2002, at least 10 HPS cases were reported in Bolivia with 6 deaths (Carroll et al. 2005). By the end of 2004, 36 cases had been reported in the country. In Brazil, the first case of HPS was reported in a family cluster in 1993 (Moreli et al. 2004), and 855 HPS cases were reported between 1993 and 2006 with a 39.3 % case fatality (Da Silva 2007). In Chile, since the first identification of HPS in 1995 (Espinoza et al. 1998), 352 cases of HPS had been reported up to 2006, with a case fatality rate of 33 %. In Uruguay, the first evidence of the circulation of these viruses came from a study of serum specimens collected from blood donors between 1985 and 1987 (Weissenbacher et al. 1996). Since then, more than 60 cases of HPS have been confirmed in (Delfraro et al. 2007). The first cluster of HPS in Central America occurred from late December 1999 to February 2000 in Los Santos Province in Panama. Through 2006, there were 85 cases of HPS reported in Panama with a case fatality rate of 17.6 % (Armien et al. 2007). In Paraguay, the first outbreak of HPS occurred in 1995 (Carroll et al. 2005), and through 2004, there had been 99 cases of HPS in that country. The overall seroprevalence of hantavirus infections in the Chaco area of Panama was 43 % (Ferrer et al. 2003).

In the Caribbean region, a single case of HPS was serologically confirmed in eastern Venezuela. A low prevalence (1.7 %) but wide distribution of hantavirus infections was demonstrated in the country (Rivas et al. 2003). Human infections in Colombia (Espinoza et al. 1998) and rodent infection with Sin Nombre-like hantaviruses in Costa Rica, Mexico, and Peru were also reported (Hjelle et al. 1995; Suzan et al. 2001; Powers et al. 1999).

Transmission largely occurs through inhalation of aerosolized urine, feces, or saliva of the rodent host. Within species, the viruses are also commonly transmitted through aggressive behavior, such as biting, especially among males, and males have a higher prevalence of infection than females (Douglass et al. 2006; Calisher et al. 2001). HPS is predominantly a rural disease, with associated risk factors of

farming, land development, hunting, and camping, because each of these activities brings humans into closer contact with the natural rodent reservoirs, which are all sylvan or agrarian in their choice of habitat. However, HPS is nearly always acquired indoors or within closed spaces, such as peridomestic buildings on farms or ranches, livestock feed containers, or the cabs of abandoned pickup trucks (Armstrong et al. 1995). Several factors contribute to the propensity for indoor acquisition by humans. Animals captured in the peridomestic environment have a higher prevalence of active infection than those captured in a sylvan environment (25 % vs. 10 %), likely because of greater supplies of foodstuffs and higher murine population densities (Kuenzi et al. 2001). Higher population densities lead to more interaction among mice and higher rates of intraspecies transmission. Likewise, humans are more likely to encounter rodent excreta when population densities are higher.

In the United States, approximately two thirds of HPS cases have been among men. The average age of patients who have HPS is 38 years, with a range of 10–83 years. There has been a striking absence of severe HPS among prepubertal individuals in the United States, although disease in 11 children aged 10–16 years had clinical courses similar to those described in adults (Kuenzi et al. 2001).

The incidence of HPS in Latin America is largely unknown but cases have been reported from Central America to southern Patagonia. The ANDV was responsible for outbreaks in Argentina and Chile and is closely related to the Bayou virus. Although most North American cases have been sporadic and isolated, most South American cases have occurred in clusters. The Patagonian outbreak in 1996 was unique in that it occurred in an area with a relatively low rodent population density, and human-to-human transmission was suspected when physicians treating infected patients became ill themselves (Enria et al. 1996). Gene sequencing of virus recovered from cases with rodent exposure and from their contacts who had no possibility of rodent exposure confirmed human-to-human transmission (Padula et al. 1998; Martinez et al. 2005).

The seroprevalence of IgG antibodies to hantaviruses differs between North and South American populations. In the United States, the Four Corners area has the highest incidence of infection; however, presence of antibodies among tested individuals in that region is less than 1 % (Auwaerter et al. 1996; Vitek et al. 1996). Childhood infection in North America is also rare. In contrast, some endemic areas in South America have a much higher rate of infection, including in children, with seroprevalence as high as 42.7 % in areas of Paraguay (Ferrer et al. 2003). In all areas studied, the seroprevalence is higher in South America than in North America, suggesting the occurrence of mild and asymptomatic infections (Pini 2004).

Based on the broad distribution of *Sigmodontinae* rodents throughout the Americas, the CDC estimates that HPS infections can be potentially detected in every county of the North and South Americas (CDC MMWR 1993).

There is currently no Food and Drug Administration-approved vaccine for the New World hantaviruses. However, several vaccine candidates are in different stages of clinical development (Schmaljohn 2009, 2012). Inactivated virus vaccines

like those used in Asia are generally not being pursued for HPS because of inadequate efficacy and concerns about the risks of mass production of a high-containment virus (Jonsson et al. 2008). Given the possible use of hantaviruses as a bioterrorism agent and its endemic status across the globe, it is clear that the development of effective hantavirus countermeasures is necessary (Hartline et al. 2013).

5.3 Inactivated Hantavirus Vaccines

Inactivated virus vaccines significantly contributed to the control of infectious diseases during the twentieth century and probably will remain an attractive strategy for vaccine development for the coming decades. Inactivated vaccines are currently widely available for poliomyelitis, influenza, rabies, hepatitis A, tick-borne encephalitis, and Japanese encephalitis (Trofa et al. 2008; Falleiros Carvalho and Weckx 2006; Webby and Sandbulte 2008; Rouraiantzeff 1988; Eckels and Putnak 2003; Schioler et al. 2007).

5.3.1 Rodent Brain-Derived Hantavirus Vaccines

The high HFRS morbidity in the 1980s in Asian countries has raised an urgent need to develop vaccines against hantaviruses. Most of these vaccines were made using either formalin or β-propiolactone inactivated rodent brain-derived hantavirus, similar (Table 5.1) to those used to prepare Japanese encephalitis and rabies vaccines (Oya 1976; Gupta et al. 1991; Acha 1967).

In the South Korea, the initial vaccines were based on the brain suspension of suckling rats infected with HTNV's strain ROK 84-105 (Lee and Ahn 1988; Lee et al. 1990). The virus strain ROK 84-105 was isolated from the blood of HFRS patient through Vero-E6 cells (French et al. 1981) and passaged 7–10 times in the brains (IC inoculation) of suckling rats (titer—7 $\log_{10}$ LD 50/mL) or mice (titer—9.2 $\log_{10}$ LD 50/mL). Brains were harvested 7–8 days after virus inoculation, and phosphate-buffered saline was added to the brains to prepare virus suspension, which was then centrifuged at 10,000 g for 15 min. At the next step, protamine sulfate was added to the supernatant to precipitate cellular proteins. The mixture was centrifuged, ultrafiltrated, and ultracentrifuged at 40,000× *g* for 2 h at 4 °C, and then 0.05 % formalin was added to the supernatant to inactivate the virus. The inactivated vaccine was then mixed with alum hydroxide (adjuvant).

The concentration of viral antigen in the vaccine preparation was determined by enzyme-linked immunosorbent (ELISA) assay. The immunogenicity of vaccine was tested in inbred BALB/c mice after intraperitoneal inoculation. The mice were bled by heart puncture 2 or 4 weeks after immunization, and hantavirus antibody titers in sera were determined by immunofluorescence (IFA), by ELISA, and by a

Table 5.1 Inactivated hantavirus vaccines

Country	Hantavirus	Substrate	Inactivation	State of development
Rodent brain-derived hantavirus vaccines				
Japan	SEOV	Suckling mouse brain	Formalin	Preclinical
South Korea	HTNV	Suckling rat brain	- " -	Clinical
- " -	- " -	Suckling mouse brain	- " -	Commercial
- " -	PUUV	Suckling hamster brain	- " -	Clinical
- " -	PUUV-HTNV	- " -	- " -	- " -
North Korea	HTNV	Suckling rat brain	Formalin	Commercial
- " -	- " -	Suckling hamster brain	- " -	Preclinical
China	HTNV	Suckling mouse brain	β-propiolactone	Commercial
- " -	SEOV	- " -	- " -	Preclinical
Russia	HTNV	Suckling mouse brain	Formalin	Preclinical
Cell culture-derived hantavirus vaccines				
China	HTNV	Golden hamster kidney cells	Formalin	Clinical
- " -	SEOV	- " -	- " -	Commercial
- " -	HTNV-SEOV	- " -	- " -	- " -
- " -	HTNV	Mongolian gerbil kidney cells	β-propiolactone	Commercial
- " -	SEOV	- " -	- " -	Clinical
- " -	HTNV-SEOV	- " -	- " -	Commercial
- " -	SEOV	Striped field mouse kidney cells	- " -	Clinical
- " -	HTNV	Chicken embryo cells	Formalin	Clinical
- " -	HTNV-SEOV	Vero cells	β-propiolactone	Commercial
South Korea	HTNV	Vero-E6 cells	Formalin	Preclinical
Russia	PUUV-DOBV	Vero cells	- " -	Preclinical

plaque reduction neutralizing test (PRNT). Protective efficacy of the vaccine was tested by challenging the mice with prototype strain 76-118 of HTNV and then measuring viral antigen in the lungs. Immunogenicity and protective activity studies showed that experimental vaccine was effective against HTNV infection in mice.

In general, in other endemic countries, the method of producing of rodent brain-derived hantavirus vaccine was similar to the protocol described above. The Japanese vaccine was based on the brain of mice, infected with SEOV (Yamanishi et al. 1988); the North Korean vaccines, on the brain of suckling rats and hamsters,

infected with HTNV (Kim and Ryu 1988; Kim et al. 1989, 1991); Chinese vaccines, on the brain of suckling mice, infected with HTNV or SEOV (Sun et al. 1992; Yu et al. 1990a); and Russian vaccine, on suckling mice, infected by HTNV (Astakhova et al. 1995). In rodent brain-derived vaccines produced in China, β-propiolactone was used for virus inactivation (Yu et al. 1990b). The experimental rodent brain-derived vaccines usually elicited good immune responses in rodent models as measured by IFA, ELISA, and neutralizing test.

A commercial South Korean inactivated HTNV ICR mouse brain-derived vaccine, named Hantavax™, was shown to be effective in protecting experimental mice and humans from HFRS (Cho and Howard 1999; Cho et al. 2002). A month after vaccination of 64 human volunteers with Hantavax™ subcutaneously (s.c.), the vaccinated individuals developed hantavirus antibody measured by IFA (79 %) and ELISA (62 %) (Cho and Howard 1999). One month after a second vaccination, the seroconversion rate increased to 97 %. Neutralizing antibody titers followed this trend, with 13 % of vaccine recipients producing neutralizing antibody 1 month after the first dose and 75 % of vaccine recipients responding 1 month after boost. Antibody titers had declined during the time and at 1 year after immunization only 37 % and 43 % of sera found to be positive by IFA and ELISA, respectively. Revaccination at this time produced a vigorous immune response, with 94 and 100 % of vaccine recipients yielding positive antibody titers. Approximately 50 % primary vaccinees produced neutralizing antibodies following the booster dose 1 year later. Another study found a neutralization response in 33 % of recipients after two immunizations (Sohn et al. 2001). It was concluded that the booster vaccination is necessary at 1 year after primary vaccination for maintaining a high level of antibodies. After the boost, antibodies persisted for 2 additional years.

During 1991–1998, more than 5 million people were vaccinated with Hantavax™ in South Korea (Cho et al. 2002). Vaccination significantly decreased the total number of hospitalized HFRS patients, from 1,234 cases in 1991 to 415 cases in 1997 (Cho et al. 2002). It seems that in addition to vaccination, some additional factors contributed to this decline (Cho et al. 2002; Hjelle 2002).

In 1996–1997, a clinical trial was conducted in endemic areas of HFRS in Yugoslavia. Vaccinees received Hantavax™ twice and boosted a year later. Twenty-five HFRS patients were documented among a control group, but none were reported among 2,000 vaccine recipients (Lee et al. 1999; Bozovic et al. 2001).

After vaccination with the Chinese inactivated HTNV mouse brain-derived vaccine (i.m.), IFA antibody were detected in 84 % and 18 % vaccinees 2 weeks and 1 year after vaccination, respectively; neutralizing antibodies were detected in 51 % and 10 %, respectively (Sun et al. 1992). 2 weeks, 1 year, and 2 years after booster revaccination, the seroconversion rates were 83 %, 42 %, and 13 % in IFA and 62 %, 41 %, and 25 % in PRNT assay, respectively. In 30 volunteers immunized with Chinese inactivated SEOV mice brain-derived vaccine, vaccination resulted in the induction of high titers of specific antibodies measured by ELISA and by PRNT (Yu et al. 1990a).

Three weeks after the boost immunization with North Korean inactivated HTNV rat brain-derived vaccine, IFA antibodies and antibodies detected in reversed passive hemagglutination inhibition assay (RPHI) were found in 78.1 % and 88.8 % of vaccinees, respectively. No neutralizing antibody data were detected. Nevertheless, in clinical trial performed in North Korea where 1.2 million people were vaccinated, the high protective efficacy (88–100 %) was reported (Kim et al. 1991).

In general, the inactivated rodent brain-derived hantavirus vaccines elicited good humoral immune responses (IFA, ELISA) in rodent models. In most cases neutralizing antibody responses were detected only after boost immunization (Cho and Howard 1999). Whereas some authors describe a high protection and significant HFRS case reduction after prime-boost immunization with these vaccines (Zhang et al. 2010; Li 2010), the clinical efficacy of these vaccines is still questionable (Hammerbeck et al. 2009; Schmaljohn 2009).

In the 1990s, formalin-inactivated suckling hamster brain-derived vaccines against PUUV were developed (Lee et al. 1997, 1999). Monovalent vaccine PUUVAX contained formalin-inactivated K-27 strain of PUUV isolated from HFRS patient from Bashkiria region of Russia. One dose of PUUVAX contained 5,120 U/ELISA of virus antigen in 0.5 mL. Antibody response of hamsters after inoculation of PUUVAX vaccine showed high titers of IFA and PRNT antibodies against PUUV (Lee et al. 1999).

Blended HTNV-PUUV vaccine contained 5,120 U/ELISA of each HTNV and PUUV antigen in 1.0 mL. Immunization of hamsters with HTNV-PUUV resulted in production of IFA and PRNT antibodies. In fact, blended HTNV-PUUV vaccine produced even higher titers of PRNT antibodies than monovalent Hantavax™ or PUUVAX vaccines (Lee et al. 1999). To study immunogenicity and efficacy of the blended vaccine, hamsters were given 0.1 mL of vaccine twice at a 1-month interval. Antibody titers were measured by IFA and PRNT against five hantaviruses: HTNV, SEOV, DOBV, PUUV, and SNV or NYV. On day 30 after the first immunization, animals had IFA antibody titers of 78.4, 68.8, 68.8, 37.9, and 15.6 and PRNT titers of 65.4, 12, 6.1, 65.6, and 0.5, respectively. On day 30 after the second shot, IFA titers were 686.9, 567.5, 550.4, 516.3, and 430.9, and PRNT titers were 710.8, 41.9, 24.3, 409.9, and 1.6 against HTNV, SEOV, DOBV, PUUV, and NYV, respectively.

None of the vaccinated hamsters challenged with infectious HTNV, SEOV, DOBV, or PUUV showed either viremia or viral RNA in lung tissues (by nested RT-PCR). In contrast, vaccinated hamsters challenged with SNV or NYV became viremic and the challenged virus was detected in lung tissues. The vaccinated hamsters challenged with HTNV, SEOV, DOBV, or PUUV did not show any significant increase in IFA and PRNT antibodies. Meanwhile, the increase in PRNT antibody against NYV was observed in vaccinated hamsters challenged with SNV or NYV (Cho and Howard 1999).

In a limited study, 10 volunteers were vaccinated with blended HTNV-PUUV vaccine and 2 volunteers received PUUVAX vaccine (3 times, s.c., 1-month intervals) with various doses. All volunteers produced relatively high IFA

(1:128–1:2,048) and PRNT (1:10–1:640) antibodies against homologous hantaviruses after the second and third vaccinations (Lee et al. 1999; Cho et al. 2002).

Formalin or β-propiolactone inactivated rodent brain-derived hantavirus vaccines induced mostly local reactions including induration and swelling. There were no serious complains and these affects were self-limiting (Lee et al. 1999; Cho et al. 2002). Nevertheless, the case of toxic epidermal necrosis (TEN) with ocular involvement associated with vaccination against HFRS was reported (Hwang et al. 2012). In general, the lack of serious side effects indicates that rodent brain-derived hantavirus vaccine appears to be well tolerated in humans.

5.3.2 Cell Culture-Derived Hantavirus Vaccines

The cell culture-derived hantavirus vaccines have been developed mainly by Chinese researchers with some contributions of scientists from South Korea and Russia. Chinese vaccines were developed based on four primary cell cultures derived from golden hamster (*Thomasomys aureus*) kidney (GHKC), Mongolian gerbil (*Meriones unguiculatus*) kidney (MGKC), striped field mouse (*Alaetagulus pumillio kerr*) kidney (SFMC), and chicken embryo (CEC) and on one continuous cell line from African green monkey kidney cells, Vero cells. Korean and Russian vaccines were based on Vero-E6 and Vero cells, respectively. Eleven cell culture-derived hantavirus vaccines were developed. Four monovalent vaccines against HTNV were produced in GHKC (Song et al. 1992a), MGKC (Sun et al. 1992), CEC (Dong et al. 2001) and in Vero-E6 (Choi et al. 2003) cells. Three vaccines against SEOV were made in GHKC (Yu et al. 1990a), MGKC (Li and Dong 2001), and SFMC (Zhao et al. 1998) cells. In addition, three blended bivalent HTNV-SEOV vaccines were produced in GHKC (Song et al. 1992b), MGKC (Liu et al. 1992), and Vero (Hang et al. 2004), and one blended bivalent PUUV-DOBV vaccine was generated in Vero cells (Tkachenko et al. 2009, 2010).

All cell culture-derived hantavirus vaccines were produced using similar technology: virus harvest, "clarification" (low-speed centrifugation), ultrafiltration, formalin or β-propiolactone inactivation, purification by zonal centrifugation or by chromatography on Sepharose column, sterilizing filtration, mixing with alum hydroxide, and final lot testing. Bivalent vaccines were blended at the initial steps.

The immunogenicity of vaccines was tested in different rodent (hamsters, mice, rats) in IFA, ELISA, hemagglutination inhibition (HI), and PRNT assays. Protective efficacy was evaluated in challenge experiments in the hamsters or gerbils. Results of these experiments showed that practically all vaccines were effective against homologues virus (Hao et al. 1996; Yu et al. 1990b).

So far, only vaccines developed and manufactured in China were tested in humans. A human clinical trial demonstrated that a three-dose vaccination regimen resulted in 90–100 % seroconversion as assayed by PRNT (Ren et al. 1996; Zhu et al. 1991; Yu et al. 1992). Two weeks after primary vaccination, PRNT conversion rates were 51–82 % and 1 year later declined to 10–12 %. After boost

immunization, PRNT conversion rates were higher, 62–80 %, and declined to 36–41 % and 23–31 % after 1 and 2 years after boost. Conversion rates of antibody detected by IFA were higher than those in PRNT assay. These results showed that hantavirus-specific antibody titers declined significantly after primary vaccination. A 1-year boost significantly increased antibody titers and resulted in slower decline of antibody titers at the end of the second year. These antibodies were still effective in virus control (Chen et al. 1998).

The GHKC-derived SEOV vaccine, MGKC-derived HTNV vaccine, and a suckling mouse brain-derived HTNV vaccine were compared in a large human trial. Vaccination protocol for GHKC-derived SEOV vaccine consisted of three vaccinations at 28- and 14-day intervals (primary vaccination) followed by a boost at 1 year. The primary vaccination with MGKC-derived HTNV included three vaccinations on day 0, 7, and 14 followed by a boost at 1 year. The SMB-derived HTNV vaccine protocol included three vaccinations at 2-week intervals followed by a boost at 1 year. Among 55,000 vaccinees who received at least three doses of vaccines, side effects were in 2.6 % of vaccinees. Suckling mouse brain-derived HTNV vaccine produced the highest side-effect rate, 7.3 %; GHKC-derived vaccine had a middle rate, 3 %; and MGKC-derived vaccine had the lowest rate of side effect, 1.9 % (Chen et al. 1998).

To date, four inactivated cell-derived and one rodent-derived vaccines against hantaviruses have been approved for commercial production in China (Table 5.2). Since 1995, the vaccines have been successfully used in highly endemic regions of the country, and in 2007, a national Expanded Program on Immunization was initiated. The massive vaccination was found to be safe and effective (Li 2010). Currently, approximately 2 million doses of inactivated rodent brain- and cell culture-derived HFRS vaccines are given annually in China (Zhang et al. 2010).

Table 5.2 Immunogenicity of Vero cell-derived blended PUUV-DOBV vaccine

	Antibody titers							
	PUUV				DOB/KURV			
	ELISA		PRNT		ELISA		PRNT	
Vaccine dilution	+/*n*	Average titer	+/*n*	Average titer	+/*n*	Average titer	+/*n*	Average titer
n/d	8/8	2,389	8/8	136	8/8	1,792	8/8	120
1/2	7/7	1,152	7/7	54.8	7/7	1,060	7/7	73.1
1/4	8/8	896	8/8	26.3	8/8	416	8/8	36
1/8	8/8	352	7/8	18.75	8/8	192	7/8	50.3
1/16	6/8	170	4/8	10	5/8	90	4/8	40
1/32	4/8	96	2/8	8	4/8	36	2/8	16
1/64	1/8	64	1/8	8	1/8	16	1/8	16
1/128	1/8	16	0/8	<8	0/8	<16	0/8	<16
1/256	0/8	<16	0/8	<8	0/8	<16	0/8	<16

The HTNV experimental vaccine was also developed in Vero-E6 cells grown on microcarriers in suspension (Choi et al. 2003). In immunized mice the Vero-E6-derived HTNV vaccine induced more than five times higher levels of PRNT antibodies than the Hantavax™ vaccine. Two immunizations with 5 μg of cell culture-based vaccine induced strong PRNT antibody production, whereas no neutralizing antibody was induced after immunization with the same amount of Hantavax™ vaccine. Mice immunized with higher doses of Hantavax™, 10 or 20 μg, induced a similar level of neutralizing antibody but showed different protection efficacy suggesting possible involvement of cell-mediated immunity.

To date, there are no HFRS vaccines approved for use in European countries. Animal studies suggest that vaccines derived from HTNV or SEOV would not protect against PUUV infection (Schmaljohn 2012; Chu et al. 1995). There have been no efforts to develop HFRS vaccine based on PUUV, in part because PUUV is difficult to produce at high titers in cell cultures. Meanwhile, rodent brain-derived vaccines are not acceptable due to EU regulation requirements.

As mentioned above, circulation of both hantaviruses, DOB/BELV and PUUV, in the same endemic areas, e.g., in European part of Russia, indicates that effective hantavirus vaccine to control HFRS in Europe has to include antigens of both viruses (Schmaljohn 2009; Tkachenko et al. 2013).

During the last decade attempts were made to develop an inactivated Vero cell-derived blended bivalent PUUV-DOBV vaccine (Tkachenko et al. 2009, 2010). The PUUV-like vaccine strain "DTK/Ufa-97" was isolated in Vero-E6 cells from an HFRS patient during an HFRS outbreak in Bashkiria region of Russia in 1997 (Dzagurova et al. 2008b) and was adapted to grow at high titers in Vero cells with serum-free medium (SFM).

The DTK/Ufa-97 strain occupies the Bashkiria-Saratov lineage of PUUV. The amino acid sequences of the S, M, and L RNA segments of DTK/Ufa-97 were 99.2–100 %, 99.3–99.8 %, and 99.8 % identical to those of the Bashkirian PUUV strain and 96.9 %, 92.6 %, and 97.4 % identical, respectively, to those of the Sotkamo strain. The DTK/Ufa-97 and other PUUV strains exhibited similar binding patterns to a PUUV panel of monoclonal antibodies. In addition, antisera against three different PUUV strains neutralized both homologous and heterologous PUUV isolates. These results suggested that DTK/Ufa-97 strain is antigenically similar to distant PUUV strains but different from other hantaviruses (Abu Daude et al. 2008).

The envelope glycoproteins of hantaviruses play a major role in the induction of neutralizing antibodies and protective immunity (Lundkvist and Niklasson 1992). The cross neutralization test confirmed that neutralizing antibodies to DTK/Ufa-97 also neutralized other PUUV strains at almost the same neutralizing titers, and antibodies to the other PUUV strains neutralized DTK/Ufa-97 as well. These findings justify development of DTK/Ufa-97-based vaccine as PUUV vaccine to control HFRS in European countries (Abu Daude et al. 2008).

The second vaccine strain, "TEA/Lipetzk-06," belongs to DOB/KURV hantavirus lineage. The TEA/Lipetzk-06 was isolated from an HFRS patient during an HFRS outbreak in the Lipetsk region of Russia in 2006, was also adapted to

replicate at high titers in Vero cells in SFM (Tkachenko et al. 2009), and was analyzed to confirm their genetic and antigenic features (Klempa et al. 2008; Dzagurova et al. 2009). Both virus strains, DTK/Ufa-97 and TEA/Lipetzk-06, were used to produce Master Virus Seeds (MVS) in Vero.

Two variants of ELISA were developed to detect antigens of DTK/Ufa-97 and TEA/Lipetzk-06 hantaviruses (Dzagurova et al. 2013). First, "Hanta-PUUV" variant was designed using monoclonal antibodies to PUUV envelope glycoprotein for detecting only PUUV antigen. The second, "Hanta-N" ELISA, was designed using monoclonal antibodies to DOBV and PUUV nucleocapsid proteins for detecting PUUV, DOB/BELV, HTNV, and SEOV. Both "Hanta-PUU" and "Hanta-N" ELISA-based assays detected specific hantavirus antigens in the blended PUUV-DOBV vaccine.

Vaccine product development included the following steps: (1) infection of Vero cells with Working Virus Seeds (WVS) of DTK/Ufa-97 and TEA/Lipetzk-06 viruses; (2) harvesting culture medium from DTK/Ufa-97-infected cultures and TEA/Lipetzk-06-infected cultures; (3) low-speed centrifugation to remove cell debris; (4) concentration by tangential flow filtration; (5) purification using Sepharose 6FF chromatography; (6) inactivation with 0.04 % formalin; (7) blending; (8) mix with adjuvant, alum hydroxide; and (9) quality control tests. Experimental blended bivalent PUUV-DOBV vaccine named "Combi-HFRS-Vac" (Tkachenko et al. 2011, 2012) was successfully tested in preclinical studies. As seen in Table 5.2, immunization of BALB/c mice with Combi-HFRS-Vac induced antibody responses against PUUV and DOBV, and these antibodies were detected by IFA, ELISA, and PRNT assays.

5.4 Recombinant Hantavirus Vaccines

Several expression systems were used to express hantavirus nucleocapsid (N), and glycoproteins (G1 and G2) and to immunize experimental animals to test immunogenicity and protective efficacy in challenge experiments. Recombinant hantavirus proteins were successfully expressed in *Escherichia coli,* yeast, transgenic plants in baculovirus system, and in viral vectors including vaccinia virus and vesicular stomatitis virus (Dargeviciute et al. 2002; Lee et al. 2006; Geldmacher et al. 2004; Khattak et al. 2002; Lundkvist et al. 1993, 1996; Schmaljohn et al. 1990; Yoshimatsu et al. 1993; Maes et al. 2006, 2008; Lundkvist and Niklasson 1992; Krüger et al. 2011; de Carvalho et al. 2002). Recombinant PUUV NP expressed in yeast induced protective immunity in experimentally immunized bank voles (Dargeviciute et al. 2002). The DOBV NP expressed in the same system induced high antibody titers after immunization of BALB/c and C57BL/6 mice (Geldmacher et al. 2004). PUUV NP was successfully expressed in transgenic tobacco and potato plants but failed to induce an antibody response in mice when administered as an oral vaccine (Kehm et al. 2001; Khattak et al. 2004).

The N protein and G1 and G2 glycoproteins were shown to induce protective immune responses in experimental rodents. Whereas this effect is explained by induction of neutralizing antibodies by the glycoproteins, the protective immune response against N, which is an internal viral protein, can be best explained by triggering cellular immunity (Krüger et al. 2011). Recombinant N protein of DOBV was tested in combination with various adjuvants for immunogenicity and protective efficacy in C57/BL6 mice. This study identified Freund's adjuvant as the additive of choice because mice that were vaccinated with this adjuvant in combination with the DOBV N showed a protection rate from challenge of 75 %, whereas the usage of other adjuvants such as Alum, which induces strong Th2-type immune responses, did not result in protective immunity (Klingstrom et al. 2004). Since the N protein is more conserved among different hantaviruses, an advantage of N protein use seems to be the induction of broader cross-reactive immunity against various hantavirus species.

Virus-like particles (VLPs) are highly structured, repetitive protein complexes that have many desirable properties as immunogens. Certain viral antigens, such as the HBV core antigen, will spontaneously form such complexes, and to varying extents, small regions of foreign proteins can be incorporated into the HBV core protein and serve as an antigen. Immunogenic epitopes of PUUV, DOBV, and HTNV NP incorporated into chimeric hepatitis B virus core particles elicited high antibody titers and protective immunity in bank voles (Ulrich et al. 1999; Geldmacher et al. 2004). These responses were strong indicating that the HBV-based VLP particles can be a promising platform for the development of hantavirus vaccines (Hjelle 2002).

In a hamster model, recombinant adenovirus expressing ANDV N and G1 or G2 protein protected vaccinated animals against homologous lethal challenge (Safronetz et al. 2009). Induction of neutralizing antibodies and protection against SEOV challenge were also observed after immunization with replication-competent recombinant canine adenovirus expressing SEOV G1 or G2 (Yuan et al. 2009, 2010).

HTNV NP, G1, and G2 expressed in baculovirus and vaccinia virus vectors were shown to induce protection after a HTNV challenge in hamster and mouse models (Yoshimatsu et al. 1993; Chu et al. 1995; Schmaljohn et al. 1990). HTNV vaccinia-vectored vaccines were shown to be efficacious and to confer cross-protection against SEOV (Chu et al. 1995; Schmaljohn et al. 1990). The vaccine was tested in a phase II, double-blinded, placebo-controlled clinical trial among 142 volunteers. Neutralizing antibodies to HTNV were detected only in 72 % of the vaccinated individuals (McClain et al. 2000). Due to limited seroconversion and the potential side effects of live vaccinia virus, the trial was terminated (Schmaljohn 2009; Hammerbeck et al. 2009).

The role of anti-N and anti-glycoprotein (G1, G2) hantavirus immunity in the protection of experimental animals was studied using DNA immunization and alphavirus replicon system. While immunogenicity of DNA vaccines varied in different animal models, these studies confirmed previous observations and showed that immune responses against G1 and G2 glycoproteins were associated with

stronger protection (Hammerbeck et al. 2009). DNA vaccines expressing glycoproteins of HTNV and PUUV were tested in 28 volunteers using DNA-loaded particles and epidermal delivery device (Schmaljohn 2009; Boudreau et al. 2010). The data showed significant levels of neutralizing antibodies against both hantaviruses.

The aerosolized DNA vaccine (PEI + rDNA) containing complexes of polyethyleneimine with recombinant DNA expressing the PUUV G1 gene under CMV promoter was prepared and used to immunize BALB/c mice in aerosolized chamber with ultrasonic generator (Filatov et al. 2007). Immunization with aerosolized vaccine was accompanied with intraperitoneal injection of adjuvant (proteoglycan of natural origin). Immunization protocol included two prime immunizations and one boost. The aerosolized DNA vaccine (PEI + DNA) induced in mice PUUV-specific IgM, IgG, and IgA antibodies assayed in ELISA.

Experimental DNA vaccines expressing the envelope glycoprotein genes of HTNV or PUUV viruses were evaluated in phase 1 study in three vaccination groups, nine volunteers/group (Boudreau et al. 2012). The volunteers were vaccinated by particle-mediated epidermal delivery (PMED) three times at 4-week intervals with the HTNV DNA vaccine, the PUUV DNA vaccine, or both vaccines (from HTNV, strain 76-118, or PUUV, strain P360). At each dosing, the volunteers received 8 μg DNA/4 mg gold. There were no vaccine-related serious adverse events. Nonspecific events were fatigue, headache, malaise, myalgia, and lymphadenopathy. Blood samples were collected on days 0, 28, 56, 84, 140, and 180 and assayed for the presence of neutralizing antibodies. In the single-vaccine groups, neutralizing antibodies to HTNV or PUUV were detected in 30 % or 44 % of individuals, respectively. In the combined-vaccine group, only 56 % of the volunteers developed neutralizing antibodies to one or both viruses (Boudreau et al. 2012).

Brocato et al. reported the synthesis of a codon-optimized, full-length M-segment open reading frame and its cloning into a DNA vaccine vector to produce the plasmid pWRG/PUU-M(s2). pWRG/PUU-M(s2) delivered by gene gun produced high-titer neutralizing antibodies in hamsters and nonhuman primates. Vaccination with pWRG/PUU-M(s2) protected hamsters against challenge with PUUV but not against infection with related HFRS-associated hantaviruses, DOBV and HTNV. Unexpectedly, the DNA vaccine protected hamsters against fatal disease caused by Andes virus (ANDV). This cross-protection was not associated with induction of ANDV cross-neutralizing antibodies. This was the first evidence of efficacy of an experimental DNA vaccine against HFRS in a hamster lethal disease model (Brocato et al. 2012).

A multi-epitope chimeric DNA vaccine against of SEOV, HTNV, and PUUV was constructed by Zhao et al. (2012). This vaccine elicited strong humoral and cellular immune responses against all targets providing feasibility for multi-epitope vaccination approach. In spite of these encouraging results, low immunogenicity in humans remains the major obstacle for development of DNA vaccines against hantavirus infections (see also Chapter 6).

5.5 Conclusion

The current status and future of hantavirus vaccines varies among different countries depending on endemic areas. The population of Eurasian countries and, first of all, the population with the high morbidity rate (China, Russia, North and South Korea, Finland, Sweden, Germany) are potentially the target for vaccination against HFRS. In the Americas, the HPS-caused morbidity rate is significantly lower and vaccination against HPS is not so obvious. Nevertheless, possible target groups can include rural residents of relatively small endemic areas, such as Western New Mexico, California's Sierra Nevada Mountains, and rural regions of the Pacific Northwest. Target groups will be persons with active outdoor occupations, for example, field biologists, forestry workers, and farmers. The at-risk populations will also include members of American Indian tribes as far east as Oklahoma and residents of the Rockies, Sierras, and Cascade mountains and in the surrounding foothills. Outside of the United States, there are many areas where the demand for vaccines is even stronger. These areas include Southern Chile from Santiago to Puerto Natales with population around 4–5 million and endemic areas of Argentina, Southern Brazil, Paraguay (Gran Chaco), and Bolivia with additional million of populations at risk. These regions are likely to remain hotspots for vaccine demand for the foreseeable future.

Based on the size of predicted population at risk, the potential market for hantavirus vaccines is likely to be in the tens of millions of doses in the western hemisphere and probably exceeds 100 million doses in Eurasia (Hjelle 2002). It seems that due to relatively high cost-benefit ratio, an HPS vaccine will be not recommended for routine use. However, in HPS endemic areas where increased contact with rodents is expected, vaccination would be advisable (Schmaljohn 2012). It is quite obvious that a commercially feasible HPS vaccine would be one that could protect against both clinical forms of hantavirus infections, HFRS and HPS. The ideal hantavirus vaccine should confer long-term protection against all epidemiologically significant hantaviruses circulated in the endemic region with no more than two or three timely close applications. The side-effect profile should be acceptable, and it would be beneficial to offer the vaccine simultaneously with vaccines against other agents that produce related symptoms, such as influenza or pneumococcus (Hjelle 2002).

The hantaviruses, causative agents of HFRS and HPS, require at least a biosafety level 3 containment to handle these viruses due to the hazardous nature of the infection and a possible aerosol transmission. The high level of containment is an additional challenge for hantavirus vaccine development. Recombinant DNA vaccine technologies provide a good opportunity to overcome this roadblock. During the last decades, several research groups published promising results in preclinical studies in small animal models using different approaches based on DNA recombinant technologies. Nevertheless, much more should be done to see feasible recombinant hantavirus vaccines for human use.

Currently, only inactivated culture cell-derived hantavirus vaccines are available for human use to control hantavirus infections in endemic areas. A few million doses of these vaccines were distributed (mostly in China and North and South Korea) without serious adverse events suggesting that these vaccines are well tolerated. However, new generation of hantavirus vaccines for HFRS and/or HPS with long-lasting humoral immune responses and increased cross-protective efficacy is needed to effectively control hantavirus infections.

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Chapter 6
Experimental DNA-Launched Live-Attenuated Vaccines Against Infections Caused by Flavi- and Alphaviruses

Peter Pushko, Peter P. Bredenbeek, and Igor S. Lukashevich

Abstract DNA vaccines represent a promising technology due to their safety, ease of production, genetic stability, no need for cold chain, and activation of innate immunity by recombinant DNA produced in bacterial cells. However, so far there are no licensed DNA vaccines for prevention of human infectious diseases. While a few DNA vaccines have been recently approved for veterinary applications, low immunogenicity in humans is the major obstacle for clinical applications. In contrast, live-attenuated vaccines are among the most cost-effective and broadly used public health interventions. They represent approximately 60 % of all licensed vaccines and provide long-term immunity following a single-dose vaccination. Live-attenuated vaccines have their own limitations including the need for bio-containment during production, cold chain requirements, and safety concerns due to the possibility of reversion, especially for RNA viruses.

Here we describe a novel infectious DNA (iDNA®) vaccine technology which combines advantages of naked DNA vaccination and live-attenuated vaccine efficacy. Using yellow fever 17D vaccine, an experimental IND vaccine TC-83 against Venezuelan equine encephalitis virus (VEEV), and live-attenuated chikungunya (CHIK) 181/25 vaccine candidate, we have provided proof-of-concept evidence that these vaccines can be launched from DNA and induce specific immune responses against pathogenic RNA viruses. The iDNA vaccine technology is based on the transcription of the full-length genomic RNA of the live-attenuated

P. Pushko
Medigen, Inc., Frederick, MD, USA

P.P. Bredenbeek
Department of Medical Microbiology, Center of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands

I.S. Lukashevich (✉)
Department of Pharmacology and Toxicology, School of Medicine, Center for Predictive Medicine for Biodefense and Emerging Infectious Diseases, NIH Regional Bio-containment Laboratory, University of Louisville, 505 South Hancock Street, Louisville, KY, USA
e-mail: isluka01@louisville.edu

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virus from plasmid DNA in vitro and in vivo. A few ng of iDNA encoding the full-length genomic RNA are required to initiate the replication of the vaccine virus in vitro. The in vivo-generated viral RNA initiates limited replication of the vaccine virus, which in turn leads to efficient immunization. Electroporation in vivo has induced specific immune responses against pathogenic viruses and protected mice against fatal disease. DNA-launched vaccine is "manufactured" in vaccinated individuals and does not require traditional vaccine manufacturing facility and technology. If successful in further testing, this technology can dramatically change the way we make vaccines as well as vaccination practice.

6.1 Introduction

Replication competent (RC) or "live-attenuated" vaccines are among the most cost-effective and widely used public health interventions. The vaccines against diseases caused by RNA viruses such as for polio, measles, mumps, and yellow fever dramatically reduced the incidence of these infectious diseases. Historically, these vaccines were generated empirically by repeated passages in cultural cells which resulted in the reduction of wild-type virulence with preservation of immunogenic potential. They induce almost lifelong protection and are easily manufactured at large scale. Progress in life science, biotechnology, and medicine changed public perception about vaccines from the preventive effectiveness to the safety. This shift has led to the irony that the present highly tight regulatory standards would probably prevent the licensure of the most successful "old" live-attenuated vaccines today.

Advances in molecular virology and rational design of RC vaccines provide new opportunities for the development of the next generation of RC vaccines that optionally balance safety and effectiveness (Lauring et al. 2010). Rationally designed live-attenuated vaccines against influenza (FluMist®, MedImmune, Inc) and rotavirus (Rotarix®, GSK Biologicals; RotaTeq®, Merck &Co., Inc.) infections are good examples of new generation of RC vaccines. Notably, these vaccines were approved for children <2 years old indicating that these vaccines are safe even for immunologically vulnerable groups.

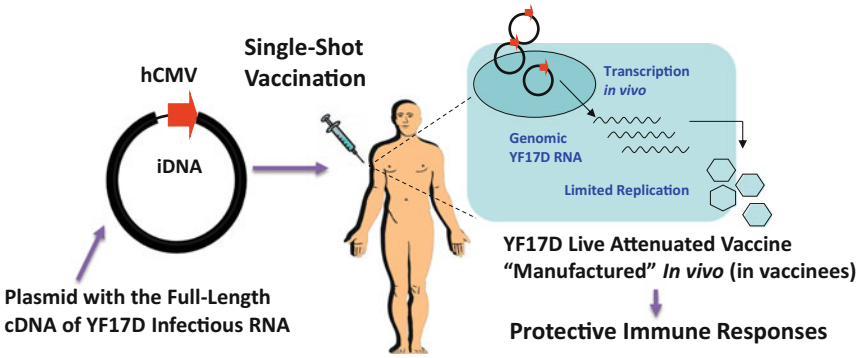
Live-attenuated vaccines provide a highly favorable benefit-risk profile and excellent cost-effectiveness. As exemplified by yellow fever 17D vaccine, in YF endemic areas, a vaccinated child is fully protected over a 50-year lifetime against different genotypes of YF for an investment of a few cents per year (Galbraith and Barrett 2009; Monath 2005). This vaccine is safe and highly immunogenic. After a single intramuscular inoculation, YF17D minimally replicates in local dendritic cells (DCs) and stimulates DC subsets via multiple Toll-like receptors (TLRs) to elicit proinflammatory cytokines (Barba-Spaeth et al. 2005; Querec et al. 2006; Palmer et al. 2007). Cytokine-activated and mature DCs become protected against

YFV-induced cytopathogenicity and migrate to regional lymph nodes to elicit a broad spectrum of innate and adaptive immune responses (Akondy et al. 2009; Martins et al. 2007, 2008; Santos et al. 2008; Miller et al. 2008). A systems biology confirmed that YF17D induces integrated multi-lineage and polyfunctional immune responses including the activation of effector mechanisms of innate immunity (complement, multiple TLRs, cytokines/cytokine receptors, and interferons), as well as adaptive immune responses through an early T-cell activation followed by robust B-cell responses (Gaucher et al. 2008; Querec et al. 2009). Computational analyses identified a gene signature, B-cell growth factor TNFRS17, predicting with up to 100 % accuracy the neutralizing antibody response, a well-established correlate of protection (Querec et al. 2009).

The inherent instability of the RNA genome is the major challenge for the development of RC live-attenuated vaccines against RNA viral diseases. The currently produced YF17D-derived vaccines (the original "YF17D" attenuated strain is not available) consists from three sub-strains, 17D-204, 17DD, and 17D-213 with a few nucleotide differences (Rice et al. 1985; Hahn et al. 1987; dos Santos et al. 1995). In 1945, a seed-lot system was introduced to assure vaccine consistency. Nevertheless, a lot-to-lot variability is a real problem during manufacturing of these vaccines. Some of them are still producing using old technology that has not been practically changed during the last 50 years. Additional disadvantages of live-attenuated vaccines include risk of contamination with adventitious agents during manufacturing, risk of reversion to pathogenic viruses, and cold-chain requirement. In tropical countries, almost 80 % of vaccination cost is associated with a cold chain.

DNA vaccination technology addresses many of these challenges (Fig. 6.1). Unlike RC live-attenuated vaccines and conventional "killed" vaccines, DNA vaccines represent recombinant bacterial plasmids encoding the gene of interest (antigen/s) under control of a strong eukaryotic promoter driving antigen expression. DNA immunization results in low levels of antigen expression in transfected somatic cells (myocytes, keratinocytes) and probably in professional antigen-presenting cells (DCs, macrophages), presentation through MHC class I and II pathways (including cross-presentation), and stimulation of humoral and cellular immune responses. Intrinsic adjuvant property of recombinant bacterial DNA (e.g., activation of TLR9 by unmethylated CpG) seems to contribute to induction of innate immunity and adjuvant effects of DNA vaccines (Li et al. 2012; Kutzler and Weiner 2008). DNA vaccines can be manufactured with clinical grade purity in a relatively inexpensive and rapid fashion; they are proved to be safe in numerous clinical trials. Recombinant DNA technology provides powerful tools for design DNA vaccines with a well-defined genetic composition to ensure genetic stability and safety profile. Rationally designed DNA vaccines can express not only gene (s) of the interest but also genes encoding cytokines and other co-stimulatory molecules to enhance immunogenicity. Notably, DNA vaccines are relatively resistant to temperature, do not require cold chain, and have a long shelf life.

Unfortunately, up to date, 25 years after discovery of DNA immunization (Wolff et al. 1990), there are no licensed DNA vaccines for human use. In spite of



Vaccine Requirements	LAV	DNA	iDNA
Genetic Stability	NO	YES	YES
Simple Manufacturing Control	NO	YES	YES
No Cold Chain	NO	YES	YES
Single Dose Application	YES	NO	YES
Min Nuclear Involvement	YES	NO	YES
Rapid Immune Response	YES	NO	YES
Effective Protection	YES	NO	YES

Fig. 6.1 Infectious DNA (iDNA®) vaccine technology. This technology combines the positive attributes of both live-attenuated (LAV) and conventional DNA vaccines. Cartoon schematically describes the cloning infectious RNA of yellow fever 17D vaccine in bacterial plasmid under control of eukaryotic CMV promoter. After several optimization steps, the recombinant plasmid (iDNA) injected into a vaccine to transfect in vivo target cells. The in vivo-generated viral RNA initiates limited replication of the vaccine virus, which in turn leads to efficient immunization. DNA-launched vaccine is "manufactured" in vaccinated individuals and does not required traditional vaccine manufacturing facility and technology. Inserted table provides advantages of iDNA technology in comparison with LAV and conventional DNA vaccines

tremendous efforts to enhance DNA vaccine immunogenicity using a wide variety of strategies, poor immune responses in humans remain the Achilles heel of DNA vaccines (Li et al. 2012). Still, several DNA vaccines have been approved in some areas of veterinary medicine. Preventive DNA vaccines were licensed to protect salmonid fish against hematopoietic necrosis virus (Apex-IHV, Novartis, Canada) (Alonso and Leong 2013) and horses against West Nile virus infection (West Nile-Innovator, CDC, and Fort Dodge Labs, USA). Two DNA vaccines have been recently available for gene therapy in dogs (canine melanoma vaccine) and in pigs to decrease perinatal mortality and morbidity (growth-hormone-releasing hormone) (Kutzler and Weiner 2008). These promising results in veterinary applications provide hope that DNA vaccine technology will overcome current limitations in humans and will be used as preventive vaccination, probably as a DNA prime/protein or vector boost approaches, in the nearest future. Recently approved preventive vaccines (Cervarix® and Gardasil®) against cervical carcinoma caused

by human papillomavirus (HPV)-16 and HPV-18 and new cutting-edge technologies renovated interest to recombinant DNA encoding one or more tumor-specific antigens to elicit immune responses (see Part II of the book). Numerous naked DNA-based vaccines against different forms of cancer are currently in clinical trials (Senovilla et al. 2013; Pol et al. 2014).

The idea to use a recombinant naked DNA to launch a live-attenuated vaccine from eukaryotic promoter in vivo came up during experiments with a full-length cDNA corresponding to the YF17D genome in attempts to use YF17D as a vector to clone and express antigens of Lassa fever, a preventable infection that touches the lives of hundreds of thousands per year in West Africa (Bredenbeek et al. 2006; Jiang et al. 2009, 2011; Carrion et al. 2012). Earlier similar approaches with partial success were applied for West Nile and foot-and-mouse infections (Gordon et al. 1997; Hall et al. 2003; Yamshchikov 2008). This idea has been tested in feasibility experiments using YF17D, Venezuelan equine encephalitis virus (VEEV) TC-83, and CHIK 181/25 vaccines and resulted in the development of "infectious DNA" (iDNA®) vaccine/vaccination technology (Tretyakova et al. 2013, 2014a; Pushko and Lukashevich 2014). Prove-of-concept studies in support of this novel technology are provided in this chapter.

6.2 A DNA-Launched Yellow Fever Virus 17D Infectious Clone

Despite the availability of a highly effective YF17D vaccine, the number of YF cases has increased over the past three decades, due to the declining immunity to YFV infection in the population, deforestation, urbanization, population movements, poor vector control, and climate change. Until recently, the vaccine was still in short supply, and vaccine coverage is low in high-risk areas. The fact is that YF is still a reemerging infection in tropical and subtropical areas of Africa and remains a major health threat in South America. Based on WHO estimations, YF affects ~200,000 individuals annually, of whom approximately 30,000 will die mostly in Africa. A major concern is the risk of introduction of the YFV into Asia where YF has not yet been reported (Monath 2004, 2005; Monath and Heinz 1996; Lefeuvre et al. 2004; Lindenbach and Rice 2001). Alternative strategies to facilitate vaccine production, transportation, or storage would therefore help accommodate the increasing needs for YFV vaccine in the nearest future.

An incubation period of the disease is 3–6 days following the infected-mosquito bite. The clinical spectrum of YF is very broad, including subclinical infection, nonspecific flu-like illness, and severe febrile disease with jaundice, renal and liver failures, hemorrhages, and fatal shock. The classic illness is characterized by three stages. During the first stage, "period of infection" (3–4 days) virus is present in the blood. Fatal cases have longer period of viremia than survivors and viremia can be as high as 5–6 log LD_{50}/ml for experimentally inoculated mice. In some cases,

period of infection may be followed by a "period of remission" which is often not obvious and very brief. In case of abortive infection, the patient can be recovered at this stage. Approximately 15 % enter the "period of intoxication" and develop moderate or severe disease characterized by jaundice. On 3–6 days after onset, fever returns and bradycardia, nausea, vomiting, jaundice, oliguria, and hemorrhagic manifestations are appeared. The subsequent progression course reflects multiple organ dysfunction including severe liver damage (viscerotropism), kidneys, and cardiovascular system. In the progressed cases lethality can be as high as 50 %. The critical period of the disease is between fifth and tenth days, when patient can die or recover (Monath 2004).

YFV, a member of the *Flaviviridae* (genus *Flavivirus*), is a small, enveloped virus with a single-stranded, positive-sense RNA genome of ~11 kb in length (Lindenbach and Rice 2001). The genome encodes a 5′-untranslated region (5′UTR), followed by a single open reading frame (ORF) and a 3′UTR. The ORF codes for a polyprotein 3,411 amino acids in length that is co- and posttranslationally cleaved to generate three structural proteins (C, capsid; prM, pre-membrane; E, envelope) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). The NS5 protein encodes flavivirus RNA-dependent RNA polymerase (RdRp). There is an experimental evidence that RdRp of YF17D-204 has a very low error rate (2×10^7 substitutions per site) in comparison with other RNA viruses (Pugachev et al. 2004).

During late steps of YFV replication, the structural proteins are incorporated in the mature virion particles, whereas the NS proteins are responsible for the replication and processing of a polyprotein. The C protein interacts with the RNA genome to form the viral nucleocapsid. The prM is a chaperone for proper folding of the E protein. The E protein contains determinants of hemagglutination and neutralization, interacts with cell receptors, and involves in membrane fusion resulting in the release of viral RNA in cytoplasm of host cells. Genetic studies have elucidated a pivotal role of the E protein in cell tropism, virulence, and immunity (Monath 2004; Lindenbach and Rice 2001). Although some potentially important mutations have been identified in E gene, it is clear that virulence is a multigenic phenotype determined by structural and nonstructural genes (Monath 2004).

The 17D vaccine is one of the safest and most effective ever developed. This vaccine was empirically developed in the 1930s by Theiler and Smith from the prototype wild-type Asibi strain of YFV by 176 serial passages in different tissues of mouse and chick embryo including minced chick embryo without brain and spinal cord. The resulting YF17D strain lost replication capability in mosquito cells as well as neurotropic and viscerotropic features while maintaining strong immunogenicity in humans. A single-dose injection induces high-affinity neutralizing antibodies, correlates of protection in over 98 % vaccinees. In a recent position paper on vaccines and vaccination, WHO has revised a 10-year booster vaccination recommendation and confirmed that a single dose of YF vaccine is sufficient to confer sustained lifelong protective immunity against YF disease; a booster dose is not necessary (Monath 2005, 2012; WHO 2014).

For last 75 years, YFV-17D vaccine has been administered to over 600 million people globally and has stood as a paradigm for a successful vaccine with a great record of both safety and efficacy (Querec et al. 2006). Currently, approximately 30 million doses of YF17D vaccine are distributed annually in endemic areas, among travelers and military personnel. Rare adverse events with either neurotropic or viscerotropic manifestations resulted in high fatality, 0.05–1.5 cases per 100,000 vaccinations (Monath 2012). Mechanisms of these rare complications seem to be associated with host responses rather than with YF17D vaccine itself.

Whereas YF17D remains a "gold standard" for successful human vaccines, our understanding of mechanisms of attenuation is still limited and nucleotide and amino acids sequences responsible for attenuation remain to be elucidated. Recent study has shown that the population of wild-type Asibi virus has a highly diverse quasispecies structure, whereas the YF17D population is very homogeneous (Beck et al. 2014; Tangy and Desprès 2014). Due to high error of RdRp, quasispecies populations are genetic features of many RNA viruses and this feature seems to be associated with pathogenic potential. Notably, attenuated mutations were not found in Asibi virus population indicating that YF17D was generated by discrete mutations rather than selection of genomes in the wild-type population.

YF17D vaccine manufacturing technology is a little change for the last decades. This vaccine is still manufactured using chicken embryos and egg allergy in vaccinees is a contraindication for vaccination. Lot-to-lot variability, possible mycoplasma and adventitious agent contamination, risk of reversion to wild-type genotype, and obligatory cold-chain requirement justify attempts to improve YF17D vaccine and vaccination practice.

With the objective of combining the commendable features of DNA vaccines with high efficacy of a live-attenuated vaccine, a DNA-launched YF17D vaccine was designed as shown in Fig. 6.2. In brief, the 5′UTR of the viral RNA was fused with a cytomegalovirus (CMV) promoter so that cellular RNA polymerase II would initiate the transcription of the YF17D genome. The hepatitis delta virus ribozyme (HDVr) was engineered precisely after the last nucleotide of YFV genome to ensure the production of an authentic 3′-end of the transcribed viral RNA. Initially the CMV promoter and HDVr cassettes were cloned into the plasmid pACNR-FLYFx (Bredenbeek et al. 2003). However, the resulting plasmid pACNR-CMVp-FLYF-HDVr was found to be genetically unstable during propagation in *E. coli* DH5α. Attempts to stabilize the plasmid by using different bacteria strains or alternative culture conditions were unsuccessful suggesting that YFV cDNA contains cryptic bacterial promoters that drive synthesis of toxic proteins affecting genetic stability and recombinant DNA yields in *E. coli*. To decrease the copy number of recombinant plasmid and to stabilize the construct, the bacterial artificial chromosome (BAC) vector (She 2003), pBeloBAC11, was used as a vector for the DNA-launched YF17D cassette. The resulting recombinant BACmid, pBeloBAC-FLYF, was shown to be genetically stable at least up to 20 passages in either *E. coli* DH5α or DH10B (Jiang et al. 2014).

Transfection of cells with recombinant BACmid launched the replication of infectious YF17D. As seen in Fig. 6.2 (panel b), ^{3}[H]-labeled viral RNA (in the

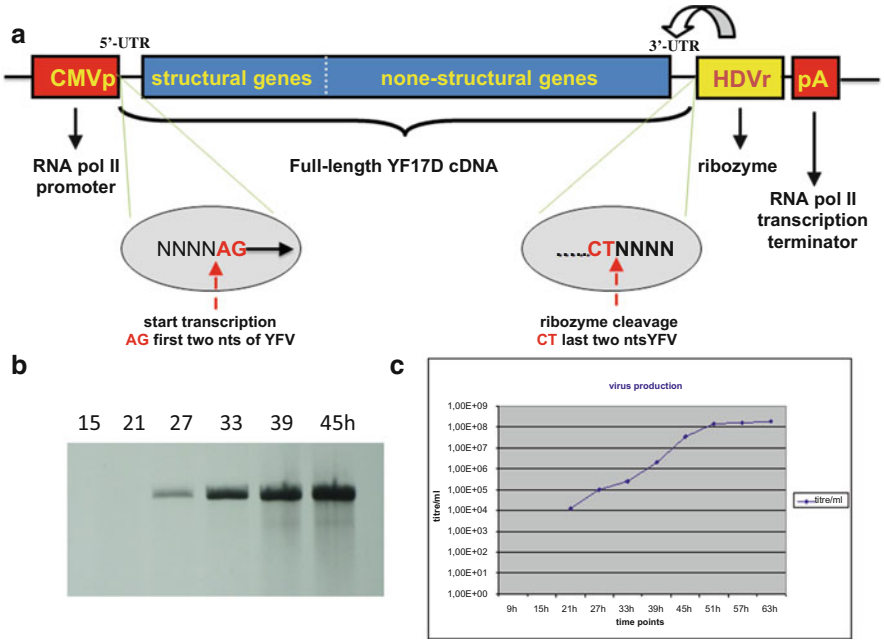


Fig. 6.2 Schematic representation of the genetic structure of pBeloBAC11-FLYF. (**a**) *Colored boxes* indicate from *left* to *right* the CMV promoter (CMVp) (*red*), the YFV-17D ORF (*blue*), the hepatitis delta virus ribozyme (*yellow*), and the RNA polymerase II transcription terminator (*red*). The 5′ and 3′ YFV UTR sequences are depicted as a *black line*. The most 5′ YFV nucleotides that originate from the CMV promoter-driven RNA polymerase II transcription and the last two nucleotides of YFV genome that are produced by cleavage of the ribozyme are also indicated. (**b**) Analysis of intracellular YF17D RNA synthesis by ^{3}[H]-uridine labeling and gel electrophoresis. BHK cells transfected with pBeloBAC-FLYF were labeled for 6 h in the presence of actinomycin D. (**c**) Kinetics of virus production in BHK-21J cells transfected with pBeloBAC-FLYF as determined by plaque assays of the supernatants collected at the indicated times post-electroporation

presence of actinomycin D) was first detected at 27 h post-electroporation and the amount of labeled RNA gradually increased over time, probably reflecting the spread of infection to cells that were initially not transfected. The replication kinetics of DNA-launched YF17D resulted in virus yields, ~8 $\log_{10}$ PFU/ml, comparable with yields of commercial YF17D-204 vaccine.

The second approach to improve genetic stability and increase recombinant plasmid yield included insertion of an 82nt-long intron at position of 9152 of the full-length YF17D cDNA (Tretyakova et al. 2014b). The site for intron insertion was chosen by predicting bacterial promoters within the cloned YF17D sequence. Putative bacterial promoters have been identified at positions between 8617 and 9012 nucleotides. The intron was inserted at position 9152 and it contained five stop codons to prevent translation of downstream putative polypeptides in *E. coli*. The resulting plasmid, YF17D-16 iDNA, was stable in *E. coli* Stbl3 and used for further

in vitro and in vivo studies. Transfection with this iDNA resulted in the replication of YF17D virus, and as low as 10 ng was required to launch YF17D replication. SDS-PAGE and Western blot analyses confirmed the expression of virus-specific structural and nonstructural proteins in iDNA-transfected cells. Plaques produced by iDNA-launched viruses appeared to be more uniform in size as compared with plaques produced by commercial YF17D-204 virus (Tretyakova et al. 2014b).

For many years experimental studies on the molecular mechanisms controlling YF pathogenesis and attenuation were limited due to the lack of a small animal model reproducing human disease. It has been shown recently that virulence of wild-type YFV in mice is IFN-α/β-dependent while attenuation of live-attenuated 17D-204 virus is not (Meier et al. 2009). Based on this discovery, subcutaneous infection of A129 mice deficient in receptors for IFN-α/β with YFV represents a biologically relevant model for studying viscerotropic infection and disease development following wild-type virus inoculation. However, the experiments with wild-type YFV are still required a high containment facility (ABSL-3). Meanwhile, additional studies have shown that intraperitoneal inoculation of AG129 mice deficient in receptors for IFN-α/β/γ with YF17D induces manifested disease in a dose-dependent manner. YFV17D-infected AG129 mice exhibit high viral titers in brain and liver suggesting this infection is both neurotropic and viscerotropic. Based on these observations, infection of AG129 mice with vaccine strain YFV17D is considered as a valuable model of human YF (Thibodeaux et al. 2012). Notably, this model does not require BSL-3 containment.

YF17D-204 and virus derived from pYF17D-16 iDNA-transfected Vero cells replicated similarly in brain tissues of AG129 mice. The viral RNA gradually accumulated starting with day 3 and peaked on days 12–15 after infection. At this time point, infected animals expressed clinical signs of neurotropic disease (paresis, high-limb paralysis) and met euthanasia criteria shortly after this time point. Replication kinetics of YF17D and pYF17D-16 iDNA-derived virus in the liver tissues differed from those in the brain. Both viruses replicated very rapidly at early stage of the infection peaking at day 6 with viral burden comparable with those in the brain on days 12–15. While replication kinetic of both attenuated viruses was similar, the replication of YF17D-16 virus in liver was more attenuated in comparison with the parental YF17D-204 (Tretyakova et al. 2014b).

Early events after YF17D vaccination are critical for induction of robust adaptive immune responses and correlate with early production of IFN-γ (Neves et al. 2009, 2013). Both vaccine viruses, YF17D and pYF17D-16 iDNA, rapidly induced strong expression of IFN-γ in spleen of AG129 mice, with no significant differences either in the scale or timing of induction. As expected, IFN-γ mRNA induction was moderate in liver tissues (less cells are susceptible for induction); however, the response in mice vaccinated with YF17D-16 virus peaked earlier in comparison with animals injected with parental YF17D (Tretyakova et al. 2014b).

The induction of cellular and humoral immune responses against YFV was investigated by electroporation of pBeloBAC-FLYF into transgenic AAD mice expressing the α1 and α2 domains from the human HLA-A2.1 and the α3 domain of the murine H-2D^{b} in the C57BL/6 background. Mice were immunized

intramuscularly with 10 ng, 100 ng, or 1 μg of endotoxin-free pBeloBAC-FLYF-17D per mouse on days 0 and 14. Mice were subsequently sacrificed and their spleens were collected and used to determine the frequency of IFN-γ producing cells after exposure to YFV peptides identified as dominant HLA-A2-restricted $CD8^+$ and $CD4^+$ T-cell epitopes in an ELISPOT assay.

A dose-dependent cellular immune response against YFV was clearly detectable in mice vaccinated with pBeloBAC-FLYF. Both $CD8^+$ and $CD4^+$ T-cell responses were highest following a dose of 1 μg of priming and boosting. Results of intracellular cytokine staining for YFV-specific $CD8^+$ T cells producing IL-2 and/or IFN-γ confirmed dose-dependent response and showed that although the majority of the $CD8^+$ T cells produced IFN-γ after stimulation with YFV-specific peptides, polyfunctional $CD8^+$ T cells were also detected (Jiang et al. 2014).

Induction of any detectable level of neutralizing antibodies is sufficient for protective immunity after vaccination with YF17D vaccines. Kinetics of neutralizing antibodies after immunization with 5 μg of pBeloBAC-FLYF was studied in A129 mice deficient for receptors for IFN α/β. Despite the fact that no virus was detected at any time point in the sera of A129 that were immunized with pBeloBAC-FLYF, these animals produced specific YF17D neutralizing antibodies. The $PRNT_{50}$ values peaked on day 11 postimmunization with titers 1:320 and 1:640 for the two subjects, respectively (Fig. 6.3). In accordance with these results, all BALB/c mice vaccinated with a single dose of pYF17D-16 iDNA (injection-electroporation) induced YFV-specific antibodies with similar $PRNT_{50}$ titers (Tretyakova et al. 2014b).

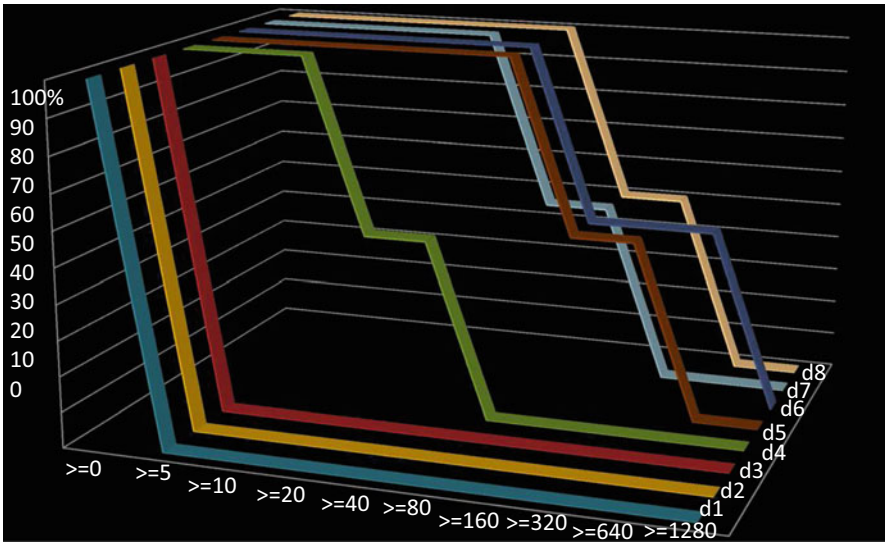


Fig. 6.3 Plaque reduction neutralization test (PRNT) results in A129 mice vaccinated with pBeloBAC-FLYF iDNA. Sera drawn from A129 mice at indicated time points were diluted and tested in $PRNT_{50}$. Percentages of subjects (mice) are plotted against $PRNT_{50}$ value

In summary, iDNA approach was applied to prepare a novel vaccine for YF. This approach resembles the traditional "infectious clone" technology but does not involve in vitro RNA transcription, thus allowing the use of iDNA for direct vaccination in vivo. The successful launch of YF17D vaccine in vivo resulted in induction of YFV-specific cell-mediated immunity and neutralizing antibodies in experimental animals. To better evaluate the potential of this iDNA-launched YF17D vaccine platform and its possible applications in human beings, the immunogenicity and efficacy as well as the safety profile should be appropriately assessed in nonhuman primates. In contrast to traditional DNA vaccination, a nanogram quantity of iDNA seems to be sufficient to launch YF17D and induce protective immune responses. Currently produced YF17D vaccine are found to contain 5×10^3–2×10^5 PFU per vaccination dose. In most successfully vaccinated individuals low viremia (<100 PFU/ml) can be detectable shortly after vaccination. However, non-viremic vaccinees are still capable to raise protective neutralizing antibodies. It indicates that even with variation in efficacy of in vivo transfection, iDNA immunization can provide protection as far as replication-competent YF17D will be launched. Nevertheless, iDNA dosage needs to be properly adjusted and novel DNA formulations and delivery routes could be explored to achieve better immunogenicity and acceptability. Although the electroporation method has been widely tested in a broad range of animals and has shown promising results in human clinical trials with DNA vaccines, intradermal delivery of DNA vaccines has started to attract more attention, due to the accessibility of the skin and the abundance of antigen-presenting cells in the epidermis and dermis. With the advances in DNA formulation and delivery technologies, the iDNA-launched YF17D vaccine may prove to be a stable and easy-to-produce/control effective alternative for the current YF17D attenuated vaccine and as a promising vector platform for the development of YF17D-based recombinant iDNA vaccines for flavivirus-related and non-related targets.

6.3 DNA-Launched Attenuated Vaccine for Venezuelan Equine Encephalitis Protects Mice Against Fatal Challenge

In contrast to YF17D, VEE TC-83 vaccine is an example of live-attenuated vaccine for limited applications. Almost for two decades this vaccine has been used to protect military forces or laboratory personnel. This vaccine was developed in the 1960s (Berge et al. 1961) and was used under Investigational New Drug protocol (IND#142, 1965) as a part of Special Immunization Programs for medical/military personnel at risk. The supply of the TC-83 vaccine is limited, as the last batch of vaccine was generated in 1972. According to the CDC, the TC-83 vaccine provides

long-lasting immunity and protection against subcutaneous and aerosol VEEV challenges. However, while TC-83 is effective, it also causes adverse effects such as headache, myalgia, fever, and fatigue in ~23 % of vaccine recipients. Another ~18 % of vaccine recipients do not develop sufficient neutralizing antibody titers and require boost with formalin-inactivated C-84 vaccine, also under IND protocol. To overcome limitations of existing investigational VEE vaccines, the development of more safer and effective vaccines is urgently needed. Novel vaccine technologies do show a promising future, and several VEE vaccine candidates based on different vaccine platforms (recombinant subunit, attenuated chimeric, alphavirus replicons, DNA vaccines) are currently in preclinical development (Carossino et al. 2014).

VEEV is a veterinary and human pathogen that causes severe neurological disease in human and horses and can be responsible for explosive outbreaks. VEE is mosquito-borne infection and humans and equids can be infected by a wide variety of mosquito vectors. The population of susceptible mosquitos is already flourishing in the US horses, and humans can act as amplifying hosts, develop a high-titer viremia, and transfer disease via mosquitos. The initial VEEV symptoms are similar to influenza and are difficult to diagnose. Clinical manifestations include fever, anorexia, depression, and signs of encephalomyelitis. Case fatality may be up to 90 % for both humans and horses. VEEV causes epizootics and epidemics in the North, Central, and South America including an outbreak in Texas in 1971. Climate, ecological changes, and international travel have increased the risk of VEEV reemergence. The virus can also be easily produced in large quantities and aerosolized as a biological weapon. The potentially threatening effects of the VEEV reemergence demand an effective vaccine (Carossino et al. 2014).

VEEV belongs to *Alphavirus* genus of *Togaviridae*. The genus contains ~30 members globally distributed, and some of them can cause a neurological disease in human and animals. Similar to YF virus, alphavirus genome is a single-stranded, positive-sense RNA encoding four nonstructural proteins (NSP1–4) which are translated from the genomic RNA and interact with host factors to form enzyme complexes involved in transcription/replication and polyprotein processing. These complexes synthesize the negative-strand RNA intermediates, new viral genomes, and the 26S sub-genomic RNA encoding structural proteins, capsid (C) and glycoproteins E1 and E2, exposed on the surface of virions. Glycoproteins are involved in attachment, endocytosis, membrane fusion, and release of genomic RNA into the cytoplasm of infected cells. Surface glycoproteins form heterodimers and induce production of neutralizing antibodies, correlates of protection.

Due to its relatively long record of clinical use, VEE TC-83 vaccine represents a logical starting point for design of safer and more immunogenic vaccine against VEEV. The iDNA technology provides attractive opportunity to make better TC-83 vaccine (Tretyakova et al. 2013; Pushko and Lukashevich 2014). The TC-83 vaccine is also a promising vaccine vector which can be used as a carrier for therapeutic cancer or preventive vaccine against infectious diseases (Pushko 2006).

The structure of pTC83 iDNA containing the full-length genome of VEE TC-83 is shown in Fig. 6.4. The full-length genome was assembled from four cDNA

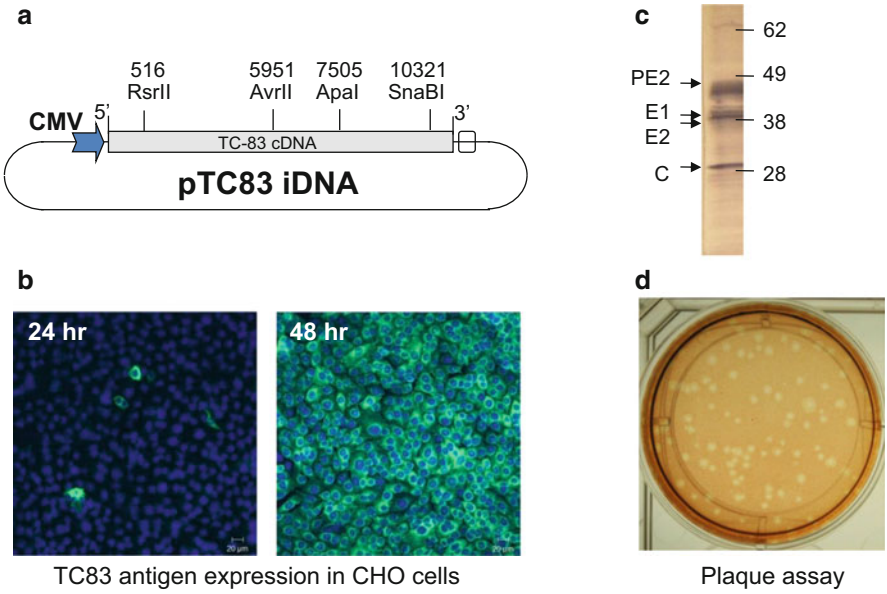


Fig. 6.4 Preparation of pTC83 iDNA containing the full-length TC-83-cloned genome and generation of TC-83 virus in transfected CHO cells. (**a**) Schematic representation of pTC83 plasmid. Restriction sites used for preparation of the full-length TC-83 clone are indicated. (**b**) Indirect immunofluorescence assay (IFA) of CHO cells transfected with pTC83 iDNA. IFA was performed at 24 h (*left panel*) and 48 h (*right panel*) post-electroporation. (**c**) Western blot of CHO cells transfected with pTC83 iDNA, 24 h post-electroporation. The blot was probed with ATCC antiserum against VEEV. (**d**) Plaque assay of the supernatant from CHO cells transfected with pTC83 iDNA

fragments initially cloned into pcDNA3.1 vector and placed downstream from the CMV major immediate-early promoter. Since the authentic 5′- and 3′-termini of RNA are critically important for alphavirus replication, the distance between the CMV promoter and the start of RNA polymerase transcription was optimized to ensure the transcription of the functional TC-83 genomic RNA. A ribozyme sequence derived from the hepatitis delta virus was inserted downstream from the TC-83 3′-terminal poly-A sequence. When this iDNA was introduced into cells, it generated the synthesis of virus-specific RNA and launched production of the TC-83 live-attenuated vaccine as evidenced by positive staining with specific antibodies and by detection of virus-specific antigens in cells. Transfection of 1 μg of iDNA and infection with 10^5 PFU of TC-83 virus resulted in a comparable number of antigen-expressing cells (Fig. 6.5a).

The TC-83 vaccine derived from in vitro transfection can be harvested from culture medium and used for vaccination according to current practices. Notably, TC-83 generated from iDNA represents homogeneous progeny virus generated from the same, well-characterized, stable DNA with genetically defined sequence. Such vaccine will have greater uniformity and lot-to-lot consistency compared to

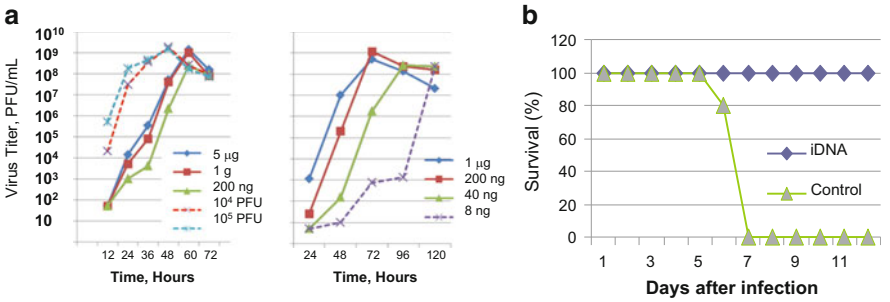


Fig. 6.5 (**a**) Growth curves of TC-83 viruses in virus-infected and in iDNA-transfected CHO cells. *Left panel*, CHO cells were either infected with 10^4–10^5 PFU of virus or transfected with 0.2–5 μg of pTC83 iDNA. *Right panel*, CHO cells were transfected with 8 ng to 1 μg of pTC83 iDNA. Plaque titer was determined in duplicates; error bars are not visible at the scale shown. (**b**) Survival of BALB/c mice after challenge with VEEV. Mice were vaccinated by electroporation with 50 μg of pTC83 iDNA. On day 28 after vaccination, mice were challenged s.c. with 10^5 PFU of VEEV virus. Mice were daily monitored for signs of illness and deaths. Uniform lethality was observed for control mice while all iDNA-vaccinated mice survived challenge

the current vaccine, which can potentially accumulate mutations during viral passages. Additionally, as it was shown for YF17D cDNA, TC-83 iDNA can be also modified to ensure sufficient attenuation and/or introduce other genes or gene fragments with desirable features (e.g., cytokines, adjuvants) to enhance immunogenicity or to serve as a vector for expression of heterologous antigens. As example of this modification, optimization of the distance between 3′-terminus of the CMV promoter and the 5′-end of the TC-83 capsid cDNA was already mentioned and 15 base pairs between the *Sac*I site (the end of CMV promoter) and the start of cDNA provided expression of functional C antigen at a maximum level. Another example is an introduction of additional 26S promoter into TC-83 iDNA between sequences encoding capsid and glycoproteins. This modification resulted in additional attenuation which can potentially improve the TC-83 vaccine and reduce adverse effects associated with this vaccine (Pushko and Lukashevich 2014).

Certainly, the most attractive application of iDNA® concept is “in vivo transfection” or immunization. Multiple prime-boost regiments with high DNA doses are one of the disadvantages of the conventional DNA immunization. It seems much less iDNA is required to lunch infectious virus (vaccine); at least transfection of cells with lowest tested concentration, 8 ng, still resulted in efficient launch of VEEV TC-83 (Fig. 6.5a). A single-shot of in vivo transfection also resulted in attenuated infection in transfected mice as confirmed by recovery of TC-83 virus from the blood of mice shortly after transfection (1–3 days). Notably, transfection in vivo resulted in very low viremia (<50 PFU/ml), and TC-83 vaccine was recovered from the blood only after biological amplification, cocultivation in Vero cells. RNA was isolated from the recovered virus and the presence of mutation in E2 gene at position 120, the major structural determinant of TC-83 attenuation, was confirmed by cDNA sequencing (Tretyakova et al. 2013; Kinney et al. 1993).

To determine if the generation of TC-83 vaccine virus from iDNA in vivo results in induction of protective immune response, BALB/c mice were vaccinated with pTC-83 iDNA intramuscularly followed by in vivo electroporation. Animals showed no adverse effects to the procedure and remained healthy after vaccinations, similarly to unvaccinated controls. By day 21, all vaccinated mice seroconverted as determined by IFA and by PRNT (Tretyakova et al. 2013). At day 28 post-transfection/vaccinations, animals were challenged s.c. with 10^5 PFU of virulent IC VEEV-3908 virus. All iDNA-vaccinated animals survived challenge (Fig. 6.5b). Furthermore, five out of ten challenged animals did not have any detectable viremia after challenge. The remaining five animals had low-level viremia as compared to unvaccinated control animals. In contrast to iDNA-vaccinated mice, all unvaccinated control animals developed high levels of viremia, lost in average 32 % of weight, and succumbed to infection and died by day 7 post-challenge.

In summary, that is the first prove-of-concept study of using iDNA to launch VEEV TC-83 in vivo and protect iDNA-immunized animals against fatal challenge with wild-type VEEV. Infectious clone technology has been broadly used for VEEV vaccine development (Carossino et al. 2014; Davis et al. 1989, 1995). Since VEEV is a cytoplasmic virus, previous efforts were focused on in vitro transcription and transfection of the full-length RNA in order to generate the virus in vitro. Although mRNA-based vaccines can address some limitations of conventional DNA immunization (Raquel et al. 2014), RNA is chemically less stable than double-stranded iDNA and poorly suitable for vaccination in vivo.

The growing body of evidence indicates that quasispecies structure of RNA virus population is associated with virulence, while loss of diversity contributes to attenuation as it has been recently shown for YF17D vaccine (see above). Deep sequence analysis of wild-type VEEV versus TC-38 has not been performed yet. Nevertheless, it is reasonable to assume that generation of TC-83 in vitro or in vivo from recombinant iDNA with well-defined genetic structure will generate more homogeneous population of vaccine and can potentially improve safety profile of TC-83. It is also obvious that the generation of iDNA-launched RNA vaccine in vivo eliminates all adverse reactions resulting from the impurities derived from traditional substrates (e.g., albumin from chick embryo) or cell-cultured vaccines (e.g., adventitious agents: mycoplasma, viruses, bacterial contamination).

6.4 Plasmid DNA Initiates Replication of Attenuated Chikungunya Vaccine and Induces Protective Immune Responses in Mice

The third prove-of-concept example of iDNA application is a live-attenuated vaccine 181/25 (TSI-GSD-218) against chikungunya (CHIK) fever. CHIK virus (CHIKV) causes disease ranging from mild febrile illness to polyarthritis and encephalitis. Classical manifestations of CHIKV infection are fever, arthralgia,

and rash. In some cases hemorrhagic manifestations have been also reported. The most significant clinical manifestation of the disease is a severe joint pain. Joints (ankles, toes, fingers, elbows, wrists, and knees) exhibit extreme tenderness and swelling which are frequently associated with paresthesia of the skin in affected areas. While mortality is not a major concern (the estimated case/fatality ration is ~1:1,000), fever and severe joint pain can incapacitate vulnerable patients, mostly children and elderly individuals, for several weeks. In most cases the disease was resolved during this period of time. However, numerous cases of chronic arthralgia and arthritis lasted for several years were also reported (Powers and Brault 2009; Simon et al. 2011).

CHIKV belongs to the *Alphavirus* genus of the *Togaviridae* and like all alphaviruses has a genome consisting of a single-stranded, positive-sense RNA molecule of ~11.8 kb encoding nonstructural and structural (capsid, surface glycoproteins) genes. The virus is transmitted to humans primarily by *Aedes aegypti* and *Aedes albopictus* mosquitos. The CHIK fever is a classic example of an emerging/reemerging viral infection. Initially isolated from a febrile patient in Tanzania in 1953, the CHIKV infection was documented in numerous countries of Central and South Africa between 1960s and 1980s. From Africa, the virus spread to the Asian continent and caused frequent outbreaks in Malaysia, Indonesia, India, and Thailand. Climate changes and urbanization result in extensive geographical expansion of CHIKV. Currently CHIKV infection has been reported in nearly 40 countries with tropical and warm climates including Europe. In 2005–2006, CHIKV infection in La Reunion islands in the Indian Ocean resulted in ~300,000 cases of CHIK fever and caused 284 deaths; at the same time, in India, ~1.5 million people were infected. Travel-associated cases have been recorded in Europe, Australia, and the USA. With the Caribbean cases of CHIKV infection and high level of transmission efficiency rates currently observed in *Aedes aegypti* and *Aedes albopictus* mosquitos, the virus is poised to invade the Americas (Simon et al. 2011; Khan et al. 2014; Vega-Rú et al. 2014).

Pathogenesis of CHIKV-induced joint damage seems to be determined by host inflammatory responses and by viral factors (persistence and virulence). From the initial site of inoculation (skin bite), the virus went to bloodstream (viremia) and efficiently disseminated at the lymph nodes, muscle, spleen, and liver. Infection activates early interferon induction, but the virus effectively evades antiviral effects of interferon-induced factors. During the acute phase, viral replication in targets tissue followed by robust inflammation responses resulted in extensive infiltration of tissue with macrophages as well as with lymphocytes, NK cells, and neutrophils. These recruited cells produce several proinflammatory cyto/chemokines (e.g., TNF-α, IL-6, IFN-γ, MCP-1). Elevated levels of these factors in circulated blood are associated with myositis, arthralgia, and arthritis. It seems that the secretion of metalloproteinases in joint tissues additionally contribute to joint damage. Chronic cases of CHIKV arthritis seem to be related to persistent infection and/or subsequent accumulation of inflammatory mediators in arthritic joints. CHIKV is highly immunogenic and induces strong antibody responses. At an early stage of the infection antibodies have no neutralizing activities which typically detected as

early as 2 weeks after infection. Neutralizing antibodies effectively control virus replication. The role of cell-mediated immunity in the protection has not been extensively studied (Powers and Brault 2009; Assunção-Miranda et al. 2013).

Currently, there are no preventive and/or therapeutic measures for CHIK fever. Several vaccine platforms including live-attenuated viruses, alphavirus replicon chimeras, recombinant vaccines, DNA vaccines, and virus-like particles have been used to develop CHIKV vaccine candidates (Weaver et al. 2012). Among these candidates, only live-attenuated vaccine named strain 181/clone 25 (TSI-GSD-218) was tested in phase II. This vaccine was derived from the virus isolated from a viremic patient from Thailand. The virus was attenuated during 18 serial plaque-to-plaque passages in MRC5 cells. The final clone 25 exhibited small-plaque phenotype, decreased neurovirulence in suckling mice, and induced low viremia in nonhuman primates. In phase I and in a randomized, placebo-controlled phase II studies, vaccine was safe and well tolerated with mild reactogenicity in 8 % of volunteers experiencing transient arthralgia. Vaccination resulted in successful seroconversion in 98 % of vaccinees. The 181/25 vaccine replicated in mosquitos and was able be transmitted by mosquitos. However, replication in arthropod vectors did not result into reversion to wild-type of CHIKV (McClain et al. 1998; Edelman et al. 2000). Recent study showed that attenuation of the 181/25 vaccine relies on two independently attenuating mutations within the E2 protein (Gorchakov et al. 2012). In at least one case, adverse effects were linked to a genetic reversion. Therefore, although 181/25 vaccine can be useful for an emergency response (Hoke et al. 2012), improvement of the vaccine is needed.

One of the attractive approaches to improve CHIKV 181/25 vaccine is iDNA® technology. The CHIKV p182/25 iDNA has been generated from the 181/25 live-attenuated vaccine TSI-GSD-218 obtained from the World Reference Center for Emerging Viruses and Arboviruses (Galveston, TX, USA). In brief, the vaccine was propagated in CHO cells, the yield was harvested, and viral RNA was isolated to generate four overlapping cDNA fragments. These fragments were assembled within the pcDNA3.1-derived plasmid under transcriptional control of CMV major immediate-early promoter and resulted in construction of ampicillin-resistant p181/25-7 iDNA plasmid. The distance between the CMV promoter and viral genome was optimized and the HDV ribozyme was placed immediately after the viral poly-A sequence (Tretyakova et al. 2014a). Notably, sequence of this plasmid revealed the presence of genetic variants within the amino-terminal region of the nonstructural polyprotein. For example, only one of seven sequenced cDNA clones contained Ile301 residue in the nsP1 that was identical to the published consensus sequence of 181/25. The remaining six clones contained Thr at position 301 which was found in wild-type CHIKV, as well as for in the CHIKV VR1 isolate from the 181/25-vaccinated viremic patient who developed mild arthralgia (Gorchakov et al. 2012). Heterogeneity was also detected at residue 314. Although neither amino acid residues 301 nor 314 are responsible for attenuation, the presence of genetic variants within the virus population may contribute to a phenotypic heterogeneity of the 181/25 vaccine.

To show the applicability of the iDNA approach for engineering of new CHIKV vaccines with improved genetic stability and safety profiles, the p181/25-39 iDNA was prepared by inserting a duplicate 26S sub-genomic promoter between the 181/25 capsid and glycoprotein genes as it was done for TC-83 iDNA (see above). Finally, the p181/25-1 iDNA variant was made by replacing the pcDNA3.1 vector backbone in the p181/25-7 with the pCRII backbone to confer kanamycin resistance to the iDNA plasmid. Thus, both p181/25-7 and p181/25-1 encoded the CHIKV 181/25 sequences and differed only in the vector backbone and antibiotic-resistance gene (Tretyakova et al. 2014a).

Transfection of CHO cells with p181/25 iDNA plasmids resulted in expression of CHIKV antigens as was assessed by IFA and Western blot analysis (Fig. 6.6). SDS-PAGE and Western blot confirmed the presence of CHIKV structural proteins, including the glycoproteins (PE2, E1, and E2) and capsid (C) protein (Fig. 6.6a). In Western blot, human convalescent serum reacted with CHIKV glycoproteins, whereas mouse antibodies (hyperimmune mouse ascetic fluid, HMAF) reacted

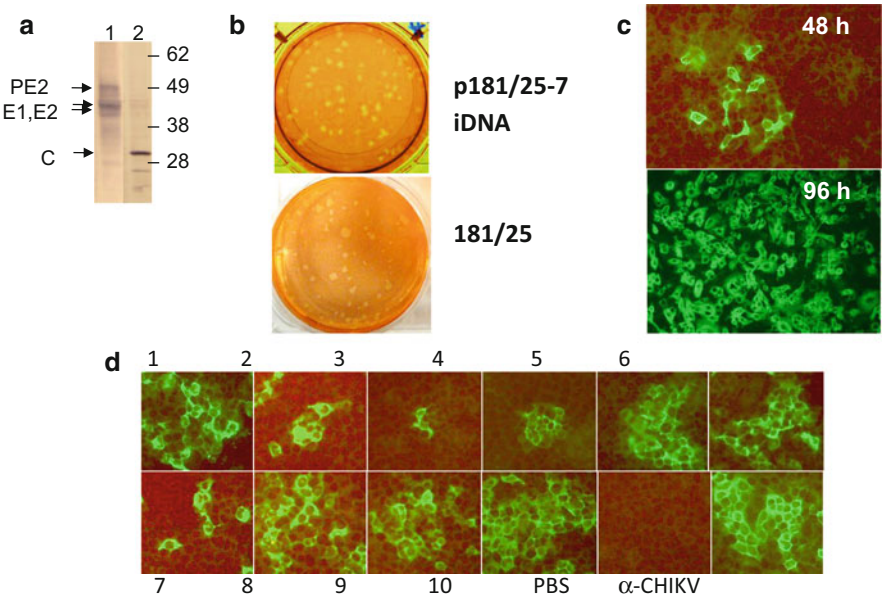


Fig. 6.6 Transfection of cells with p181/25-7 in vitro and in vivo. (**a**) Detection of CHIKV antigens in transfected CHO cells by Western blot. The blot was probed with human convalescent-phase CHIKV-specific serum (lane 1) and CHIKV HMAF (lane 2; see text). Positions of PE2, E2, E1, and C are indicated by *arrows*. (**b**) Plaque phenotypes of p181/27-7-derived CHIKV and original 182/25 vaccine. (**c**) Expression of CHIKV antigens detected by IFA after transfection of cells with iDNA plasmid. Aliquots of transfected cells were seeded in 8-well chamber slides, fixed at indicated times in cold acetone, and stained with mouse CHIKV-specific antibody, followed by fluorescein isothiocyanate-conjugated secondary antibody. (**d**) Mice ## 1–10 were vaccinated intramuscularly by electroporation with 10 μg of p181/25-7 iDNA. Sera were taken at day 21 after vaccination and probed at a dilution of 1:10 with mouse CHIK-specific antibodies, followed by fluorescein isothiocyanate-conjugated anti-mouse staining

only with capsid. This can be explained by the fact that mouse HMAF has been prepared against the S27 isolate of CHIKV, which has 39 amino acid substitutions in the capsid and glycoprotein region, compared with the 181/25 virus.

The presence of the virus in transfected cell culture supernatants was assessed by direct plaque assay. Both small and larger plaques were detected in the iDNA-derived virus, potentially reflecting assay variation or genotypic differences within the virus/vaccine population. However, the largest plaques were detected in the parental 181/25 virus (Fig. 6.6b).

In Vero cells infected with either 181/25 vaccine or transfected with iDNA, the virus titers reached 10^8–10^9 PFU/ml. All three iDNA clones, p181/25-7, p181/25-1, and p181/25-39, successfully initiated the replication of vaccine viruses when cells were transfected with 1 μg of the respective plasmids. The virus was successfully launched in transfections containing 1 μg, 100 ng, or 10 ng of p181/25-39 DNA but not in transfection containing 1 ng of p181/25-39 DNA. Virus replication in the cells transfected with 1 μg or 100 ng of p181/25-39 showed comparable titers, whereas a delay in the peak titer was observed when cells were transfected with only 10 ng of p181/25-39 iDNA (Tretyakova et al. 2014a).

Immunogenicity and efficacy of iDNA-launched 181/25 vaccine was tested in two groups of experiments in BALB/c mice. In the first study (immunogenicity), mice were vaccinated with either a single intramuscular injection of 10 μg of iDNA followed by electroporation or injected subcutaneously with 10^5 PFU of 181/25 virus (vaccine control). After immunizations, all mice remained healthy, with no detectable pathology at the site of injection or adverse effects due to vaccinations. Consistent with the results of phase II clinical trial, no viremia was detected on days 2 and 4 in both iDNA- and virus-vaccinated groups either by direct plaque assay or by incubating pooled sera with Vero cells. On day 21, all experimental and virus control mice successfully seroconverted, as shown by Western blot, IFA, and PRNT, suggesting the replication of vaccine virus in vivo. Notably, the titers of neutralizing antibodies were approximately sixfold higher in the iDNA-vaccinated animals, compared with the virus-vaccinated controls (Tretyakova et al. 2014a).

In the immunogenicity-efficacy study, BALB/c mice were similarly injected/electroporated intramuscularly with 10 μg of p181/25-7 iDNA. On day 21, all mice were successfully seroconverted as determined by IFA and PRNT. On day 28 iDNA-vaccinated mice were challenged intranasally with 6×10^6 PFU in 20 μL of neuro-adapted strain Ross of CHIKV. Since Ross CHIKV in BALB/c mice does not result in fatal disease when mice exceed 6 weeks of age, level of viremia was used as a vaccination endpoint (Table 6.1). On day 1, viremia was not detectable in any vaccinated mice, whereas all unvaccinated controls had high levels of viremia. Similarly, viremia was not detectable in vaccinated animals on day 2 after challenge, whereas virus was still detectable in two of five animals in the unvaccinated group at levels of 100 PFU/ml, thus demonstrating a significant ($P < 0.05$) protective efficacy of experimental p181/25 iDNA vaccine in immunocompetent BALB/c mice.

In summary, as in case of vaccine against YF and VEEV, an iDNA® technology allowed to launch experimental vaccine against CHIK fever from recombinant

Table 6.1 Immunogenicity and efficacy studies of p181/25-7 CHIKV iDNA in BALB/c mice

CHIK vaccine[a]	Animals, no.	Seroconverted/ total (%)[b]	Virus neutralization $PRNT_{80}$ range (mean)	Viremic/total (%); titer, log_{10} PFU/ml	
				Day 1	Day 2
p181/25-7 iDNA	10	10/10 (100)	160–1,280 (367.580)	0/10 (0); <0.9	0/10 (0); <0.9
Mock PBS	5	0/5 (0)	Not detected	5/5 (100); 3.04 ± 0.53	2/5 (40); 2.00

PBS phosphate-buffered saline, *PFU* plaque-forming units

[a]BALB/c mice were vaccinated by intramuscular injection-electroporation of 10 μg of p181/25-7 iDNA. On day 28 vaccinated and control (mock PBS) animals were challenged intranasally with 6×10^6 PFU of CHIKV-Ross virus in a 20 μL volume

[b]Detected by immunofluorescence assay

plasmid, p181/25 iDNA. CHIKV infection is a growing global threat and safe and efficacious vaccine is urgently needed. Among available vaccine candidates, only live-attenuated vaccine 181/25 was tested in phase II with promising results. The Indian Immunologicals Ltd. (IIL), a leading vaccine manufacturer in the country, expressed interest and commitment for commercialization of this vaccine. Genetic instability and possible reversion to wild-type genotype is a major concern for 181/25 as well as for other live-attenuated vaccines. The attenuation of the current 181/25 is based on small number of attenuating point mutations and this vaccine is at risk of being unstable or even sensitive to reversion. The iDNA technology is well positioned to address this concern by providing powerful tools to optimize genome of vaccines and enhance genetic stability. The possibility of genetic manipulation with 181/25 genome encoding structural genes has been already demonstrated in this section of the chapter. It seems that nonstructural genes are an additional promising target for stable attenuation. In fact, it has been shown that introduction of large deletion in nsP3 results in genetically stable attenuation and it does not affect immunogenicity (Hallengärd et al. 2014). A CHIKV DNA-launched vaccine with a large nsP3 deletion raised protective immunity after a single immunization providing an additional argument in support of iDNA vaccine technology. As a platform technology and a reverse genetics system, iDNA can also be used to engineer other CHIPV vaccines, including those containing live chimeric alphaviruses (Tretyakova et al. 2014a).

6.5 Conclusion

The iDNA approach is being developed to combine the positive attributes of both live-attenuated and plasmid DNA vaccines. Many successful live-attenuated vaccines against RNA viruses offer clear advantages over inactivated, subunit, or virus-like particles providing rapid and long-lived humoral and cell-mediated immunity after a single dose at favorable risk-to-benefit ratio. However, in the

current highly tight safety requirements, novel approaches are required to preserve technologies based on the concept of RC vaccines. iDNA immunization provides a powerful technical tool to address some crucial disadvantages of live-attenuated vaccines such as genetic instability and temperature vulnerability. If successful, iDNA technology will completely change manufacturing process because iDNA-based live-attenuated vaccines will be "manufactured" in vaccinated individuals eliminating traditional technology of production in cell substrates. The iDNA-based vaccines will no longer require a cold chain and might be potentially needle-free administered. The iDNA technology combined with rational attenuation and advanced DNA immunization may lead to a new generation of safer and more widely applicable live-attenuated vaccines. Certainly, this iDNA approach requires further testing in advanced animal models including nonhuman primates before progression to human testing.

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Chapter 7
Sugar-Based Immune Adjuvants for Use in Recombinant, Viral Vector, DNA and Other Styles of Vaccines

Nikolai Petrovsky

Abstract Highly attenuated viral vectors, purified protein antigens and DNA vaccines all suffer from problems of low immunogenicity providing a major challenge to find the best way to address this problem. A convenient solution is to identify a suitable adjuvant to add to the vaccine formulation to enhance its immunogenicity. Adjuvants come in many shapes and flavours, with no single unifying theme to explain why such a diversity of compounds should share the ability to enhance vaccine action. Hence adjuvant selection remains an empiric exercise of trial and error, largely based on comparisons of adjuvant potency in animal models plus assessment of safety and tolerability. In addition, there are unique challenges with successfully adjuvanting nonprotein-based vaccine technologies such as viral vectors or DNA vaccines for which existing adjuvant technologies such as aluminium salts or oil emulsions are likely to be unsuitable or incompatible. Amongst the many different groups of potential adjuvant compounds, carbohydrate-based adjuvants have received relatively little attention despite having favourable properties including compatibility for formulation with live vector vaccines, safety, tolerability and ease of manufacture and formulation. These properties may make them ideal for use across all vaccine platforms including live viral vectors and DNA vaccines. This chapter will review the various carbohydrate-based adjuvants and the science that underpins their activity and will highlight the potential for sugar-based adjuvants to replace more traditional adjuvant such as aluminium salts and oil emulsions across a wide variety of human and veterinary vaccine applications using traditional antigens, viral vectors or DNA to protect against infectious disease and for treatment of cancer and allergy.

N. Petrovsky (✉)
Department of Diabetes and Endocrinology, Flinders Medical Centre, Flinders University, Adelaide 5042, SA, Australia

Vaxine Pty Ltd, Bedford Park, Adelaide 5042, Australia
e-mail: nikolai.petrovsky@flinders.edu.au

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7.1 Introduction

The goal of vaccination is to generate a protective immune response of sufficient strength and duration to prevent or attenuate the virulence of pathogenic organisms. This is achieved by immunisation using either inactivated or recombinant proteins or sugars, live attenuated vectors or DNA plasmid vaccines targeting key antigens expressed by the pathogen or, in the case of cancer vaccines, the particular tissue being targeted. The aim is to induce a protective immune response that may comprise a neutralising antibody response or cytotoxic T-cell response against the relevant target. In the early days of vaccinology, inactivated whole cell antigens being impure and containing immunologically active contaminants generated strong immunity without the need for an added adjuvant. Thus, older-style attenuated virus vaccines such as the yellow fever vaccine were highly immunogenic on their own. On the negative side, early vaccines suffered from major reactogenicity and safety issues. Attempts in recent years to improve the quality and purity of vaccine antigens and vectors to reduce their reactogenicity and improve their safety have had the unfortunate consequence that vaccine immunogenicity has been correspondingly reduced. This has necessitated a search for ways to improve vaccine immunogenicity. The most commonly adopted approach has been to find an adjuvant that when added to the vaccine improves its immunogenicity and thereby its ability to protect against the relevant infectious disease or cancer.

An adjuvant is defined as any compound that enhances the immune response against a vaccine antigen; the word adjuvant comes from *adjuvare*, meaning to help or to enhance. Adjuvants can be used for multiple purposes: to enhance immunogenicity, provide antigen-dose sparing, accelerate the immune response, reduce the need for booster immunisations, increase the duration of protection, improve efficacy in poor responder populations or in the case of cancer vaccines to help break self-tolerance (Petrovsky and Aguilar 2004). To this day, alum-based adjuvants hold a relative monopoly over approved human vaccines although oil emulsion adjuvants are used to a limited extent in influenza vaccines. The reason for alum's dominance effects its long record of safe use, with newer adjuvants facing major regulatory hurdles to gain approval. Regulatory requirements require any new adjuvant to prove its benefits outweigh any safety or tolerability issues, making it difficult for new adjuvants to challenge alum's supremacy (Petrovsky 2013). However, approved adjuvants have largely proved unsuitable for use in viral vector vaccines, necessitating development of novel adjuvant approaches for enhancement of such vaccines. An additional factor driving the need for new adjuvants is that viral vector vaccines are not just administered through traditional parenteral injection approaches, but also through alternative routes such as intranasal or transdermal delivery for which parenteral adjuvants such as alum are not suited. Furthermore, safety concerns have been raised even with regard to alum (Passeri et al. 2011) and squalene emulsion adjuvants (Miller et al. 2013), making it critical that any new adjuvant have strong human safety and tolerability data

(Petrovsky 2008). Carbohydrate compounds such as polysaccharides are generally regarded as safe with minimal risk of toxic metabolites or long-term tissue deposits. From a safety aspect, this makes them ideal candidates amongst which to search for adjuvant-active compounds. Interestingly, many sugars play major signalling roles within the immune system, and hence it is not surprising that many carbohydrate compounds, when tested, have been found to have adjuvant activity (summarised in Table 7.1).

7.2 Fructan Adjuvants

One of the first polysaccharides recognised to have immunological effects was β-D-[2-1] poly(fructo-furanosyl)-D-glucose, more commonly known as inulin, a natural plant-derived storage carbohydrate of plants of the Compositae family. Inulin is a polymer comprising linear chains of fructosyl groups linked by β(2-1) glycosidic bonds terminated at the reducing end by an α-D-(1-2)-glucopyranoside ring group. Inulin's immune activity was first identified when inulin solutions being administered to human subjects to measure kidney function were noted to activate complement through a nonclassical antibody-independent pathway, thereby leading to the discovery of the alternative complement pathway. Complement activation was found to be due to minute inulin crystals contaminating inulin solutions (Cooper and Carter 1986). These insoluble inulin crystals with immunological activity need to be distinguished from the more soluble alpha- and beta-inulin forms that do not have immunological activity (Cooper et al. 2013). The most advanced inulin crystalline form, delta inulin, developed under the trade name, Advax™, was shown to have potent adjuvant activity (Cooper and Petrovsky 2011). Advax adjuvant has been demonstrated to enhance the immunogenicity of a wide variety of protein antigens drawn from viral, bacterial and protozoan pathogens, toxins, cancer antigens and allergens when administer to a broad range of animal species including mice, rats, guinea pigs, rabbits, chickens, dogs, sheep, monkeys, horses and camels. Advax has been shown to enhance vaccine immunogenicity in murine models of H1N1 influenza (Honda-Okubo et al. 2012), Japanese encephalitis (Larena et al. 2013), West Nile virus (Petrovsky et al. 2013), hepatitis B virus (Saade et al. 2013) and human immunodeficiency virus (Cristillo et al. 2011), a ferret model of avian (H5N1) influenza (Layton et al. 2011), a horse model of Japanese encephalitis (Lobigs et al. 2010) and camel models of African horse sickness and glanders (Eckersley et al. 2011). In the Japanese encephalitis and West Nile virus models, the enhanced protection obtained with Advax adjuvant was shown to be mediated by its ability to induce a protective memory B-cell population, whereas in other models such as influenza protection was shown to be mediated by both memory B-cell and T-cell populations. More recently, Advax adjuvant was effective in human trials where it significantly boosted seroconversion and seroprotection to a recombinant pandemic influenza vaccine (Gordon et al. 2012). A notable feature of Advax adjuvant is its lack of reactogenicity and

Table 7.1 Examples of carbohydrate-based adjuvants

Adjuvant name	Chemical structure	Modified forms	Source	Receptor(s)	Immune actions/mechanism of action
Delta inulin	β-D-[2-1]Poly (fructo-furanosyl) α-D-glucose	Epsilon, omega	Compositae plants	Not known	Noninflammatory, TLR-independent, activates complement, chemokine secretion, costimulation
Dextran	α-1,6-Glucan with α-1,3-branches	Dextran sulphate, DEAE-dextran, acetylated dextran	Lactobacilli	Glucan receptor	Proinflammatory, activates inflammasome, complement, NFkB, induces IL1β, IL-6, TNF-α
Lentinan	β-1,3-Glucohexaose with β-1,6-branches	Sulphated lentinan	Shiitake mushroom	Glucan receptor	Proinflammatory, activates inflammasome, complement, NFkB, induces IL1β, IL-6, TNF-α
Zymosan	β-1,3-Glucan	Glucan particles	*Saccharomyces cerevisiae*	GR, TLR2, Dectin-1, ASC	Proinflammatory, activates inflammasome, complement, NFkB, induces IL1β, IL-6, TNF-α
Beta-glucan	β-1,3-Glucan	Glucan particles	*Saccharomyces cerevisiae*	GR, TLR2, Dectin-1, ASC	Proinflammatory, activates inflammasome, complement, NFkB, induces IL1β, IL-6, TNF-α
Mannan	1,4-Polymaltose	Oxidation, reduction, acylation, mannosylated niosomes	*Aloe barbadensis*	MBL, mannose receptor	Proinflammatory, activates NFkB, induces IL1β, IL-6, TNF-α
Chitin	*N*-Acetyl-D-glucosamine	Acetylation (chitosan)	Crustaceans	Dectin-1, MMR, TLR-2	Proinflammatory, phosphorylates MAPK, induces TNF-α, COX-2 , prostaglandin E2, DC maturation
Muramyldipeptide	*N*-Acetyl muramyl-L-alanine-D-isoglutamine	D-Murapalmitine, GMDP, murabutide	Mycobacteria, *Lactobacillus bulgaricus*	NOD2	Proinflammatory, activates NFkB, induces IL1β, IL-6, TNF-α
Cord factor	Trehalose-6-6-dimycolate	Multiple	*M. tuberculosis*	Mincle	Proinflammatory, activates monocyte Syk-Card9 signalling, activates NFkB IL1β, IL-6, TNF-α

LPS	Lipopolysaccharide	Monophosphoryl lipid A (MPL)	Gram negative bacteria	TLR4	Proinflammatory, activates NFkB, induces IL1β, IL-6, TNF-α
QS21	Triterpenoid glycosides	GPI-0100	Bark of *Quillaja saponaria*	Inflammasome	Proinflammatory, activates inflammasome and NFkB

safety as demonstrated in many animal and human studies. This supports the hypothesis that polysaccharides on the whole are extremely well tolerated. Advax adjuvant induces robust CD4 and CD8 T-cell immunity against co-administered antigens (Honda-Okubo et al. 2012), raising potential for its use in cancer vaccines and vaccines for problematic infectious diseases such as HIV and tuberculosis where T-cell immunity is critical for protection. An interesting feature of Advax adjuvant is its ability to enhance adaptive immune responses even when injected separately in time to the antigen. Thus, the adjuvant action of Advax was maintained even when it was injected 24 h prior to injection of hepatitis B surface antigen, a feature not shared by alum adjuvant (Saade et al. 2013). Unlike alum, Advax does not work by binding antigen and forming a tissue depot, meaning formulation is a simple matter of mixing the antigen with Advax. The combined formulation can then be immediately injected if desired. Formulation is a particularly important aspect of preparation of viral vector vaccines as it is critical that virus viability not be adversely affected during storage with adjuvant. Recent studies confirmed that co-formulation with Advax did not adversely affect the viability of live vector vaccines including a live modified vaccinia Ankara (MVA) smallpox vaccine and a live respiratory syncytial virus (RSV) vaccine. Enhancement of vaccine immunogenicity was observed in both studies, confirming that delta inulin adjuvant can indeed enhance the immunogenicity of live viral vector vaccines. A critical question is how delta inulin is able to mediate these beneficial effects while not causing the reactogenicity and toxicity observed with other adjuvants. The difference may be that delta inulin does not work through activation of innate immune receptors such as the TLRs, Dectin-1 or the inflammasome and thereby does not induce proinflammatory and pyrogenic cytokines such as interleukin (IL)-1 that mediate side effects of others adjuvants. Delta inulin modulates antigen-presenting cell function, inducing phenotypic changes associated with enhanced antigen presentation to T and B cells, while bypassing innate immune activation thereby explaining the lack of reactogenicity.

7.3 Glucan Adjuvants

Glucans are plant- or microorganism-derived polysaccharides made up of repeating D-glucose units joined by glycosidic bonds in various alternative conformations. Alpha-glucans include dextran (α-1,6-glucan), glycogen (α-1,4- and α-1,6-glucan), pullulan (α-1,4- and α-1,6-glucan) and starch (α-1,4- and α-1,6-glucan). Beta-glucans include cellulose (β-1,4-glucan), curdlan (β-1,3-glucan), laminarin (β-1,3- and β-1,6-glucan), chrysolaminarin (β-1,3-glucan), lentinan (purified β-1,6:β-1,3-glucan from *Lentinus edodes*), lichenin (β-1,3- and β-1,4-glucan), pleuran (β-1,3- and β-1,6-glucan isolated from *Pleurotus ostreatus*) and zymosan (β-1,3-glucan from *Saccharomyces*). Each type and source of glucan can be of widely varying quality and purity and may contain mixtures of polymer chain

structures with differing amounts of branching and variation in chain length. Because these polymer variables are influenced by the source of the glucan, glucans are named according to their plant or microbial source. Hence while zymosan is predominantly β-1,3-glucan extracted from yeast cell walls, it also contains variable amounts of other sugars and yeast proteins that also have immunological activity. Hence, it is not always clear which component of complex glucan formulations is responsible for their adjuvant activity. As detailed in Table 7.1, glucans and other carbohydrate adjuvants modulate immune responses through the action of specific innate immune receptors known as lectins that bind sugars. These include the β-glucan receptor, mannan receptor, Dectin-1, toll-like receptors (TLR) and other receptors expressed on monocytes and antigen-presenting cells (APC). Binding of sugars to the relevant innate receptor(s) results in monocyte activation, with nuclear translocation of nuclear factor kappa-B (NFkB) leading to proinflammatory cytokine production that then amplifies the adaptive immune response to a co-administered antigen.

7.4 Alpha-Glucan Adjuvants

Dextran is a branched microbial polysaccharide made up of α-1,6-glucan with α-1,3-branches. Dextran sulphate has marked proinflammatory effects as highlighted by its ability to induce inflammatory colitis in mice. Diethylaminoethyl-dextran (DEAE-dextran) a polycationic derivative of dextran was shown in rhesus monkeys to enhance the antibody response to formalin-inactivated Venezuelan equine encephalomyelitis virus vaccine (Houston et al. 1976). DEAE-dextran similarly enhanced responses to whole cell cholera vaccine in mice (Kaistha et al. 1996). Acetylated dextran (Ac-DEX) microparticles have been used to deliver the TLR7 agonist, imiquimod, to immune cells thereby enhancing TLR7 potency (Bachelder et al. 2010).

7.5 Beta-Glucan Adjuvants

Zymosan consists of β-1,3-glucan protein complexes from yeast cell wall extracts. Zymosan binds to TLR-2 and Dectin-1 on monocytes, thereby activating NFkB. It also activates the alternative complement pathway, contributing to its potent inflammatory action (Schorlemmer et al. 1977; Sato et al. 2003; Dillon et al. 2006). Zymosan also directly activates the inflammasome through ASC and cryopyrin resulting in caspase-1 activation and IL-1β secretion, a feature that it shares with mannan and may be responsible for its high reactogenicity (Lamkanfi et al. 2009). Through these mechanisms zymosan induces nonspecific resistance to bacterial and fungal infection as well as inducing tumouricidal activity in polymorphonuclear cells (Williams et al. 1978; Morikawa et al. 1985; Emod and Joo 1990). When

added to a nasal inactivated influenza vaccine administered to mice, zymosan enhanced the mucosal adjuvant activity of the TLR3 agonist, poly(I:C), through a TLR2-mediated mechanism (Ainai, Ichinohe et al.). Zymosan has also been shown to enhance the response to DNA vaccines through a complement-dependent mechanism (Ara et al. 2001). Oxidised beta-glucan derived from zymosan was able to substitute for Freund's complete adjuvant in induction of collagen-induced arthritis (Hida et al. 2006), consistent with zymosan's proinflammatory effects being sufficiently potent to break immune tolerance. Lentinan is another beta-glucan that is made up of β-1,3-glucan with β-1,6-branches purified from plant sources including shiitake mushrooms (*Lentinus edodes*). Intranasal lentinan induced an enhanced respiratory burst, nitric oxide and IL-6 production by bronchoalveolar macrophages resulting in nonspecific resistance against virus infection (Irinoda et al. 1992). As seen with other immunologically-active polysaccharides, lentinan also exhibits antitumour (Chihara et al. 1987; Jeannin et al. 1988) and antibacterial activities (Drandarska et al. 2005). Addition of lentinan increased the efficacy of a vaccine prepared by transfection of adenovirus-mediated melanoma-associated antigen gene (gp100) into bone marrow-derived dendritic cells for treatment of B16 melanoma in mice, with enhancement of cytotoxic T lymphocytes (CTL) and increased tumour inflammation and necrosis (Wang et al. 2007). Sulphated lentinan enhanced the serum antibody titre and T-cell proliferative response to a Newcastle disease vaccine and reduced mortality of challenged chickens (Guo et al. 2009). Lentinan also increased HIV env-specific Th1 cytokine production and CTL activity to an orally administered recombinant vaccinia virus (rVV) vector expressing gp160 but had no effect on humoral responses (Wierzbicki et al. 2002). Yet another β-1,3-glucan is algal glucan, extracted from *Euglena gracilis*. Algal glucan enhanced humoral and cellular immunity to co-administered herpes virus glycoprotein D peptide antigens and was not toxic, even when administered intravenously at doses up to 25 mg/kg body weight (Mohagheghpour et al. 1995). Beta-glucan particles are purified cell walls of *Saccharomyces cerevisiae* treated so as to remove mannans and yeast proteins, leaving a skeleton primarily made of β-1,3-D-glucans (Huang et al. 2009). Glucan particles bind dendritic cells via the Dectin-1 receptor thereby inducing inflammatory cytokine production. The hollow porous structure of GP allows them to be loaded with antigens including viral vectors resulting in enhanced dendritic cell phagocytosis, upregulation of maturation markers and increased potency on antigen-specific T cells (Huang et al. 2010).

7.6 Mannan Adjuvants

Mannan is a 1,4 linkage polymer of the sugar mannose used as a storage polysaccharide by yeast, bacteria and plants. Binding of mannan by mannan-binding lectin and other C-type lectins of the mannose receptor family leads to complement

activation, opsonisation, phagocytosis, inflammasome activation, caspase 1 activation and inflammatory cytokine production (Takahara et al. 2004; Thiel and Gadjeva 2009; Lamkanfi et al. 2009). The ability of mannan to mature dendritic cells was shown to be mediated through a TLR4-dependent mechanism (Sheng et al. 2006). Mannan and its derivatives including oxidised and reduced mannan have been extensively used to target antigens to dendritic cells, particularly in the area of human cancer vaccines. Mannan when oxidatively coupled to recombinant protein antigen and given intranasally was shown to enhance the production of antigen-specific serum and secretory antibodies (Stambas et al. 2002). A phase 2 clinical study of Muc-1 antigen conjugated to oxidised mannan showed a >4-fold lower rate of tumour recurrence (Vassilaros et al. 2013). Conjugation of myelin basic protein (MBP) to reduced mannan was able to switch the anti-MBP immune response from Th1 to Th2 and protect against experimental allergic encephalomyelitis (Katsara et al. 2009). In a mouse model of Alzheimer's disease, mannan conjugated to Aβ antigen enhanced anti-Aβ antibody production in otherwise hyporesponsive transgenic mice, suggesting an ability of mannan adjuvant to break self-tolerance (Petrushina et al. 2008). Polymannose purified from the *Aloe barbadensis* plant enhanced antibody titres in coxsackievirus B3-immunised mice (Gauntt et al. 2000). Mannose has also been used to target plasmid DNA-containing liposomes to macrophages (Kawakami et al. 2000). Coating of cationic liposomes with mannan significantly enhanced the ability of a DNA vaccine to induce HIV-specific cellular immunity and also the activity of a DNA vaccine against melanoma (Toda et al. 1997; Lu et al. 2007). Mannosylated niosomes have been used as oral DNA vaccine carriers for the induction of mucosal immunity. Niosomes carrying DNA encoding a hepatitis B antigen and composed of Span 60, cholesterol and stearylamine (all coated with the modified polysaccharide O-palmitoyl mannan) were able to induce protective serum titres, cellular immune responses and IgA in mucosal secretions when given orally to mice (Jain et al. 2005). A similar approach was used for topical vaccine delivery using an O-palmitoyl mannan coating to target niosomes to Langerhans cells in the skin (Jain and Vyas 2005). Mannan has similarly been used to enhance delivery of live virus vector vaccines. A recombinant adenovirus vector modified with mannan was used to deliver vascular endothelial cadherin antigen as an antitumour vaccine in mice. Mannan was shown to enhance vaccine responses, resulting in prophylactic and therapeutic inhibition of tumour growth and prolonged survival (Zhao et al. 2011). Similarly, when cDNA of human telomerase reverse transcriptase was inserted into an adenovirus vector and the recombinant adenovirus modified with mannan, the expression of adenovirus in mice was restricted to splenic dendritic cells, consistent with efficient targeting by the surface mannan (Ding et al. 2009). The above data shows that mannan can be used as both an adjuvant and also a dendritic cell-targeting tool for enhanced delivery of live vector vaccines.

7.7 Chitosan Adjuvants

Chitin, a linear β-1-4-linked polymer of D-glucosamine and *N*-acetyl-D-glucosamine extracted from shrimp and chitosan obtained by partial deacetylation of chitin, exhibit a range of immunological effects including macrophage activation and production of inflammatory cytokines resulting in enhanced antibody titres to co-administered antigens (Nishiyama et al. 2006). These effects are mediated by binding of chitin to receptors including Dectin-1, macrophage mannose receptor and TLR-2 (Arca et al. 2009). The addition of chitosan to an intramuscular inactivated influenza vaccine resulted in an increase in antibody titres in mice (Ghendon et al. 2009) and enhanced protection against lethal challenge (Chang et al. 2010). Chitosan particles produced by cross-linking with a counter ion were shown to entrap antigen and enhance its immunogenicity in mice (Prego et al. 2010). By virtue of their mucoadhesive qualities, chitin and its derivatives have been extensively tested as nasal adjuvants. Chitin derivatives such as *N*-trimethyl chitosan chloride enhance the absorption of proteins at mucosal surfaces by inducing transient opening of tight junctions (Kotze et al. 1997). The concomitant use of chitosan microparticles and the mucosal toxin-based adjuvant, LTK63, significantly enhanced the immunogenicity of an intranasal group C meningococcal polysaccharide vaccine (Baudner et al. 2003). Similarly, intranasal alginate-coated chitosan nanoparticles loaded with antigen and CpG adjuvant boosted antibody and cellular responses in mice (Borges et al. 2008). Intranasal plasmid DNA vaccine loaded chitosan nanoparticles induced potent humoral, cellular and mucosal responses (Khatri et al. 2008). In a study with live viral vectors, chitosan improved the immunogenicity in cattle of an intranasal replication-defective adenovirus type 5 vaccine expressing bovine herpesvirus 1 glycoprotein D. The best protection was obtained with vector adjuvanted with glycol-chitosan (Gogev et al. 2004). Similarly, increased immunity was seen when an apathogenic enterotropic live Newcastle disease vaccine was administered by oculonasal route together with chitosan adjuvant to 1-day-old chickens (Rauw et al. 2010). Enhanced protection was also seen with a live virus vaccine against Newcastle disease encapsulated in chitosan nanoparticles (Zhao et al. 2012). Microencapsulation of adenoviral vectors into a chitosan microparticle for mucosal delivery not only protected the virus but also made its release dependent on cell contact (Lameiro et al. 2006). Chitosan also enhanced the protective efficacy of a live attenuated influenza vaccine with the chitosan adjuvant significantly increasing the levels of influenza-specific antibodies and IFN-γ-secreting T cells (Wang et al. 2012). Human phase 1 studies of a chitosan and MPL-adjuvanted intranasal Norwalk virus-like particle vaccine derived from norovirus GI.1 genotype as a mucoadhesive successfully induced high anti-norovirus IgG and IgA titres (El-Kamary et al. 2010), consistent with the ability of chitosan to adjuvant human vaccines. Nevertheless, negative effects of chitosan were observed in cancer vaccine studies. A chitosan-adjuvanted murine adenovirus

cancer vaccine provided minimal protection against tumour challenge, with evidence of reduced antigen-specific CD8+ T cell, IFN-γ and CTL activity (Lemke et al. 2011). This was due to the chitosan inhibiting adenovirus-mediated transgene expression and antigen-presenting cell activation.

7.8 Lipoarabinomannan Adjuvants

Mycobacteria are the major ingredient in Freund's complete adjuvant, which remains the gold standard in terms of adjuvant potency, but also toxicity. Many of the microbial compounds contributing to this adjuvant activity have been progressively identified. Amongst the adjuvant compounds discovered in microbial extracts, many turn out to be carbohydrate-containing structures, including oligosaccharides, glycoproteins and glycolipids with mycobacteria recognised by immune cells through various pathogen-associated molecular pattern (PAMP) receptors, including the TLRs and C-type lectins (e.g. mannose receptor, Dectin-1 and DC-SIGN), with many of these interactions being dependent on carbohydrate structures within the mycobacterial cell wall (Gringhuis et al. 2009). Lipoarabinomannan (LAM) is a major mycobacterial structural cell surface component. LAMs have varying immune activities depending upon their structure. LAMs from nonpathogenic mycobacteria bind TLR2 on macrophages and activate NFkB and induce inflammatory cytokines (Doz et al. 2007). By contrast, mannosylated-LAM from pathogenic *M. tuberculosis* binds to the mannose receptor and DC-SIGN resulting in stimulation of anti-inflammatory cytokines (Doz et al. 2007). Hence, LAMs can be either pro- or anti-inflammatory, depending on their origin, and which innate receptors, e.g. mannose receptor, TLR1/TLR2, TLR4 or DC-SIGN, they signal through, highlighting the subtleties of carbohydrate signalling (Doz et al. 2007). Nevertheless, LAM-derived arabinomannan oligosaccharides from *M. tuberculosis* covalently conjugated to the mycobacterial antigen, Ag85B, protected animals against mycobacterial challenge when administered with Eurocine L3, a monoglyceride adjuvant (Hamasur et al. 2003). No data is available on the use of LAM as an adjuvant for live virus vector vaccines.

7.9 Muramyl Dipeptide Adjuvants

MDP (*N*-acetyl muramyl-L-alanine-D-isoglutamine) was first identified from a mycobacterial peptidoglycan fraction known to have potent adjuvant activity (Yamamura et al. 1976). MDP has been tested on its own and as a component of more complex adjuvant formulations. MDP binds and activates innate immune receptors, including NOD2 (Uehara et al. 2005), and TLR receptors (Takada and Uehara 2006). This leads to potent activation of NFkB, induction of inflammatory cytokines and dendritic cell maturation. The carbohydrate moiety of MDP is critical

to its adjuvant activity as shown by studies in which carbohydrate analogues of MDP were tested in the induction of delayed-type hypersensitivity in guinea pigs (Azuma et al. 1981). This confirmed a structural requirement of the carbohydrate moiety of MDP as only D-mannosamine, D-galactosamine and D-glucose analogues of MDP were active as vaccine adjuvants (Azuma et al. 1981). Many analogues of MDP have been tested as vaccine adjuvants. MTP-PE (*N*-acetyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1,2-dipalmitoyl-sn-glycero-3-(hydroxy-phosphoryloxy)) ethylamide) was included as an immuno-stimulator in the original MF59 squalene emulsion adjuvant (Valensi et al. 1994). However, the MTP-PE component was abandoned because of excessive reactogenicity (Ott et al. 1995). MDP analogues can be made that are either lipophilic or hydrophilic with the lipophilic variants being more immunologically active. When formulated in saline, MDP analogues predominantly stimulate humoral immunity, whereas when incorporated into emulsions or liposomes, they induce cellular immunity. For example, *N*-acetylglucosaminyl-*N*-acetylmuramyl-L-Ala-D-isoGlu-L-Ala-glyceroldipalmitate (DTP-GDP) when formulated as an adjuvant in liposomes induced remission in human metastatic colorectal cancer although it shared MDP's toxicity, producing fever, chills and hypotension at high doses (Vosika et al. 1990, 1991). While the utility of MDP analogues as human adjuvants is restricted by their high reactogenicity, they are found in many veterinary adjuvants. Gerbu adjuvants, for example, are veterinary adjuvants based on GMDP (*N*-acetyl-glucosaminyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine), a glycopeptide from the cell wall of *Lactobacillus bulgaricus* (Schwarzkopf and Thiele 1996). Gerbu adjuvants are complex mixtures that may also contain cimetidine, saponin, paraffin, dimethyldi-(stearoylhydroxyethyl)ammonium chloride, mannide monooleate, glycerol, L-proline and ciprofloxacin. MDP was shown to enhance the cellular immune response to a water-in-oil adjuvanted live bovine rotavirus administered intramuscularly to calves (Archambault et al. 1988). Murabutide a less toxic analogue of MDP that has previously been tested in multiple human clinical trials was also shown to act as a mucosal adjuvant and enhanced the immunogenicity of Norwalk virus-like particles administered intranasally to mice (Jackson and Herbst-Kralovetz 2012). The MDP analogue LK415 when tested on chickens immunised with a live vaccine against infectious bursal disease significantly enhanced antibody titres consistent with an adjuvant effect of MDP on live virus vaccines (Rojs et al. 2000).

7.10 Trehalose Dimycolate Adjuvants

Trehalose-6-6-dimycolate (TDM) was previously known as *M. tuberculosis* cord factor and is a potent inducer of inflammatory cytokines with effects including antitumour activity and stimulation of host resistance against infections (Masihi et al. 1983; Sueoka et al. 1995). A number of TDM analogues were synthesised for structure-activity studies, and some attenuation of TDM's toxicity was possible while still retaining adjuvant activity (Fujita et al. 2007). TDM augments both

humoral and cell-mediated immune responses when combined with vaccine antigens, with a comparable efficacy to MDP (Ribi et al. 1975, 1982; Koike et al. 1998). Given its high reactogenicity, TDM is most likely unsuitable for human vaccines but is a component of the long-standing veterinary Ribi Adjuvant System® where it is formulated with squalene oil, monophosphoryl lipid A (MLP) and other components (Ribi et al. 1975, 1982). No data is available on the use of TDM as an adjuvant for live virus vector vaccines.

7.11 Lipopolysaccharide Adjuvants

Bacterial lipopolysaccharide (LPS) is a potent inducer of macrophage activation and inflammatory cytokine production. LPS itself is too toxic to be used as a human adjuvant leading to the development of less reactogenic analogues focusing on the lipid A component that consists of a β-(1,6)-linked D-glucosamine disaccharide phosphorylated at 1-O and 4′-O-positions. In low-acid conditions, lipid A can be hydrolysed to remove the 1-phosphate group, and subsequent mild alkaline treatment leads to removal of the fatty acid at position 3 resulting in monophosphoryl lipid A (MPL). MPL has lower toxicity than LPS but retains immuno-stimulatory activity (Masihi et al. 1986). MPL signals via TLR4 with preferential signalling through the downstream TRIF adaptor rather than MYD88 adaptor, explaining its reduced reactogenicity when compared to LPS (Cekic et al. 2009). MPL has been used in a variety of proprietary adjuvant formulations, including GSK's AS02 and AS04 adjuvants where MPL is used in combination with QS21/oil-in-water emulsion or aluminium hydroxide, respectively. AS04 adjuvant is contained in licensed vaccines against hepatitis B (Fendrix®) and human papilloma virus (Cervarix®) (Tong et al. 2005; Schiller et al. 2008). MPL has also been shown in mice to act as a mucosal adjuvant for influenza virus-like particles with a similar efficacy to alum or CpG adjuvants (Quan et al. 2013). No data is available on the use of MPL as an adjuvant for live virus vector vaccines, which may reflect formulation incompatibility with viruses given the highly hydrophobic nature of MPL.

7.12 Saponin-Based Adjuvants

Saponins from Rhamnaceae, Araliaceae, Polygalaceae and Fabaceae plant families have all been reported to have adjuvant activity (Lacaille-Dubois and Atta ur 2005). The most extensively characterised saponin adjuvant is QS21. QS21 is a saponin derived from Quil A, a mixture of triterpenoid glycosides derived from the bark of the South American soap bark tree, *Quillaja saponaria* (Kensil et al. 1995). QS21 is an acylated saponin at the 4-hydroxyl position on fucose with two linked 3,5 dihydroxy-6-methyloctanoic acids (Kensil et al. 1996). QS21 induces inflammatory cytokines and imparts a Th1 bias in vaccine responses (Kensil et al. 1995; Meraldi

et al. 2005). QS21 has been used in numerous vaccine trials in the cancer field, alone, complexed with cholesterol as ISCOMS (Pham et al. 2006) or mixed with MPL in an oil-in-water emulsion such as GSK's AS02 adjuvant. Because of its ability to lyse cell membranes, adverse reactions including injection site pain and hemolysis can be major limiting factors in use of QS21 (Sun et al. 2009). To overcome these problems a range of chemically modified variants of QS21 have been created. GPI-0100 is a variant with incorporation of the C-12 alkyl chain through a stable amide bind at the carboxyl group of the glucuronic acid residue of deacylated saponin (Marciani et al. 2000; Quenelle et al. 2008). GPI-0100 was shown to be 20 times less lethal in mice than QS21 and stimulates a Th2-like immune response, whereas QS21 stimulates a Th1-like response (Ragupathi et al. 2002). A number of completely synthetic isomers of QS21, called QS21-Xyl and QS21-Api, have been developed (Deng et al. 2008). Other plant-based saponins also have adjuvant activity. For example, glycyrrhizin, a triterpenoid saponin glycoside of glycyrrhizic acid, the main sweet-tasting compound in liquo rice root, activates NFkB and induces inflammatory mediators in murine macrophages and has adjuvant activity when co-administered with antigen (Dai et al. 2001; Raphael and Kuttan 2003). Due to their chemical nature, saponin adjuvants are unsuitable for use as adjuvants with live viral vectors due to their propensity to disrupt cell membranes thereby restricting their use to inactivated or recombinant antigens (Newman et al. 1997) or DNA vaccines (Sasaki et al. 1998).

7.13 Mechanisms of Carbohydrate Adjuvant Action

Many of the sugar-based adjuvants described above including zymosan, mannan, MDP, TDM and LPS work by binding and activating innate immune receptors including TLRs, NOD2 and C-type lectins, resulting in inflammatory cytokine production and thereby enhancement of vaccine immunogenicity (Lee et al. 2001; Nishiyama et al. 2006; Huang et al. 2009). Many carbohydrate adjuvants including MDP, LPS, zymosan, mannan, β-glucan and delta inulin activate complement, and this may also contribute to their adjuvant activity (Ray et al. 1979; Kawasaki et al. 1987; Bohana-Kashtan et al. 2004; Rawal et al. 2009). Chemotaxis induced by IL-8, MCP-1, MIP-1α and MIP-1β, may also play a role in carbohydrate adjuvant action. Phosphomannosyl structures, for example, potently induce lymphocyte migration (Weston and Parish 1991; Dong et al. 2007). Some carbohydrate compounds such as zymosan, mannan and QS21 directly activate the inflammasome, thereby contributing to their adjuvant activity (Lamkanfi et al. 2009). Other adjuvant actions of polysaccharides such as mannan include ability to target antigens directly to dendritic cells. QS21 may have a direct action on T cells as the aldehyde group at C4 of the aglycone unit of QS21 may form a Schiff base with the amino groups of receptors on the T-cell surface thereby providing co-stimulatory signals (Kensil et al. 1996). The odd man out amongst

the carbohydrate adjuvants is delta inulin as it does not activate TLRs, the inflammasome or other innate immune receptors and thereby does not induce inflammatory cytokine production. Nevertheless, it still shares potent adjuvant activity as measured by enhancement of antigen-specific T- and B-cell memory responses. This appears to be through a unique ability to enhance antigen-presenting cell function without activation of inflammatory cytokine genes.

7.14 Conclusions

Sugar structures play a critical role in immune function, and it is not surprising, therefore, that sugar-containing compounds should be a fertile ground for discovery of new immune adjuvants. Carbohydrates are generally safe and well-tolerated, critical attributes for a human adjuvant. Amongst carbohydrates with adjuvant activity, the polysaccharides stand out as promising. The propensity of some polysaccharides such as dextran, zymosan and mannan to potently induce inflammatory cytokines may limit their use in prophylactic human vaccines. Delta inulin is a unique polysaccharide adjuvant that works via TLR- and inflammasome-independent mechanisms and does not induce inflammatory cytokine production. This results in a favourable tolerability and safety profile making it a strong candidate for future human prophylactic vaccine development. As a group, the polysaccharide adjuvants are all compatible for formulation with live virus vector vaccines, and many have positive efficacy data in this context. Given the difficulties of adjuvanting live vector vaccines, this makes polysaccharide adjuvants prime candidates for development in this role.

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Part II
Viral-Vectored Therapeutic Cancer Vaccines

Chapter 8
Adenovirus-Based Vectors for the Development of Prophylactic and Therapeutic Vaccines

Anton V. Borovjagin, Jorge G. Gomez-Gutierrez, Haval Shirwan, and Qiana L. Matthews

Abstract Emerging and reemerging infectious diseases as well as cancer pose great global health impacts on the society. Vaccines have emerged as effective treatments to prevent or reduce the burdens of already developed diseases. This is achieved by means of activating various components of the immune system to generate systemic inflammatory reactions targeting infectious agents or diseased cells for control/elimination. DNA virus-based genetic vaccines gained significant attention in the past decades owing to the development of DNA manipulation technologies, which allowed engineering of recombinant viral vectors encoding sequences for foreign antigens or their immunogenic epitopes as well as various immunomodulatory molecules. Despite tremendous progress in the past 50 years, many hurdles still remain for achieving the full clinical potential of viral-vectored vaccines. This chapter will present the evolution of vaccines from "live" or "attenuated" first-generation agents to recombinant DNA and viral-vectored vaccines. Particular emphasis will be given to human adenovirus (Ad) for the

A.V. Borovjagin
Institute of Oral Health Research, University of Alabama at Birmingham School of Dentistry, 1919, 7th Avenue South, SDB716, Birmingham, AL 35294-0007, USA
e-mail: aborov@uab.edu

J.G. Gomez-Gutierrez
The Hiram C. Polk Jr MD, Department of Surgery, University of Louisville School of Medicine, 505 South Hancock St., Louisville, KY 40202, USA
e-mail: jgguti01@louisville.edu

H. Shirwan
Department of Microbiology and Immunology, University of Louisville, Baxter Bldg. I, Suite 404E, 570 South Preston St., Louisville, KY 40202-1760, USA
e-mail: haval.shirwan@louisville.edu

Q.L. Matthews (✉)
Division of Infectious Diseases, Department of Medicine, University of Alabama at Birmingham, Birmingham, AL 35294, USA
e-mail: qlm@uab.edu

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development of prophylactic and therapeutic vaccines. Ad biological properties related to vaccine development will be highlighted along with their advantages and potential hurdles to be overcome. In particular, we will discuss (1) genetic modifications in the Ad capsid protein to reduce the intrinsic viral immunogenicity, (2) antigen capsid incorporation for effective presentation of foreign antigens to the immune system, (3) modification of the hexon and fiber capsid proteins for Ad liver de-targeting and selective retargeting to cancer cells, (4) Ad-based vaccines carrying "arming" transgenes with immunostimulatory functions as immune adjuvants, and (5) oncolytic Ad vectors as a new therapeutic approach against cancer. Finally, the combination of adenoviral vectors with other non-adenoviral vector systems, the prime/boost strategy of immunization, clinical trials involving Ad-based vaccines, and the perspectives for the field development will be discussed.

List of Abbreviations

Wt	Wild type
CTL	Cytotoxic T lymphocyte
DC(s)	Dendritic cell(s)
Ag(s)	Antigen(s)
TAA	Tumor-associated antigen(s)
HPV	Human papillomavirus
HBV	Hepatitis B virus
PEI	Preexisting immunity
Ab(s)	Antibody (antibodies)
Nab	Neutralizing antibody
AAV	Adeno-associated virus
Ad	Adenovirus
Ad5	Adenovirus serotype 5
CRAd	Conditionally replicating adenovirus
HVR	Hypervariable region
His6	A molecular tag (motif) containing 6 histidine residues
VLP	Virus-like particles
IM	Intramuscular
IV	Intravenous
IP	Intraperitoneal
SC	Subcutaneous

8.1 Introduction

Emerging and reemerging infectious diseases as well as cancer pose great global health impacts on the society. Vaccines have emerged as effective treatments to prevent or reduce the burdens of already developed diseases. This is achieved by means of activating various components of the immune system to generate systemic inflammatory reactions targeting infectious agents or diseased cells for control/

elimination. DNA virus-based genetic vaccines gained significant attention in the past decades owing to the development of DNA manipulation technologies, which allowed engineering of recombinant viral vectors encoding sequences for foreign antigens (Ags) or their immunogenic epitopes as well as various immunomodulatory molecules. Having emerged as a result of the parasite–host coevolution, viruses as vehicles for delivery and expression of Ag-encoding sequences may also function as immune adjuvants, enhancing immune responses to the transgene-expressed Ags. Despite tremendous progress in the past 50 years, many hurdles still remain for the development of virus-based vaccines and their effective clinical use.

Human adenovirus (Ad) emerged as one of the top candidate viral vectors for vaccine development owing primarily to its relatively low pathogenicity, genetic safety, and the lack of host genome integration step in its replication cycle. Other attractive features of Ad for vaccine application include its strong immune-adjuvant properties, highly efficient infection of various cell types, and vast transgene incorporation/cloning capacity. Although this chapter primarily focuses on the use of human Ad for the development of prophylactic and therapeutic vaccines, application of other viral vectors for vaccine development will also be briefly discussed. We will present vaccines first according to delivery systems/viral vector types and then with regard to their clinical applications, i.e., prophylactic or therapeutic. This will lead to detailed description of Ad as a vaccine vector, its taxonomy (serotype diversity), as well as the genomic and molecular structures. Ad biological properties related to its potential role as a vaccine vector will be highlighted along with advantages and potential hurdles for vaccine development. Along these lines, we will discuss in detail the "Ag capsid incorporation" strategy aimed at generating robust humoral Ag-specific immune responses while circumventing vector-specific preexisting immunity.

Substantial attention will be devoted to Ad vectors carrying "arming" transgenes with immunostimulatory functions as a vaccine adjuvant strategy and illustrate its use for the development of therapeutic vaccines against cancer. Retargeting of Ad vectors to specific cell surface receptors (de-targeting from natural Ad5 receptor "coxsackie/adenovirus receptor") will be discussed along with the underlying molecular technologies as a means of augmenting Ad infectivity for cancer cells and improving its gene transfer efficiency. We will also describe vaccination strategies involving combinations of Ad vectors with other (non-Ad) vaccine systems as well as the prime/boost strategy of immunization and commonly used immunocompetent animal models for immunotherapy studies and preclinical evaluation of adenoviral vaccines. Lastly, we will briefly discuss clinical trials involving Ad-based vaccines and the perspectives for the field development.

8.2 Virus-Based Recombinant Vaccines

Invention of virus-based vaccines dates back more than 200 years when Edward Jenner made a groundbreaking discovery that pus from cowpox-infected patients can elicit cross-protection of naïve subjects from much more virulent human disease smallpox (Jenner 1904). The term "vaccine" comes from the Latin word *vaccinus*, translated as "pertaining to cows," which reflects the history of the discovery. Although by the beginning of the twentieth century vaccination had already been widely used for prevention of other infectious diseases, such as diphtheria, rabies, and plague, that had nothing to do with cows, the original term remained.

Traditional concept of vaccination applies to induction of protective immunity against a given pathogen in a host achieved through intentional exposure to the natural or surrogate pathogen or Ags derived from such pathogens. The source of Ags can vary from well-defined recombinant proteins to natural proteins isolated from the pathogen or the whole pathogen. The first-generation vaccines included "live" or "attenuated" vaccines, prepared from pathogenic viruses, such as yellow fever, measles, mumps, and rubella, or bacteria, such as *Salmonella enterica enterica*, with reduced virulence. Treatment of virulent strains with temperature or chemicals allows for the generation of "inactivated" or "killed" vaccines. Those include influenza, cholera, polio, hepatitis A, and rabies vaccines as well as "toxoids," formaldehyde-inactivated toxins naturally produced by some pathogens and represented by diphtheria and tetanus vaccines. While pathogens/toxins in such vaccines are no longer infectious/toxic, i.e., biologically safe, their immunogenic properties are retained.

Advances in basic knowledge in immunology along with the development of recombinant DNA technology in the past 50 years prompted extensive efforts in vaccine development and led to second-generation vaccines, including recombinant vaccines. Recombinant DNA technology substantially expanded the repertoire of vaccine types, which may differ with regard to the form of immunological target (immunogen) and route of delivery. Recombinant vaccines comprise several distinct classes: (1) "subunit" vaccines that are typically obtained by genetic cloning and expression of immunogenic proteins or their individual subunits/domains using various expression systems, such as bacterial, yeast, or mammalian; (2) "conjugate" vaccines, a special type of "subunit" vaccines using Ags or toxoids conjugated to polysaccharides, enhancing their immunogenicity, such as *Haemophilus influenzae* type B vaccine; (3) DNA or "naked" DNA vaccines; and (4) viral-vectored vaccines. In the latter instances, foreign Ags are expressed in the host cells upon the delivery of recombinant DNA molecules coding for such Ags, such as West Nile virus experimental vaccine (Alarcon et al. 1999). In contrast to "naked" DNA vaccines, also known as "third-generation vaccines," virus-based vaccines use recombinant viral vectors as natural vehicles to deliver foreign Ag-encoding sequences. The term "vectored vaccine" is generally used for live recombinant viruses or bacteria representing a natural carrier or "vector" capable of

incorporating transgenes from a pathogen and expressing it in the host without itself causing illness. Viral vectors provide a substantially more efficient Ag delivery method, known as "transduction," as compared to "naked" DNA. Besides, naked DNA-based vaccines were found to be unable to induce strong cellular immune responses in humans. By contrast, Ags delivered in the context of viral vectors are more immunogenic than the same Ags delivered as proteins or the Ag-encoding naked DNA. This is primarily due to the ability of mammalian innate immune system to recognize viruses as danger signals through Toll-like receptors, leading to the generation/integration of innate and adaptive immune responses for a more pronounced immune efficacy against the virus.

Most virus-based vaccines contain Ag-coding sequences, replacing genes required for viral replication, and thus are replication deficient. Recently, vaccine design expanded towards replication-competent viral vectors. The advantage of replicative viruses as vectors is the dramatic (~10,000-fold) amplification of transgene and its expression because of excessive viral DNA replication. This enhances immunization efficiency and allows decreased vaccine dosing. The disadvantages include cell lysis, which typically occurs upon infection as the ultimate step in viral life cycle and may reduce the duration of transgene expression and vaccine efficacy. Furthermore, development of vaccines based on replication-competent viral vectors requires adequate animal models that should not only be immunocompetent but also fully permissive for replication of a given virus, which may not be available or cost permissive.

An important benefit of using virus-based vaccines is the ability to modulate the efficiency and cell-type specificity of an Ag-encoding transgene delivery. This can be achieved through alteration of natural tropism of viral vectors, making possible an efficient and specific transduction of specific cell population/cell type, such as cancer or Ag-presenting cells (APCs). Various strategies for transductional retargeting of viral vectors have been developed in the recent years. A large variety of viral vector systems, each with its advantages and disadvantages, have been used for vaccine development. A more detailed description of those vector systems is provided in Sect. 8.3.

Natural viral infections or viral vectors are capable of inducing strong innate as well as adaptive immune responses and establish long-term immunological memory. Preexisting immunity (PEI), particularly Ag-specific antibodies (Abs) against many human viruses typically found in patients, significantly compromises clinical efficacy of the corresponding viral vectors when used for vaccine or gene therapy applications. Many strategies have been developed to circumvent PEI to virus-based vectors, some of which will be described in other sections. Low immunogenicity of some Ags may require a more complex regimen of vaccination involving a "prime–boost" approach that may combine two different vaccine types for repeated delivery of the same Ag: a DNA vaccine followed by a viral vaccine or vice versa. Many contemporary vaccines are designed using a polyvalent approach allowing immunization against more than one Ag, serological variant (serotype) of a given pathogen, or even several different pathogens simultaneously. Optimal vaccination protocols are developed by considering various factors, such as dose, route of

administration, type of priming and boosting vectors, number of boost immunizations, and their timing.

Conventional prophylactic, i.e., preventive, vaccines against infectious diseases have been highly effective at eliminating or drastically reducing incidence of many life-threatening diseases, such as smallpox and poliomyelitis. These vaccines are administered to the host prior to an encounter with pathogenic infectious agents, such as viruses or bacteria. A single exception is the rabies vaccine, developed by Pasteur more than 100 years ago, which is administered only after exposure to the virus. However, the application of therapeutic vaccines has recently been on the rise owing to significant technological developments and comprehensive understanding of the immune system. Unlike prophylactic vaccines that achieve their disease-preventive effect by generating humoral immune responses, therapeutic vaccines often function through robust T-cell responses against key viral Ags. The contribution of humoral responses to the therapeutic efficacy of a vaccine depends on the target pathogen or disease. Therapeutic vaccines have been developed or under development for various indications, including persistent infections, such as hepatitis B and C viruses (HBV, HCV), human papillomavirus (HPV), human immunodeficiency virus (HIV), tuberculosis, malaria, as well as noninfectious chronic diseases, such as autoimmune diseases, gastric ulcers, prion-caused mad cow disease, Creutzfeldt–Jakob disease, Alzheimer's disease, and various cancers.

Immunologic control of persistent viral infections, such as HPV, HBV, and HCV causing a long-term damage to the host or the infected organ, has become the primary goal of therapeutic vaccine efforts. While HPV does not cause obvious disease, it sets the infected host at risk of developing cancers similar to HBV and HCV. While HPV, HBV, and HCV can be eliminated by the immune system after acute infection or sometimes in chronic phase, persistent HIV infection has proved more challenging. Although there are various contributing factors, the integration of HIV DNA into the genome of the virus-targeted immune T cells appears to play a determining role (Finzi et al. 1999). Unlike prophylactic vaccines where the induction of effector immune responses against pathogens is the primary requirement for vaccine efficacy, therapeutic vaccines against chronic infections and cancer are required to overcome two major obstacles to achieve efficacy: (1) induction of effector immune responses in a host that may have developed tolerance to the pathogen or cancer Ags and (2) overcoming various immune evasion mechanisms employed by the chronic infection and progressing cancer. Furthermore, therapeutic vaccines are administered to patients with suppressed immunity due to prior chemo- and/or radiation therapy treatments and in already developed disease background.

Vaccine development has gone a long way to achieving success in controlling or fully eradicating a number of fatal infectious diseases. However, despite the rapid progress in recombinant DNA technology, effective immunization against many human infectious and noninfectious diseases remains a challenge due to immune evasion achieved by the corresponding pathogens or their disabling of the immune system components crucial for vaccination mechanisms. This warrants further efforts in improving recombinant vaccines and vaccination strategies. In this

regard, viral vectors provide important advantages over recombinant DNA-based vectors.

8.3 Viral Vector Systems Used for Vaccine Development

There are two major types of vector systems for delivery of recombinant DNA (rDNA) to human tissues: nonviral and viral (Luo et al. 1999). Viral vectors provide the highest gene transfer and transgene expression efficiencies in vivo, which is the main impetus for the use of virus-based vectors in ~75 % of reported rDNA-based clinical protocols (Luo et al. 1999). A wide variety of animal viruses have been employed for the development of viral vector systems (Table 8.1). The extensive knowledge of replication, packaging, and assembly requirements for various viruses have allowed the generation of both replication-competent and replication-deficient (non-replicative) viral vectors. Furthermore, genetic manipulation of viral genomes aimed at modification of (1) viral natural tropism to achieve tissue- or cell-type specificity of viral infection and replication, (2) timing and efficiency of transgene expression, and (3) intracellular trafficking of transgene-encoded products has recently become possible. The combination of safety, specificity, and high levels of production makes viral vectors a leading choice for the expression of foreign genes in experimental and commercial applications (Levine 1987).

8.3.1 Adeno-associated Vectors

Adeno-associated virus (AAV) is a member of the *Dependovirus* genus of the *Parvoviridae* family, which includes a vast series of small viruses with a single-stranded DNA genome (Fig. 8.1). These viruses infect numerous species of mammals, including humans (Berns and Linden 1995). AAV virions are the smallest among gene therapy vectors. They have a capsid with icosahedral symmetry with a diameter of 18–25 nm, composed of only 60 proteins encoded by a single gene (the cap gene). The encapsidated AAV genome is a linear single-stranded DNA of either positive or negative polarity. A typical AAV preparation is a 50:50 mixture of virions containing DNA with positive or negative polarity (Berns 1990).

Comparative studies using lung cancer cell lines identified AAV2/1 as the most effective transducer among five adeno-associated virus serotypes: AAV2/1, AAV2/2, AAV2/4, AAV2/5, and AAV2/8 (Chen et al. 2013). AAV2 is the most commonly used serotype for transgene delivery. Although, the majority of the human population is seropositive for AAV, no significant adverse events during either pretrial efficacy studies or clinical trials involving AAV were observed (Maguire et al. 2009; Bainbridge et al. 2008). The wild-type (wt) AAV integrates into a specific region of the human chromosome 19 (between q13.3 and q13.4) upon

Table 8.1 Features of viral vectors used in gene therapy

Viral vector	Advantages	Disadvantages
Retroviral vectors	Insertion capacity for transgene <7–8 kb; stable integration into host DNA; recombinant virus titers within 10^6–10^7 pfu/ml; broad cell tropism, relatively easy manipulation of viral genome for vector engineering	Limited ability for targeting of viral infection; unable to infect nondividing cells; random integration into host genome; vector instability
Lentiviral vectors	Infect dividing and nondividing cells; stable transgene expression; transgene insertion capacity up to ~10 kb, no cytopathic effect associated with virus delivery	Can induce insertional mutagenesis; presence of regulatory (tat, rev) and accessory protein sequences in the packaging constructs
Herpesvirus vectors	Infect a wide variety of cell types, high insertion capacity (up to 50 kb); natural tropism to neuronal cells; stable viral particles allow propagation to high virus titers (10^{12} pfu/ml)	Possible toxicities; risk of recombination; no viral integration into host DNA
Poxvirus vectors	High cloning capacity allowing insertion of large DNA fragments; high transgene expression level; suited for live recombinant vaccine	Potential cytopathic effects (CPE)
Baculovirus vectors	Large insertion capacity (15 kb, up to 100 kb); very high levels of heterologous protein expression (~1 mg of protein per 1×10^6 of infected cells); production scale-up capability using high-density suspension cultures; no need for plaque purification; can be modified for transduction of mammalian cells (do not replicate but able to express the gene of interest; a very safe system)	Low cultivation temperature of insect cells (27 °C) may not be suitable for some proteins; improperly folded proteins; intracellular protein aggregates due to expression late in the infection cycle; improper glycosylation as reported for some glycoproteins
Sendai virus vectors	Capable of infecting human cell lines; low pathogenicity; powerful capacity for gene expression and a wide host range; cytoplasmic gene expression	Excessive immune responses associated with this virus administration in vivo
Epstein–Barr virus (EBV) vectors	Infects dividing and nondividing cells with preference for B cells; high transgene insertion capacity (<150 kb)	Limited access to packaging cell lines
Vaccinia virus vectors	Cytoplasmic replication mode; excellent experimental model system; broad host range; supports large insertions of foreign DNA (~25 kb)	Not suitable for large-scale, long-term expression of foreign proteins in continuous cell cultures
Adeno-associated virus (AAV) vectors	Infect dividing and nondividing cells; broad cell tropism; capability for targeted integration; low immunogenicity and pathogenicity	Limited capacity for transgene insertion (4 kb); difficulty in obtaining high titer preparations; require Ad or herpesvirus as helpers for the viral replication

(continued)

Table 8.1 (continued)

Viral vector	Advantages	Disadvantages
Adenovirus (Ad) vectors	High infectious titers (10^{12} pfu/ml); high level of transgene expression; large foreign DNA insertion capacity (7–8 kb); infects dividing and nondividing cells; safety of gene therapy applications, owing to the lack of integration in human genome	Immune response to viral proteins; lack of integration into host genome; transient gene expression

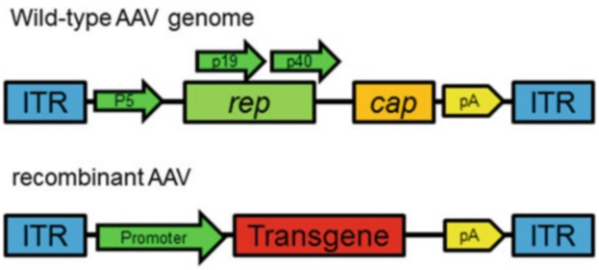

Fig. 8.1 Structure of adeno-associated virus (AAV) vectors. The wild-type AAV consists of the viral genes *rep* and *cap* coding for the different rep genes and cap (VP1, VP2, VP3) proteins, the AAV promoters (p5, p19, p40), the polyadenylation site (pA), and the inverted terminal repeats (ITR). In rAAV vectors, a transgene cassette carrying the promoter, the transgene, and the pA site are in place of the viral *rep* and *cap* genes. Figure is extensively modified from previously published work. Adapted from Walter W. and Stein U. Viral Vectors for Gene Transfer A Review of Their Use in the Treatment of Human Diseases. Drugs. 60 (2): 249-271, 2000. Adis International Limited

host cell infection (Kotin et al. 1992), whereas this ability for site-specific integration is lost in rAAV, possibly due to deletion of the viral *rep* gene. Although the integrating gene delivery systems allow for a more stable transgene expression than the episomal ones, integration of foreign DNA in the cellular chromosomal DNA is associated with the risk of insertional mutagenesis. This, in turn, may cause malignant cell transformation (Romano 2012). Therapeutic transgenes and internal promoters, regulating transgene expression in rAAV-based vectors, replace the viral *rep* and *cap* genes (Fig. 8.1) (Bartel et al. 2012). Although AAV vectors are less immunogenic than adenoviral vectors, low transduction efficiency for certain tissues, inability for targeted delivery to specific cell types, relatively low transgene-carrying capacity (~4 kb), and dependence on a helper virus for propagation limit utility of these vectors for human clinical applications.

However, several clinical trials with AAV have been performed. For example, a clinical trial in advanced cancer patients conducted in China evaluated the safety of adoptive cytotoxic T lymphocytes (CTLs) generated by the coculture with dendritic cells (DCs) transduced with rAAV encoding carcinoembryonic Ag (CEA). This study demonstrated that infusion of CEA-specific CTL was well tolerated and showed no severe adverse reactions in cancer patients (Di et al. 2012). Two phase

I clinical trials (one for rAAV2 and the other for rAAV1) and one phase II clinical trial (with rAAV1) for the alpha-1 antitrypsin gene therapy have shown promising results. However, levels of alpha-1 antitrypsin were only 3–5 % of the target range, indicating the need to increase the dose of the vector and/or gene expression levels to achieve a therapeutic range (Mueller and Flotte 2013).

8.3.2 *Adenovirus Vectors*

Ads, discovered in 1953 in human adenoid tissue (Enders et al. 1956), are non-enveloped DNA viruses carrying linear double-stranded DNA of about 35 kb in size (Fig. 8.2). Currently, over 100 types/serotypes of the *Adenoviridae* family composed of 5 genera and capable of infecting humans and a large number of different animal species are known. Human Ads belong to the *Mastadenovirus* genus with 57 characterized serotypes (Ad1–Ad57) and 7 distinct species/subgroups (A–G). They are responsible for 5–10 % of acute respiratory diseases in children and a variable number of epidemic conjunctivitis and gastroenteritis (Giacca and Zacchigna 2012). The natural tropism of human Ads for the respiratory epithelium and the conjunctiva is mainly determined by their mode of transmission rather than the molecular characteristics of the virus. Indeed, the CAR receptor-mediating cell infection by Ads is ubiquitously expressed, and most human cell types can sustain adenoviral infection and replication regardless of their proliferative state (Law and Davidson 2005). The most extensively characterized Ad types are type 2 (Ad2) and type 5 (Ad5), which are members of the C subgroup. These Ad

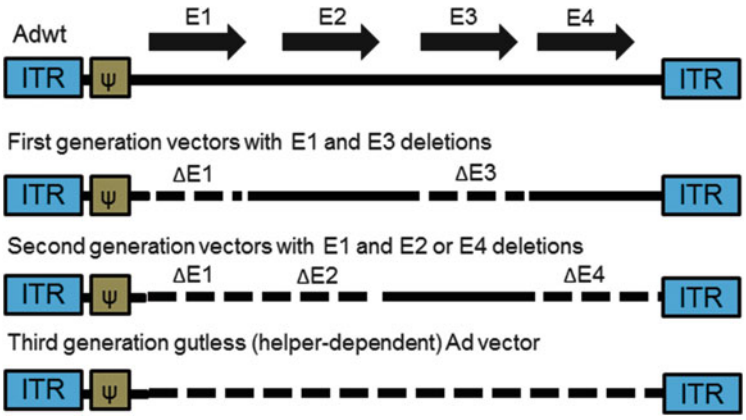

Fig. 8.2 Three generations of recombinant Ad vectors. The Ad wild type contains all early genes. The first generation lacks *E1* and *E3*; the second generation is devoid of *E1*, *E2*, or *E4*; and the third generation lacks all early genes. ITR, inverted terminal repeats; ψ, packaging signal. Figure is extensively modified from previously published work. Adapted from Walter W. and Stein U. Viral Vectors for Gene Transfer A Review of Their Use in the Treatment of Human Diseases. Drugs. 60 (2): 249-271, 2000. Adis International Limited

serotypes were used for engineering the first-generation Ad vectors since they are not associated with severe disease in humans and, therefore, suitable for in vivo applications. Another attractive property of Ads is the high efficiency with which they exploit the cellular machinery to synthesize viral mRNAs and virus-specific proteins. Given these considerations, it is not surprising that Ad vectors have become the focus of a vast series of both animal and clinical experimentations since the second half of the 1990s (Giacca and Zacchigna 2012). The attributes of Ad as well as other viral vectors have been described in detail in Table 8.1.

Three generations of replication-incompetent Ads have been described to date (Fig. 8.2). The first-generation (FG) Ads have deletion of *E1* or both *E1* and *E3* genes, which become substituted with an expression cassette typically consisting of a promoter driving a therapeutic gene and a polyadenylation signal (Danthinne and Imperiale 2000). Helper cells, which contain a genomic copy of the entire *E1* region (*E1A* plus *E1B* genes), provide in trans the *E1* proteins essential for the initiation of viral replication (Louis et al. 1997). The human embryonic kidney 293 (HEK293) cell line, stably transfected to express the viral *E1* proteins, is widely used as a helper cell line for the production of recombinant replication-deficient Ad stocks (Danthinne and Imperiale 2000). The second-generation (SG) Ad vectors were developed to lack the *E2A* and *E4* genes in addition to *E1* and *E3* genes (Fig. 8.2). The SG vectors were expected to show prolonged transgene persistence/expression due to fewer encoded Ad-specific Ags, eliciting vector-associated immune responses.

Immune responses to virus-specific genes decrease the duration of Ad-delivered therapeutic transgene expression by CTL-mediated elimination of Ad-transduced host cells (Bessis et al. 2004; Liu and Muruve 2003). The SG vector propagation depends on a helper cell line, providing in trans the missing function of the *E4* genes, which is required for Ad DNA replication and transcriptional regulation of Ad genes. The helper cell-provided functions also include transition from early to late phase of viral gene expression, viral mRNA transport, the host protein synthesis shutoff, and the assembly of the virions (Morsy and Caskey 1999). Deletion of the additional genomic regions in the SG Ad vectors did not, however, circumvent the problem of short-term transgene expression, plausibly due to the immunogenic and inflammatory potential of the residual Ad gene products in the SG vectors. Furthermore, expression of the therapeutic gene from an SG vector was reduced compared to that from a FG vector, probably due to the missing regulatory functions of some *E2* and *E4* gene products directly or indirectly upregulating expression of other virus-specific genes in the FG or the wt Ads (Giacca and Zacchigna 2012).

The third-generation of replication-deficient Ad vectors (Fig. 8.2) is characterized by the complete elimination of all viral coding regions, leaving only the inverted terminal repeats (ITRs), the transgene expression cassette, and the *psi* (ψ) packaging signal. The advantage of such vectors lies in a substantially lower immunogenicity and production of high viral titers in the 293 cell line. However, these vectors are named "gutless" or "helper dependent" as their replication depends entirely on coinfection of the packaging cell line with a helper vector

producing in trans all the required Ad proteins. These vectors are also described as "high capacity" as they can accommodate DNA sequences of up to 37 kb, thus allowing delivery of large or multiple transgenes (Alba et al. 2005; Brunetti-Pierri and Ng 2008).

Another type of Ad vectors used particularly in cancer gene therapy approaches is the conditionally replicating Ads (CRAds), which are also referred to as oncolytic Ads. CRAds infect, replicate, spread, and kill cancer cells by a natural lytic mechanism referred to as oncolysis. The general principle of these vectors is based on rendering viral replication cancer selective by mutations introduced in the Ad *E1*genes, such as a deletion of the *E1B* gene or a partial [24 base pair (bp)] deletion in the *E1A* gene. The Ad *E1B* gene product triggers proliferation of the infected cells (essential for Ad replication function) by inducing degradation of tumor suppressor p53 and the resulting block in p53-dependent apoptosis along with the activation of cell cycle signaling. The *E1B* mutation would, therefore, make an Ad unable to productively replicate in normal cells carrying a functional p53. In contrast, such a CRAd would replicate in many cancer cells where p53 signaling is disabled and the normal tumor suppression mechanism is inactive. A high percentage of tumor cells possess defective p53 and, thus, are susceptible for killing by such a CRAd (Bischoff et al. 1996), known as ONYX-015 (originally called dl1520) (Barker and Berk 1987). Another type of CRAd, carrying the abovementioned 24 bp deletion in the *E1*A pRb-binding domain, shows impaired replication in normal cells. The *E1*A pRb-binding function is required to displace the cellular transcription factor E2F from its complex with tumor suppressor pRb to induce the proliferative state of the infected cell. However, the *E1*A defect is complemented by pRb signaling defects in a number of pRb-defective cancers, making this CRAd, known as AdΔ24 (Fueyo et al. 2000) and another CRAd dl922-947 with similar *E1*A deletion (Heise et al. 2000), cancer selective.

The clinical utility of Ad-based vectors has further been improved through the generation of vectors that lose their native tropism and/or acquire cell and tissue specificity of transduction through genetic modification of viral capsid proteins. Besides targeting, Ad capsid modifications showed utility for imaging and vaccine development applications. Table 8.2 summarizes the features of Ad capsid modifications and their applications.

8.3.2.1 The "Antigen Capsid Incorporation" Strategy in Vaccine Development

Of the identified human Ad serotypes, Ad5 and Ad2 have been the most extensively used for gene therapy applications. Scientists have taken an alternative approach to conventional expression of immunization Ags in the context of viral vectors. This approach embodies genetic incorporation of Ags into viral capsids. This innovative paradigm is based on presenting Ags as components of viral capsids, achieved through genetic fusion to or incorporation in viral capsid protein(s), rather than vector-expressed transgenes. Vaccine design using Ad vectors displaying Ags on their capsid surface is known as the "Ag capsid incorporation" strategy. Whole Ags

Table 8.2 Adenovirus genetic capsid modifications and their applications in vector targeting

Capsid modification	Effect/application	References
Ad5/Ad3 serotype chimera fiber (F5/3): Ad5 fiber C-terminal "knob" domain is replaced with the Ad3 counterpart. The virus carrying such modification is referred to as Ad5/3	Enhances tumor transduction and multiple steps of Ad replication by targeting DSG2 and/or CD80/CD86; ablates CAR tropism	Stevenson et al. (1995), Krasnykh et al. (1996), Kanerva et al. (2002), Kawakami et al. (2003)
An Arg-Gly-Asp (RGD) motif incorporated into the HI loop of the fiber knob in the form of CDCRGDCFC ("RGD4C") peptide	Enhances tumor transduction via αvβ3 and/or αvβ5 integrin binding, but does not ablate CAR tropism	Dmitriev et al. (1998), Suzuki et al. (2001), Murugesan et al. (2007)
A polylysine (pK7) motif at the C-terminus of the Ad5 fiber knob alone or together with the RGD motif bearing an RGD4C peptide, incorporated into the HI loop of the Ad5 fiber knob. Vectors are referred to as Ad5pK7 and Ad5pK7/RGD, respectively	Enhanced transduction of various cell types including cancer cells through heparan sulfate proteoglycans (HSPG) or simultaneous targeting of HSPG and α_v integrins (pK7/RGD)	Wickham et al. (1997), Bouri et al. (1999), Wu et al. (2002b), Stoff-Khalili et al. (2005), Borovjagin et al. (2011)
Genetic modification of the minor capsid protein IX (pIX) by its fusion (C-terminal) to various size ligands including fluorescent (EGFP, mRFP1, mCherry) and other imaging reporter proteins such as HSV-tk or its fusions to luc and mRFP1 (HSV-tk-luc, tk-mRFP1) and metallothionein (MT)	Provides some infectivity enhancement and retargeting to various receptors (generally less efficient than corresponding fiber modifications); exposes ligand on the capsid surface and tolerates incorporation of large molecules and fusions that can be used as imaging reporters	Dmitriev et al. (2002), Vellinga et al. (2004), Le et al. (2004), Meulenbroek et al. (2004), Campos and Barry (2006), Matthews et al. (2006), Kimball et al. (2009), Borovjagin et al. (2010), Mathis et al. (2011)
Replacement of the Ad5 hexon gene with the Ad3 or Ad7 counterparts. vectors Ad5/H3 (H7)	Escape from the host neutralizing Abs against Ad5	Wu et al. (2002a), Short et al. (2010), Tian et al. (2011)
Ad5 fiber modifications replacing both the shaft and the knob domains by their counterparts from other serotypes including Ad9, Ad35, Ad41	Reduces the natural Ad5 liver tropism and in vivo (factor X-mediated) liver uptake	Shayakhmetov and Lieber (2000), Shayakhmetov et al. (2000), Nicol et al. (2004)
Incorporation of the integrin-binding peptide RGD4C at the C-terminus, in the HI loop or both locales of the Ad3 knob, in the context of F5/3 chimera fiber	Enhances cancer cell transduction, de-targets from CAR (ablated CAR tropism). Improves gene transfer to glioma	Borovjagin et al. (2005), Tyler et al. (2006)

(continued)

Table 8.2 (continued)

Capsid modification	Effect/application	References
Fiber modification by incorporation of the integrin-binding peptide RGD4C in the Ad5/35 chimera fiber knob domain	To enhance Ad5 transduction for CD46 receptor-expressing cancer cells	Murugesan et al. (2007), Matsui et al. (2011)
Ad5 fiber modification replacing its knob and part of the shaft with T4 phage-derived fibritin fused to CD40 ligand (CD40L)	To selectively target CD40-expressing cells (DC cells) for immunotherapy applications	Krasnykh et al. (2001), Belousova et al. (2003)
Fiber modification using a fiber–fibritin chimera fused to a single-chain Ab, an affibody or the *S. aureus* protein A Fc-binding domain	For targeting of Ad gene transfer to cells expressing a particular surface marker directly or via an Ab or an adapter molecule	Hedley et al. (2006a), Belousova et al. (2008), Korokhov et al. (2003)
Fiber modifications by incorporation of various size ligands in the HI loop of the Ad5 knob with or without ablation of CAR tropism	For retargeting of Ad gene transfer to cell surface markers/receptors other than CAR; to de-target from CAR	Belousova et al. (2002), Magnusson et al. (2007)
Modification of hypervariable loop 5 (HVR5) in the capsid protein hexon	To reduce/prevent Ad infection of hepatocytes; for vaccination approach	Wu et al. (2005), Vigne et al. (1999)
Modification of hypervariable loop 2 (HVR2) in the capsid protein hexon	For Ad5-based vaccine applications	Matthews et al. (2010)
Single point mutation in the hexon capsomer designed to enable defined chemical capsid modifications	To permit both de-targeting from and targeting to hepatocytes with evasion from Kupffer cell scavenging	Prill et al. (2011)

or immunogenic peptides incorporated into the viral capsid offer a potential advantage for vaccine applications. Owing to processing of the capsid-incorporated Ags through the exogenous pathways native to the Ad capsid proteins, the Ags could accrue their immunostimulatory potential. A strong humoral response against the incorporated Ags, similar to the one induced against the Ad capsid proteins, could result from the adjuvant function of the Ad vector.

The "Ag capsid incorporation" strategy has also been applied to the human rhinovirus as a vector for vaccination against HIV. A chimeric human rhinovirus HIV was shown to stimulate immunity against HIV-1 (Smith et al. 1994). In addition, combinatorial libraries of human rhinovirus capsid-incorporated HIV-1 glycoprotein 41 (gp41) epitopes were shown to induce Abs with activity that can mimic the NAb effect (Arnold et al. 2009). Preclinical and clinical development of Ad-based HIV vaccines has progressed faster than the development of other vector systems, such as human rhinovirus, owing to the tremendous flexibility of Ad vectors generally exceeding that of the rhinovirus systems. For instance, since human rhinovirus is a relatively small RNA virus, the human rhinovirus platform

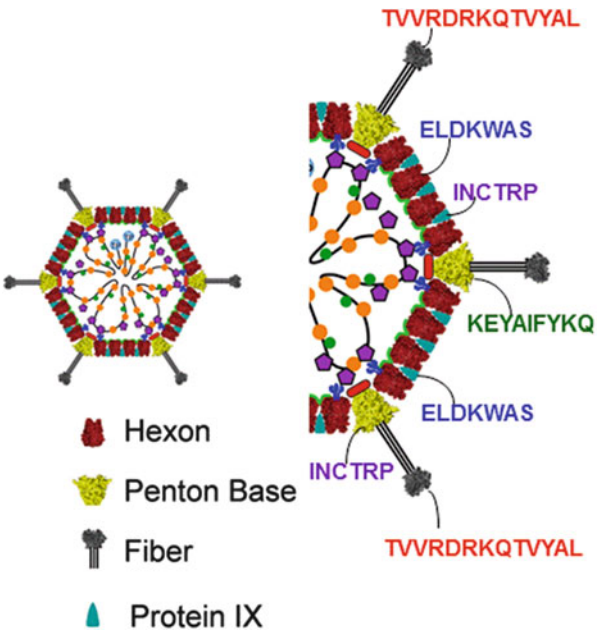

Fig. 8.3 Antigen incorporation in adenoviral structural proteins. Adenoviral capsid consists of hexon, penton base, fiber, and pIX. Antigenic epitopes can be incorporated into these capsid (structural) proteins to induce Ag-specific immune responses. For example, this figure depicts the incorporation of HIV Ags from the HIV-1 variable region 2 (TVVRDRKQTVYAL), (KEYAIFYKQ), glycoprotein 41 (ELDKWAS), and glycoprotein 120 (INCTRP). This figure is adapted from Nemerow et al. 2009. *Virology 384 (2009) 380–388,* copyright Elsevier

can only display 60 copies of a single antigenic epitope (Smith et al. 1994). In contrast, the Ad vector capsid platform could allow incorporation of HIV-1 epitopes into four distinct structural proteins/locales including hexon (Abe et al. 2009), fiber, penton base, and pIX capsid proteins (Fig. 8.3) (Matthews 2011; Nemerow et al. 2009; Matthews et al. 2013).

Although fiber (Krause et al. 2006; Shiratsuchi et al. 2010; Sharma et al. 2013), penton base (Krause et al. 2006), and pIX (Krause et al. 2006; Seregin et al. 2010a, b) have been utilized for "Ag capsid incorporation," the majority of "Ag capsid incorporation" strategies have been endeavored for the major capsid protein hexon (Fig. 8.3). Hexon is the most abundant structural protein of the Ad capsid, accounting for 63 % of its total protein mass (Rux et al. 2003; van Oostrum and Burnett 1985). Sequence analysis of hexon proteins from different Ad species revealed that, in addition to the evolutionarily conserved regions, there are also non-conserved hypervariable regions (HVRs), containing serotype-specific epitopes (Rux et al. 2003; Crawford-Miksza and Schnurr 1996). The loops at the top of the HVRs are the most pliable to modification by genetic engineering. Short heterologous peptides can be incorporated within the HVRs of the hexon without affecting the virion's stability or biological characteristics. A subset of the modifiable loops is exposed on the surface of the Ad5 capsid. HVRs 1, 2, and 5 have been utilized for

peptide or Ag incorporation (Vigne et al. 1999; Wu et al. 2005; Worgall et al. 2005, 2007; McConnell et al. 2006; Matthews et al. 2008; Palma et al. 2011).

Ad-based vaccines developed using the "Ag capsid incorporation" strategy have been tested preclinically in various disease settings (Crompton et al. 1994; Worgall et al. 2004, 2005; Krause et al. 2006; McConnell et al. 2006; Matthews et al. 2010; Matthews 2011; Gu et al. 2013). One of the first examples of "Ag capsid incorporation" used in the context of Ad was a study (Crompton et al. 1994) where an eight-amino acid sequence of the VP1 capsid protein of poliovirus type 3 was inserted into two distinct regions of the Ad2 hexon protein without affecting the propagation efficiency of one of the resulting chimeric vectors in tissue culture. Antiserum raised against the recombinant Ad vector with the polio epitope insert specifically recognized the VP1 capsid protein of polio type 3. A similar study demonstrated that His_6 epitopes could be incorporated into Ad5 hexon HVRs 1–7 (now reclassified as 1–9) without perturbing viral viability and any major biological characteristics such as replication, thermostability, or native infectivity. According to this study, His_6 appeared to be surface exposed when incorporated within HVR2 or HVR5 (Wu et al. 2005).

In an effort to create multivalent vaccine vectors using the "Ag capsid incorporation" strategy, the HVR2 and HVR5 of Ad5 have been targeted for incorporation of antigenic epitopes. To compare the flexibilities and insertion capacities, the Ad5 hexon's HVR2 and HVR5 were modified to incorporate identical epitopes of incrementally increasing sizes, ranging from 33 to 83 amino acids, within those locales. Viable vectors were produced with incorporation of 33 amino acids plus a 12-amino acid linker at HVR2 or HVR5. In addition, viable vectors were produced with incorporations of up to 53 amino acids plus a 12-amino acid linker at HVR5. The HVR5 was found to be more permissive, allowing incorporation of peptides of up to 65 amino acids. These model Ags were surface exposed as evidenced by a whole-virus ELISA analysis. In vivo immunization with these vectors demonstrated an Ag-specific immune response (Matthews et al. 2008). Although, in vivo responses were not evaluated in the context of PEI, it is possible that these vectors would indeed escape Ad5 PEI (Matthews et al. 2008).

Along these same lines, a study evaluated the ability of Ad5-based vectors expressing an HIV transgene to induce Ag-specific immune responses in the presence of Ad5 pre-immune conditions. To overcome limitations imposed by PEI to Ad5, the authors constructed vectors carrying modifications in the hexon's HVR5. This study characterized various immunological parameters associated with these vectors, such as vector neutralization, acquisition of adaptive immune response, and comparison of protective immunity. In this regard, Ad-Luc (luciferase protein expressed as a transgene in the Ad *E1* region), Ad-HisLuc (His_6 epitope presented in HVR5 region and luciferase protein expressed as a transgene), or Ad-END/AAALuc vector (containing three amino acid mutations in HVR5 and expressing luciferase protein) were administered to mice IM. The hexon-modified vector (Ad-HisLuc) generated the lowest Ad5-specific neutralizing activity, which was significantly lower than that generated by Ad-Luc at weeks 6 and 8 and by Ad-End/AAALuc vector at week 8. The individual neutralizing activity in response

to Ad-HisLuc immunization was significantly lower than that in response to immunization with Ad-Luc. Further studies support the concept that modified hexon thwarts Ad5 NAbs and promotes cellular immune responses. These findings indicate that a change in the immunogenic epitope is necessary to avoid neutralization by preexisting NAbs (Abe et al. 2009).

In a study, the first of its kind, the membrane proximal ectodomain region (MPER) of HIV gp41 protein, was incorporated in the Ad5 hexon HVR2 alone or in combination with genomic incorporation of the Gag transgene. Characteristics of the resulting vector Ad5/HVR2-MPER-L15(Gag) with respect to growth kinetics and thermostability remained unchanged as compared with peptide or Ag capsid-incorporated vectors (Matthews et al. 2006; Li et al. 2010), demonstrating that incorporation of the MPER epitope within HVR2 was not substantially detrimental to the vector's biology (Matthews et al. 2006; Li et al. 2010). This study was the first demonstration that a disease-specific Ag could be incorporated within Ad5 HVR2. Most importantly, the data demonstrated a humoral anti-HIV response in mice immunized with the hexon-modified Ad vector. Immunization with the MPER-displaying vector allows boosting, in contrast to immunization with AdCMVGag vector, possibly because the Ad5/HVR2-MPER-L15 (Gag) vector elicits less of an anti-Ad5 immune response. It is likely that the incorporation of the MPER epitope within this vector reduces the immunogenicity of the Ad5 vector. This finding is notable because HVR2 has not been fully explored for its potential use in "Ag capsid incorporation" strategies.

In a follow-up study, generation of Ad-based multivalent vectors with potential utility for vaccination against several strains of an organism or two or more distinct organisms was attempted. A multivalent vaccine based on Ad was generated that displayed Ags within hexon HVR1 and HVR2 or HVR1 and HVR5. This study focused on the generation of proof-of-concept vectors that can ultimately result in the development of multivalent vaccine vectors displaying dual Ags within the hexon of a single Ad virion/particle. These novel vectors utilize Ad5 hexon's HVR1 as an incorporation site for a seven amino acid epitope (ELDKWAS) of the HIV's gp41 MPER region (Fig. 8.3) in combination with His_6 incorporated in HVR2 or HVR5. The multivalent capsid-based vaccines incorporating HIV Ag along with His_6 within a single Ad virion/particle generated His_6 and HIV epitope-specific humoral immune responses in mice (Gu et al. 2013). This report illustrated that multivalent capsid-based vaccines are viable and immunogenic and can present different antigens within a single Ad virion/particle.

8.3.2.2 Chimeric and Rare Serotype Adenoviral Vectors for Vaccine Development

In the immediate future, it is likely that viral vector-based vaccination will become a common clinical intervention. Therefore, it has become increasingly important to design vectors that can overcome Ad5 PEI (Thacker et al. 2009; Seregin and Amalfitano 2009). Towards this end, rare and nonhuman Ad serotypes have been

genetically modified for vaccine development. Chimeric Ad vectors could consist of a subportion of the Ad5 vector genome that is replaced with genomic portions of another alternative serotype, thus creating "chimeric" Ad vectors. Alternatively, in a more extreme approach, the entire Ad vector genome could be composed of genes derived solely from alternate Ad serotypes (Noureddini and Curiel 2005; Seregin and Amalfitano 2009; Abbink et al. 2007; Youil et al. 2002; Roberts et al. 2006; Liu et al. 2009; McCoy et al. 2007). Ad hexon and fiber proteins have been manipulated genetically in chimeric strategies, primarily because these proteins are known to be the target of vector NAbs (Molinier-Frenkel et al. 2002; Roy et al. 1998; Wu et al. 2002a; Gall et al. 1996). Several chimeric fiber- and hexon-generating strategies have been employed (Table 8.2) (Seregin and Amalfitano 2009). NAbs generated against hexon HVRs account for 80–90 % of the anti-Ad NAb response, which plays a critical role in vector clearance, thereby reducing therapeutic efficacy of the vaccine vectors (Sumida et al. 2005). The importance of the HVRs as NAbs epitopes remains unclear as it relates to Ad5 and other serotypes (Yuan et al. 2009). Therefore, exact mapping of the NAb epitopes in these HVRs may be necessary to improve chimeric Ad5-based vectors (Alba et al. 2009).

One of the first studies on Ad5-based chimeric vector construction reported the replacement of Ad5 native hexon gene with the counterpart sequence of the Ad2 (Gall et al. 1998). This study was the launching point for development of other chimeric vectors. In another study, a chimeric adenoviral vector (Ad5/H3) was constructed by replacing the Ad5 hexon gene with that of the Ad3. The chimeric vector was successfully "rescued" in the HEK293 helper cell line. Ad5/H3 had a significantly slower growth profile as compared to the wt Ad5/H5 vector, indicating that the Ad3 hexon is capable of capsid incorporation and supporting encapsidation of the viral genome but with lower efficiency than the native (Ad5) hexon. The gene transfer efficiency of Ad5/H3 in HeLa cells was also lower than that of Ad5/H5. The host neutralization studies demonstrated that the NAbs against Ad5/H3 and Ad5/H5 generated in immunized C57BL/6 mice did not cross-neutralize each other in the context of in vitro infection of HeLa cells. Therefore, pre-immunization of C57BL/6 mice with one of the two types of vectors did not prevent subsequent infection with the other type, clearly demonstrating that substitution of the Ad5 hexon with the Ad3 hexon can circumvent the host neutralization of the Ad5 (Wu et al. 2002a).

More recently and along the same lines, another chimeric Ad vector, Ad3/H7, generated by replacing the Ad3 hexon gene with the hexon gene (H7) of the Ad7 was reported (Table 8.2) (Tian et al. 2011). The chimeric vectors were successfully generated in HEp-2 cells, and the Ad7 hexon-containing particles were able to encapsidate the viral genome, functioning as efficiently as the Ad3. The host vector neutralization response studies demonstrated that up to 97 % of the NAbs produced in BALB/c mice infected with both Ad7 and Ad3/H7 vectors were specific for the H7 protein in vitro. Therefore, pre-immunization of mice with one of the vectors (Ad7 or Ad3/H7) significantly prevented subsequent INL infection with the other vector in vivo. In marked contrast, pre-immunization of mice with either Ad3 or

Ad3/H7 did not prevent subsequent infection with the other vector (Tian et al. 2011).

Replacing sequences of seven HVRs in the Ad5 hexon with those of rare serotype, Ad48, resulted in a chimeric vector, Ad5HVR48 (1–7), capable of evading most of the Ad5-specific PEI in preclinical studies in mice and rhesus monkeys (Roberts et al. 2006). Ad5-based chimeric vectors in which all seven HVRs were substituted induced the same level of anti-Ag immune responses in mice with Ad5 PEI as in naïve mice. In contrast, replacing a single HVR in H5 provided only a slight enhancement of anti-Ag immune responses over those of non-chimeric Ad5 vector. Studies are underway to determine epitopes of NAbs for this vector.

Recent studies suggested that Ad5 NAb responses may be focused on one specific HVR, such as HVR1 or HVR5 (Abe et al. 2009; Crawford-Miksza and Schnurr 1996). Chimeric Ad5 vectors with subsets of H5 HVRs substituted for the Ad48 hexon HVRs were constructed and used to assess the potential of individual hexon HVRs as epitopes for Ad5 NAbs. These partial HVR-chimeric vectors were evaluated by NAb assays and immunogenicity studies with and without Ad5 PEI. Through various studies, it was demonstrated that Ad5-specific NAbs are targeted against several HVRs, indicating the need for replacing all HVRs to optimize evasion of Anti-Ad5 immunity (Bradley et al. 2012).

Liver sequestration of Ad5-based vectors is another substantial drawback that hinders systemic applications of Ad5-based therapies. Previous studies demonstrated that the human coagulation factor X (FX) binds to the Ad5 hexon through a direct interaction between its Gla domain and the hexon HVRs, leading to Ad5 liver uptake/sequestration following its systemic delivery (Waddington et al. 2008; Kalyuzhniy et al. 2008). The binding affinities for FX vary among Ad serotypes and may account for the differences in their hepatocyte transduction efficiencies previously observed in vivo. While Ad2 and Ad5 bind factor X with the highest affinity, weak or no binding was observed for Ad9, Ad35, Ad48, and Ad51. The hexon–FX interaction has been demonstrated for multiple human Ad serotypes, showing diversity in the affinity levels. The domains and amino acid sequences in the HVRs are integral for high-affinity interaction with FX; however, several aspects of this binding and its mechanism remain uncertain (Alba et al. 2009).

Utilization of vectors derived entirely from alternative human Ad serotypes (including Ad26 and Ad35) have also shown great promise, particularly in terms of ability to deliver transgenes (Abbink et al. 2007; Liu et al. 2008a, 2009; Barouch et al. 2004). Vectors based on Ads, which normally infect nonhuman species, have also shown a great promise. These nonhuman Ad vectors have been developed from numerous species, including canine, bovine, porcine, and chimpanzee (Bangari and Mittal 2006). For example, vectors have been recently developed from chimpanzee Ads C1 or C8 (AdC). Initially AdC vectors gained attention since human sera fail to significantly neutralize them (Roy et al. 2004). Notably, unlike some other serotypes, the *E1*-deleted mutant of AdC7 is easily propagated in vitro (Kobinger et al. 2006). An AdC7 vector expressing the SARS coronavirus Ag elicited higher B- and T-cell responses as compared to Ad5 vector carrying the same transgene in

mice with Ad5 PEI (Zhi et al. 2006). Additional promising results were seen with an AdC7 vector for another infectious disease. In this regard, a single injection of AdC7 encoding the *Ebola* glycoprotein provided protection from a lethal challenge, unlike the corresponding Ad5 vector (Kobinger et al. 2006).

It is important to note that several Ad epitopes recognized by T cells are conserved among a broad range of human and nonhuman primate-derived Ads. Therefore, it is possible that T cells in patients with Ad5 PEI will also recognize vectors derived from the alternate Ad species (Calcedo et al. 2009; Leen et al. 2004; Tang et al. 2006; Joshi et al. 2009). Since NAbs against bovine Ad3 (BAd3) have not been reported in humans, BAd3 have been evaluated for vaccine applications. In a mouse model, a single immunization of BAd3 encoding the HA Ag of H5N1 influenza induced greater levels of Ag-specific cellular immunity than the corresponding Ad5 vector, and this property was not compromised by Ad5 PEI (Singh et al. 2008). Importantly, mice with Ad5 PEI that received a prime–boost regimen of BAd3-Ad5 vectors encoding HA were fully protected from lethal flu virus challenge. In contrast, mice treated by a homologous Ad5-Ad5 prime–boost regimen were not protected. Consequently, Ads of nonhuman species may induce strong responses and provide immune protection comparable or superior to those of Ad5, while retaining protective potential in the presence of Ad5 PEI. The use of alternative serotype Ads thus allows for improved induction of immune responses to vector re-administration in host that have Ad5 PEI (Abbink et al. 2007; Liu et al. 2009; McCoy et al. 2007). As a result of these earlier studies, alternative serotypes vectors have been now tested in patient populations for HIV vaccine development (Barouch et al. 2011). In addition, human clinical trials evaluating Ad26 as a vaccine agent against HIV have been initiated.

In spite of the benefits associated with the use of alternative serotype Ad vectors, those vectors have several limitations including the likelihood of causing adverse effects in humans. One limitation of alternative Ad serotype usage is that some of them are unable to induce adequate levels of transgene expression in infected cells and are less amenable to large-scale purification (Thacker et al. 2009). With respect to Ad immune response, humans have evolved under continuous exposure of their populations to human Ad species, as opposed to Ad species infecting nonhuman hosts. Consequently, it is possible that the human system of innate immunity reacts to the capsid proteins of nonhuman Ad species in a different way. It is also plausible that the human immune system induces a more robust innate response when challenged with different xenotype or rare serotype Ads than upon exposure to Ad serotypes native to humans (hAd2/5). Recently it has been demonstrated that the innate immune response to capsid proteins of alternative serotype Ads has not only been substantially more robust as compared to Ad5 but in some cases can cause toxicity in animal models (Abbink et al. 2007; Appledorn et al. 2008; Hartman et al. 2008; Hensley et al. 2007). Alternative serotype vectors have intrinsically different tropism than Ad5 resulting in quite different biodistribution of these vectors in vivo. Over the last decade, Ad5-based vectors have been proven to be safe in humans and animals. The knowledge gained from such safety studies must be taken into account when testing chimeric or alternative serotype-based vectors.

8.3.2.3 Replicating Ad Vectors as Vaccines

The majority of the current Ad vaccine candidates are represented by transgene-expressing vectors, commonly engineered to express a foreign gene inserted into early (*E*) regions 1, 3, or 4 of the Ad genome (Small and Ertl 2011). Genes of the Ad5 *E1* and *E4* regions are essential for viral replication, and the majority of Ad vectors lacking those are replication deficient (Huang and Hearing 1989; Weinberg and Ketner 1986; Jones and Shenk 1978). A substantial amount of research has been performed with replication-deficient vectors in humans and animals, showing promise in several cases (Sullivan et al. 2000). Study of replicating Ad vaccines is difficult because the host/animal model system must support Ad replication if the vaccines are to be evaluated under conditions recapitulating their intended use in humans (Deal et al. 2013). Mice do not support human Ad replication. However, Syrian hamsters (Thomas et al. 2006a), cotton rats, dogs, pigs, monkeys, and chimpanzees partially support replication of some human Ad vectors. These animals, therefore, offer fully immunocompetent model systems that can be exploited to evaluate replicating vaccines (Thomas et al. 2006a; Pacini et al. 1984; Chengalvala et al. 1991; Jogler et al. 2006; Lubeck et al. 1989; Klessig and Grodzicker 1979). While cotton rats and Syrian hamsters have been extensively utilized for evaluation of replication properties of oncolytic Ad vectors (Thomas et al. 2006a; Toth et al. 2005), dogs have been typically used to characterize Ad-based vaccines (Chengalvala et al. 1991). With respect to evaluation of replicative vectors in the context of primate models, it has been difficult to assess vaccine efficacy and translate in vivo findings to the human clinical context due to the differences between primate and human immune systems.

Replication-competent Ad serotypes 7 (rAd7) and 4 (rAd4) expressing the HBV surface Ag (HBsAg) were used to prime (rAd7 HBsAg) and then boost (rAd4 HBsAg) two Ad4/Ad7-seronegative chimpanzees via an oral administration (Lubeck et al. 1989). After primary immunization, both chimpanzees shed vaccine virus for 6–7 weeks and developed Ad7-specific Abs, suggesting successful Ad7 replication in the animals. One chimpanzee developed transient seropositivity for HBsAg after prime, and both chimpanzees developed modest Ab titers after the boost immunization. A third chimpanzee primed with wt Ad7 and then boosted with rAd4HBsAg did not develop HBsAg Abs. Both rAd7/rAd4 HBsAg chimpanzees were protected from acute clinical disease, but not from infection, as evident by development of Abs against the HBV core protein in response to an HBV challenge. The animal that did not seroconvert (wtAd7/rAd4HBsAg), along with an unimmunized control, became clinically infected with HBV (Lubeck et al. 1989).

Three human participants of a phase I vaccine trial were immunized with the rAd7 HBsAg vaccine and exhibited no adverse effects. They shed the virus between days 4 and 13 post-vaccination with no evidence of person-to-person spread. Despite the fact that all the patients showed a significant increase in Ad7 Abs, none generated Abs to HBsAg (Tacket et al. 1992). Protection from the disease, if

not infection, in chimpanzees, regardless of lack of seroconversion in humans, suggests a potential value of using oral enteric vaccination with rAd to induce humoral immune responses to foreign pathogens.

8.4 Adenovirus-Based Prophylactic and Therapeutic Vaccines for Infectious Diseases

8.4.1 Prophylactic Vaccines Against Pandemic and Seasonal Influenza

Vaccines against infectious diseases that have been currently licensed for use in patients include heat-inactivated or killed whole organism vaccines, microbial extracts, purified or recombinant proteins, DNA vaccines, virus-like particles (VLPs), or recombinant viruses. While many diseases have been controlled or essentially eradicated owing to vaccination, the goal to generate community immunity for a wide variety of diseases remains elusive due to a number of problems associated with current vaccines (Zhang et al. 2011). A substantial amount of time and research efforts are directed towards determining which strains of influenza (flu) to include in the flu vaccine each year. In most years the vaccine matches quite closely to the current disease-causing strains of flu, while sometimes, despite all of the research efforts, the vaccine is not a good match and fails to provide adequate protection (Duda 2014). Since fewer flu B cases have been identified as compared to flu A cases, there are two strains of influenza A, H1N1 and H3N2, and one strain of influenza B used in the current flu vaccine. The H3N2 strain of influenza A used in the vaccine is a good match to the H3N2 strain causing the majority of flu illnesses in the United States. During the 2012/2013 flu season, there have been two illness-causing influenza B strains. One of those strains was well matched by the flu vaccine, but the other was not. Therefore, patients who were vaccinated with this season's flu vaccine are likely to be well protected, while those who happened to contract the other strain of flu B that was not part of the vaccine may not have a good protection. According to the Centers of Disease Control and Prevention, the flu vaccine has been about 61 % effective at protecting people from flu during the 2013–2014 mid-season for all age groups (CDC 2014). This number represents quite an impressive success rate for a vaccine. However, considering the emergence of new seasonal strains, the battle against flu should continue by changing the vaccine strain composition. In this regard, the development of a universal vaccine, capable of providing a broader spectrum of protection against the disease, would greatly benefit the community and is a task of highest priority.

Traditional inactivated or live attenuated vaccines are fairly effective in protecting the population against seasonal flu by targeting a viral Ag, hemagglutinin (HA). However, in case of a pandemic caused by a new strain of influenza with high mortality rate in humans, such as H5N1, it is difficult to produce adequate amounts

of an effective vaccine in a timely manner using the conventional egg-based production system because: (1) it takes at least 4 months to produce the first vaccine after the identification of a new potential strain (Emanuel and Wertheimer 2006); (2) H5N1 viruses are extremely lethal to personnel, requiring biosafety level 3 containment facilities for the vaccine production; and (3) H5N1 viruses do not replicate well in chicken embryos, resulting in low yield of the virus for vaccine development (Smith et al. 2008). In addition, the supply of eggs for vaccine production might be compromised during an H5N1 pandemic due to high mortality of the affected chickens. Overall, both inactivated and live attenuated H5N1 vaccines are only mildly immunogenic in humans, requiring high doses of Ag, multiple cycles of vaccination, and/or the use of adjuvants, further complicating the process (Adams and Sandrock 2010; Rockman and Brown 2010; Clayville 2011). Furthermore, the live attenuated influenza vaccine is only approved for healthy people of ages 2 through 49 and excludes high-risk populations. Generally, the licensed platforms for manufacturing of the existing seasonal influenza vaccines are not ideal for an H5N1 pandemic scenario as experienced in the 2009 H1N1 influenza pandemic (Haaheim et al. 2009). Therefore, it is urgent to explore alternative vaccine strategies for pandemic influenza, capable of preventing or controlling H5N1 infection in a timely manner. Numerous vaccine types with egg-independent production technologies, such as mammalian cell-based vaccines, recombinant protein-based vaccines, virus-like particle-based vaccines, DNA vaccines, bacterial-vectored vaccines, and viral-vectored vaccines, have been comprehensively studied as alternative options (Haaheim et al. 2009; Singh et al. 2010; Horimoto and Kawaoka 2009). Included in the list of possible alternative strategies are recombinant Ad vector H5N1 vaccines, which are promising candidates capable of inducing a rapid and long-term cross-protective immunity against frequently evolving H5N1 viruses (Tutykhina et al. 2011; Tang et al. 2009; Vemula and Mittal 2010; Toro and Tang 2009; Lambe 2012).

Comprehensive studies have shown that Ad vector-based vaccines induce humoral and cellular immune responses superior to those of recombinant protein vaccines, plasmid-based DNA vaccines, and other recombinant vector systems currently available (Abbink et al. 2007; Naslund et al. 2007; Barefoot et al. 2008). The natural tropism of Ad5 vectors for the respiratory tract makes them particularly desirable for intranasal (INL) vaccination against pathogens (e.g., influenza virus) that preferentially invade the mucosa. INL immunization with Ad-based vector vaccines through the use of a nasal spray could be advantageous since immunization becomes simple, practical, economical, and well suited for mass vaccination plans. In this context, Ad-based vectors are well suited for vaccine development against influenza that infects upper respiratory track. The effectiveness of Ad-based influenza vaccines has been extensively evaluated against various influenza virus subtypes with major focus on H5N1.

Immunization with Ad-based vaccines using different routes and dose regimens has been shown to have a significant effect on the type and strength of the induced immune responses (Holst et al. 2010; Kaufman et al. 2010; Suda et al. 2011; Steitz et al. 2010). For example, parenteral administration is associated with diminished

vaccine efficacy due to the presence of preexisting anti-Ad immunity. In marked contrast, vaccination via alternative routes was shown to overcome PEI against Ad5-based vectors (Van Kampen et al. 2005; Song et al. 2010; Holman et al. 2009; Appledorn et al. 2011; Xiang et al. 2003; Pandey et al. 2012). INL immunizations can overcome some effects of PEI against Ad5 vectors, inducing substantial immune response against encoded Ags and providing protection against challenge pathogens in mice, rabbits, and nonhuman primates (Xiang et al. 1996; Shi et al. 2001; Yu et al. 2008; Croyle et al. 2008; Xu et al. 2009; Richardson et al. 2011). INL and IM immunization with an Ad, expressing HA protein from A/Hong Kong/156/97 virus, has been shown to confer effective protection against lethal challenges with the heterologous (A/Hong Kong/483/97) and (A/Vietnam/1203/04) H5N1 influenza viruses in the absence of strong humoral neutralizing responses against A/Vietnam/1203/04 virus (Hoelscher et al. 2006). These studies thus demonstrate that Ad-vectored vaccines generate a robust cellular immunity against influenza that has the benefit of conferring broader protection against continuously evolving H5N1 viruses. The cellular immune response likewise plays an important role in virus clearance and promotes early recovery from infection (Thomas et al. 2006b; Lin et al. 2010).

An Ad vaccine encoding the influenza HA gene is able to stimulate cross-protective immunity between different subtypes of avian influenza virus (Wei et al. 2010; Toro et al. 2008; Shmarov et al. 2010). This suggests that Ad-based vaccination may induce secretion of Abs against conserved epitopes of the HA molecules from different strains and/or subtypes (Du et al. 2010), providing cross-protection against divergent influenza viruses. Mounting evidence suggests that Ad-based nasal influenza vaccines induce greater Ag-specific IgA and IgG responses in the respiratory tract. These vaccines could also provide more virus-specific activated T cells in the lung and better protection than IM-administered Ad vaccines (Shmarov et al. 2010; Park et al. 2009; Price et al. 2009). This is significant because mucosal immunity can potentially provide cross-protection against different strains of influenza (Hasegawa et al. 2009; Tamura et al. 2005; Ichinohe et al. 2007; Perrone et al. 2009; Lau et al. 2012; Gustin et al. 2011). More importantly, immune responses have been found in human clinical trials where human subjects could be safely and effectively immunized with Ad-vectored nasal influenza vaccines in the presence of preexisting anti-Ad5 immunity (Van Kampen et al. (2005) and data from a recent and yet unpublished Ad H5N1 nasal vaccine phase I clinical study involving 48 patients).

The HA glycoprotein is the primary flu vaccine target that stimulates higher levels of HA inhibition and neutralizing Ab (NAb) titers and stronger cellular immune responses and confers better protection against homologous or heterologous H5N1 virus challenge compared to neuraminidase-, nucleoprotein-, and matrix protein 1/2-based flu vaccines (Rao et al. 2010; Patel et al. 2009; Chen et al. 2009b; Nayak et al. 2010). Nevertheless, a monovalent HA Ad-based flu vaccine may not provide adequate protection against a broad range of heterologous strains of H5N1 influenza viruses that are currently classified into more than ten antigenically unique clades on the basis of phylogenetic analysis of their HA genes

(Continued evolution of highly pathogenic avian influenza A (H5N1): updated nomenclature 2012).

Several strategies have been undertaken to broaden the protection against pandemic flu strains, potentially pathogenic to both animals and humans, and combat the disease (Zhang 2012). These strategies include: (1) co-immunization with multivalent Ad vectors expressing HA glycoproteins or other Ags derived from different clades, (2) generation of Ad vectors expressing HA protein with NA protein and/or other highly conserved influenza Ags, (3) the use of rare Ad serotypes, and (4) different combinations of prime–boost vaccination with Ad-based H5N1 vaccines. The above strategies are not mutually exclusive and have been tested in animals to evaluate the efficacy of Ad-based vaccines against H5N1 isolates from various clades.

There has been a significant progress during the past decade in the field of Ad vector H5N1 flu vaccines. However, these vaccines must circumvent several challenges before they can be considered a suitable alternative to current licensed vaccines. One major drawback that candidate vaccines must overcome before their licensure is the lack of information related to correlation between the vaccine-induced immunity and their disease protection efficacy (Madore et al. 2010). In addition, the standardization of immune assays used in the assessment of innate and adaptive immune response is essential for the comparative analysis of such vaccines. Recently, Ad5 PEI has been thought to diminish vaccine efficacy. Emerging data from clinical trials suggest that this limitation can be overcome by increasing the vaccine dose (Catanzaro et al. 2006) or by using INL route of vaccination (Van Kampen et al. 2005). However, more clinical data is needed to clarify the influence of PEI on the Ad-vectored vaccines.

8.4.2 Prophylactic Vaccines Against Human Immunodeficiency Virus

Since the beginning of the HIV epidemic, nearly 70 million people have been infected with the virus, and approximately 35 million have died of AIDS. The estimates are that 34.0 million people were living with HIV at the end of 2011. After decades of efforts, we have seen dramatic progress in treating AIDS, but the disease has still not been eradicated (Li et al. 2013; Duerr et al. 2006). Current treatment options involve combinations (or "cocktails") of at least three different kinds of antiretroviral agents; however, the results are far from being satisfactory. Therefore, there is an urgent need to develop effective prevention and treatment options for AIDS. In this context, development of effective vaccines has been the main focus of the governments, academic institutions, and the industry worldwide.

There has been a rapid increase in preventive candidate vaccines against HIV utilizing various strategies, including DNA, protein, and viral vector-based vaccines (Gamble and Matthews 2010). Initial efforts were focused on the

development of traditional (first-generation) vaccines using inactivated or attenuated HIV virions. However, the advancements in molecular engineering and gene-based vector development have led to the emergence of viral vector-based vaccines, eliciting robust immune responses against HIV (Casimiro et al. 2003). The generation of humoral as well as cellular immunity, particularly involving CTL with long-lasting memory, is the desired features of HIV vaccines. The role and the importance of NAbs as well as CTL responses in preventing and controlling HIV infection have already been demonstrated (Sha et al. 2004). Among all viral-vectored vaccine candidates (Table 8.1), replication-defective Ad5 is the most widely used due to its proven efficacy in producing high titers of Ag-specific Abs and strong CTL responses, as well as high safety of intramuscular (IM) or subcutaneous (SC) administration (McElrath and Haynes 2010; McElrath et al. 2008).

There have been several clinical trials using Ad5 vector-based vaccines against HIV. A multiclade vaccine (MRKAd-5 HIV-1) containing clade B gag/pol/nef genes was tested in both phase I (Merck 16) (Priddy et al. 2008) and phase IIb (HVTN 502/Step) clinical trials (Buchbinder et al. 2008). In the phase I trial, the MRKAd5 trivalent vaccine was generally well tolerated and induced cell-mediated immune responses against HIV-1 peptides in most healthy adults. Another multiclade and multivalent recombinant Ad5-vaccine, VRC-HIVAD014-00VP, which contained a clade B gag-pol gene insert as well as the envelope gene inserts from three major HIV clades (A, B, and C), was tested in a phase I clinical trial (HVTN 054) (Peiperl et al. 2010). The VRC-HIVAD014-00VP vaccine was determined to be safe and highly immunogenic following a single-dose immunization in human volunteers without preexisting Ad NAbs.

While the Merck vaccine was highly immunogenic, it induced only narrow responses generating a median ≤ 1 T-cell response per participant (McElrath et al. 2008). The HVTN 502/Step phase IIb trial was terminated after an interim analysis showing that the tested vaccine neither reduced the rate of HIV-1 incidence nor plasma viremia after infection (McElrath et al. 2008; Buchbinder et al. 2008). Extensive work has been performed to elucidate potential causes of vaccine failure. Although preliminary results suggested a reverse correlation between the natural Ad5 Ab titers and the vaccine efficacy, subsequent analyses failed to support such a correlation (Hutnick et al. 2009; O'Brien et al. 2009). The results of the abovementioned vaccine trials (using two vaccines) have been extensively evaluated to determine if (1) vaccine-induced immune responses are arbitrarily distributed across vaccine inserts or clustered into immunodominant epitope hotspots; (2) the immunodominance patterns, observed in these trials, differed from each other; (3) vaccination-induced epitope hotspots overlap with those induced by a natural HIV-1 infection; (4) immunodominant hotspots correspond to the evolutionarily conserved regions of the HIV genome; and (5) epitope prediction methods can be used to identify these hotspots. It was concluded that the observed immune responses clustered into the epitope hotspots in all three vaccine trials, while some of these hotspots were not the same as in natural chronic infection. There were significant differences between the immunodominance patterns revealed in each trial. Furthermore, in some trials such differences were observed even between

different groups of participants receiving the same vaccine. Some of the vaccine-induced immunodominant hotspots were found in highly variable regions of the HIV genome. The latter was most evident for the MRKAd-5 HIV vaccine. Finally, epitope prediction methods can partially estimate the region of vaccine-induced epitope hotspots. These findings have potential implications for vaccine design and suggest a framework by which different vaccine candidates can be compared in early phases of evaluation (Hertz et al. 2013). Additional post-trial data analyses would be informative for the design of effective vaccines, in general, and Ad-based HIV vaccines, in particular.

In spite of tremendous progress, the design of effective Ad-based vaccines still has major drawbacks. In particular, PEI to Ad vectors facilitates induction of strong Ad-specific Ab and CTL responses following IM or SC vaccine administration, which result in diminished expression of transgene-encoded Ags and compromised Ag immunity. To circumvent the effects of PEI, various techniques have been employed individually or collectively. For instance, a prime–boost heterologous vaccination approach involving priming with DNA vaccine and boosting with Ad-vectored vaccine has shown a great promise (Nabel 2002; Lo et al. 2008; Park et al. 2003; Qiu and Xu 2008). The superior efficacy of heterologous prime–boost strategy over homologous prime–boost approach in eliciting immunity against a variety of pathogens and tumors has been established (Park et al. 2003; Kim et al. 2007; Schneider et al. 1998) and demonstrates the ability of heterologous prime–boost regimen to induce $CD4^+$ Th1 responses (Park et al. 2003).

8.4.3 *Adenovirus-Based Vaccines Against Potential Bioterrorism Agents*

A number of highly contagious infectious diseases with limited or no preventive or therapeutic treatments can be potentially used as weapons for bioterrorism (Boyer et al. 2005). A vaccine (AdsechPA) based on *E1*/*E3*-deleted human Ad vector has been developed to encode *B. anthracis's* protective Ag (PA) for inducing host immune defenses against the corresponding pathogen. AdsechPA was assessed for its ability to evoke anti-PA immune responses to protect mice against lethal dose of the *B. anthracis* toxin by comparison with a vaccine based on purified PA protein combined with a commonly used adjuvant Alhydrogel (rPA/Alhydrogel). AdsechPA vaccine developed protective immune response in immunized mice faster than the rPA/Alhydrogel vaccine. By 11 days, 27 % of the AdsechPA-immunized mice demonstrated protective immune responses, whereas no rPA/Alhydrogel-immunized mice survived the challenge. This study was able to demonstrate that an Ad vector encoding a secreted PA can elicit a rapid, robust protective immune response to lethal toxin, the primary mediator of anthrax pathogenesis (Tan et al. 2003).

In 2006 the "Ag capsid incorporation" strategy (see Sect. 8.3.2.1) was utilized to construct anthrax-specific Ad vaccine vectors. Vectors with chimeric hexon incorporating *B. anthracis's* PA induced formation of PA-specific Abs in mice but failed to yield protection against the anthrax toxin (lethal factor) challenge (McConnell et al. 2006). In contrast to these findings, a prior study employing an "Ag capsid incorporation" strategy demonstrated protection against *P. aeruginosa* challenge (Worgall et al. 2005, 2007). Briefly, this study described incorporation of a neutralizing epitope from the *P. aeruginosa's* outer membrane protein F (OprF) into the HVR5 of the Ad5 hexon, which increased the Ab response, consisting of both IgG1 and IgG2a subtypes in BALB/c mice (Worgall et al. 2005). Furthermore, the mice immunized with the virus containing the OprF epitope on the capsid achieved 60–80 % survival rate upon pulmonary challenge with *P. aeruginosa*.

An Ad-based vaccine has also been constructed to protect against plague-causing bacteria *Y. pestis*. One critical requirement for a vaccine against the plague is its ability to prevent the *Y. pestis's* effector proteins from entering macrophages. Because "V" Ag of the bacteria plays a key role in this process, an Ad-based vaccine vector (AdsecV) was designed to encode the Ag as a fusion with a signal sequence for its extracellular secretion. High anti-V IgG titers were induced in immunized mice 2 weeks following a single IM administration of the vaccine and continued to rise through 4 weeks post-immunization. A single IM dose of AdsecV protected mice from a lethal INL challenge with *Y. pestis*, whereas no mice were immunized with the control vector (Chiuchiolo et al. 2004). Recently the "Ag capsid incorporation" strategy was utilized to construct vaccine vectors for protection against *Y. pestis* infections. Ad vectors displaying *Y. pestis*'s V Ag or F1 capsular Ag on the capsid surface elicited a higher V- or F1-specific response, allowing boosting and better protection against a lethal challenge than that produced by vaccination with V or F1 proteins and conventional adjuvants (Boyer et al. 2010).

8.4.4 Therapeutic Vaccines Against Human Immunodeficiency Virus

To date, there has been one semi-successful HIV prophylactic trial, (RV144). The tested vaccine was comprised of four doses of recombinant canary pox priming immunogen, ALVAC-HIV (vCP1521), and two doses of AIDSVAX B/E, recombinant HIV-1 gp120 proteins from HIV-1 subtype B and circulating recombinant form 01_AE (CREF01_AE). The RV144 HIV-1 trial was the first to demonstrate evidence of protection against HIV-1 infection with an estimated vaccine efficacy of 31.2 % (Rerks-Ngarm et al. 2009). In the absence of a vaccine that can prevent HIV-1 infection, there are still many benefits to be realized from generation of a therapeutic vaccine. A therapeutic vaccine would be beneficial if it were able to increase the viral threshold titer necessary for infection, increase the time to clinical

manifestation of virus, control viral load after infection, and reduce the chance of secondary transmission (McMichael 2006; Emini and Koff 2004; Thorner and Barouch 2007; Robinson and Amara 2005; Sekaly 2008; Gamble and Matthews 2010). A therapeutic HIV vaccine could induce this type of response and would invariably decrease contagiousness, the need for costly and potentially dangerous antiretroviral treatments, and the number of opportunistic infections in patients. While the effect of controlling the normal HIV-1 pathology with therapeutic vaccines would be favorable for individual patients as well as society at large, the effect of preventing HIV-1 infections in humans with a prophylactic vaccine is also broadly appealing and of the utmost importance (Beena et al. 2013).

Therapeutic HIV vaccines are designed specifically for HIV-positive individuals, who have an uncompromised immune system that is capable of generating effective anti-HIV immune responses. Therefore, clinical trials for therapeutic vaccines are recruiting volunteers with $CD4^+$ T-cell counts greater than 250 cells/mm^3, and most studies require a $CD4^+$ T-cell count greater than 350 cells/mm^3. Patients with compromised immune system may be unable to produce a good immune response to a therapeutic HIV vaccine and are therefore not eligible for these trials. Furthermore, most of the trials require that therapeutic vaccine recipients continue taking antiretroviral drugs during the study.

While multidrug therapy has improved the prognosis for subjects infected by the virus, it has not eliminated the infection. Immunological therapies, including therapeutic vaccines, are needed to complement drug therapy in the search for a "functional cure" for HIV. DermaVir (Genetic Immunity Kit, Budapest, Hungary and McLean, Virginia, USA), an experimental HIV/AIDS therapeutic vaccine, combines three key elements of a rational therapeutic vaccine design: a single pDNA immunogen expressing 15 HIV Ags, a synthetic pDNA nanomedicine formulation, and a DC-targeting topical vaccine administration. DermaVir alone or in combination with antiretroviral drugs was evaluated in chronically SIV-infected macaques. DermaVir provided virological, immunological, and clinical benefit for SIV-infected macaques during chronic HIV infection and AIDS. In combination with antiretroviral drugs, DermaVir augmented SIV-specific T-cell responses and enhanced control of viral load rebound during treatment delays. The data also indicated the feasibility of therapeutic immunization even in immune-compromised hosts and suggested that DermaVir can supplement antiretroviral drugs to sustain suppression of HIV-1 replication (Lisziewicz et al. 2005). DermaVir's novel mechanism of action involves vaccine transportation by epidermal Langerhans cells to the lymph nodes to express the pDNA-encoded HIV Ags and induce precursor/memory T cells with high proliferation capacity. This effect has been consistently demonstrated in mouse, rabbit, primate, and human subjects. Safety, immunogenicity, and preliminary efficacy of DermaVir have been clinically observed in HIV-infected human subjects. The DermaVir technology platform for DC-based therapeutic vaccination might also offer an innovative treatment paradigm for cancer and infectious diseases (Lori 2011).

8.5 Adenovirus-Based Prophylactic and Therapeutic Vaccines for Cancer

8.5.1 Adenoviral Vectors in Cancer Therapy

Antitumor potential of human Ad was discovered soon after its isolation in 1953 (Rowe et al. 1953) by observation of tumor regression in clinical cases of cervical carcinoma following Ad inoculation (Huebner et al. 1956). However, Ad emerged as a potential therapeutic agent for cancer only decades later, after the groundbreaking developments in recombinant DNA technology. Several clinical studies using Ad vectors for cancer therapy have been conducted in the past decade. Some of those led to the clinical development of Ad vector-based products for treatment of various cancers, such as Advexin® and Gendicine® encoding wt tumor suppressor p53 under control of cytomegalovirus or Rous Sarcoma Virus promoters, respectively (Raty et al. 2008). Remarkably, in 2003 Gendicine® was approved in China as the first gene therapy medicine in the world (Toth et al. 2010). Onyx-015, an Ad2/Ad5 hybrid oncolytic virus replicating selectively in p53-defective cancers, became the first engineered replication-selective virus to be used in humans (Liu et al. 2008b). In 2005 a similar virus (H101) in combination with chemotherapy was approved by the Chinese State Food and Drug Administration for the treatment of refractory nasopharyngeal (head and neck) cancer (Liu and Kirn 2008).

The current use of human Ad as a vector for cancer therapy applications can be divided into six major categories: (1) suicide gene therapy also known as gene-directed enzyme prodrug therapy (GDEPT), (2) suppressor replacement gene therapy, (3) RNA interference (RNAi)-based gene therapy, (4) anti-angiogenic therapy, (5) oncolytic virotherapy, and (6) cancer immunotherapy. Briefly, the GDEPT approach utilizes the concept of foreign (bacterial or viral) enzyme-mediated intracellular conversion of nontoxic compounds (prodrugs), compatible with systemic administration, into cytotoxic metabolites capable of blocking DNA replication or transcription/protein synthesis in target (cancer) cells upon vector-mediated delivery of the foreign enzyme-encoding transgenes ("suicide" genes). Due to inability of cellular enzymes to use such prodrugs as substrates, the cytotoxic effect is achieved specifically in target (cancer) cells expressing the delivered therapeutic transgenes. Suppressor replacement and RNAi-based therapies represent variations of the gene therapy approach aimed at replacing mutated/inactivated tumor suppressors or disrupting tumor-promoting factors, respectively. In the former instance, a tumor suppressor gene p53 mutated in many cancer cell types is replaced by its functional (wt) counterpart, whereas in the latter instance disruption of mRNA encoding a tumor-promoting factor (hTERT, HER2, etc.) by RNAi, delivered in the context of an Ad vector, is aimed at inhibiting tumor progression. Similarly, the anti-angiogenic therapy utilizes a gene therapy approach for systemic or intratumoral delivery and expression of anti-angiogenic factors, inhibiting tumor vasculature formation and thereby growth of primary tumors and metastases (Sharma et al. 2009).

In contrast to the above gene delivery-based strategies, the oncolytic virotherapy approach utilizes natural ability of replication-competent Ad for lytic destruction of infected cells, whereby intracellularly produced viral progeny gets released from the infected cells and spread around a tumor mass in consecutive rounds of Ad replication cycle. This is achieved by cancer targeting of Ad replication process. Cancer selectivity of Ad replication is rendered by two major genetic strategies of blocking Ad DNA replication in normal (non-cancer), but not in malignant cells. Those involve engineering mutations (deletions) in DNA replication-controlling immediate early (*E1*) Ad genes to inactivate their crucial biological function of inducing proliferation of infected cells (see also Sect. 8.3.2) (Bischoff et al. 1996; Heise et al. 2000; Fueyo et al. 2000) or replacing the native promoter of *E1* genes with tumor-specific promoters (TSPs) selectively activated in cancer cells (Ko et al. 2005).

The ultimate goal of cancer immunotherapy is to elicit endogenous immune responses against developing or well-established tumors. The ineffectiveness of antitumor immune responses, caused by the establishment of immunosuppression in the tumor microenvironment during oncogenesis, indicates that the immune system needs to be modulated in a very specific way to effectively suppress tumor formation. Immunotherapy relies on preformed effector mechanisms and is well suited for the treatment of established tumors. In this context, cancer vaccines based on whole tumor cells, DCs pulsed with tumor-associated Ags (TAAs), or TAA-based subunit vaccines with immune adjuvants have recently emerged as important modalities to combat cancer. In summary, viral vector-based vaccines represent an important treatment modality owing to their high practical value, versatility in Ag delivery, robust natural adjuvanticity, and intrinsic capability for oncolysis.

8.5.2 Adenovirus-Based Cancer Vaccines

Increased understanding of the immune system and developments in recombinant DNA technology led to the emergence of innovative vaccine strategies and the notion that cancer vaccines could become a common treatment modality in the next decade. The recent licensure of DC-based vaccine Provenge® for the treatment of prostate cancer in humans along with the approval of Oncept™ as therapy for oral melanoma in dogs lends support to the clinical promise of cancer vaccines (Aurisicchio and Ciliberto 2011). Ad-based cancer vaccines represent an important and rapidly developing branch of the cancer vaccinology field. Ad vectors emerged as promising gene therapy vectors and recombinant vaccine carriers owing to their well-characterized molecular genetics, high yield propagation capacity amenable to pharmaceutical scale production, and biological characteristics (Choi and Yun 2013). Many studies have shown that Ad-based vaccines are more efficient in generating antitumor immunity than vaccines based on other delivery systems (Basak et al. 2000; Okur et al. 2011). However, PEI to human Ad2 and 5 and

their natural liver tropism substantially compromised the utility of these viruses for vaccine development. These hurdles justify implementation of various strategies for "shielding" of hAd vectors from PEI, including utilization of alternate Ad serotypes, xenotypes, or chimeric Ad vectors (Hedley et al. 2006b) described in previous Sects. 8.3.2.1 and 8.3.2.2). The "shielding" approach involves hAd capsid genetic and chemical modification strategies as well as the use of hAd vectors in conjunction with cell-based therapies (ex vivo pre-loaded DCs). Administration of hAds through mucosal surfaces, preventing rapid vector clearance by immune system, represents a powerful alternative to the vector "shielding" approach.

To bypass the problem of PEI to hAd2/5 in human populations, alternative serotypes from humans or nonhuman primates have been employed for vaccine development. Human Ad35 has been proposed as an alternative to hAd5 for vaccine delivery because of its low seroprevalence. However, hAd35-based vectors demonstrated lower immunological potency as compared to hAd5 vectors in mice and nonhuman primates. Similar results were obtained for hAd11, hAd24, and hAd34 (Barouch et al. 2004; Lemckert et al. 2005). Since PEI in humans does not cross-neutralize ovine, chimpanzee, canine, porcine, or fowl Ads (Aurisicchio and Ciliberto 2011), nonhuman Ad species, capable of efficiently transducing some types of human and murine cells in culture, gained attention as potential vectors for cancer vaccine development. Most importantly, cancer vaccines based on chimpanzee Ads, such as ChAd3, have been shown to induce immune responses comparable to hAd5 serotype-based vectors, break tolerance to self-tumor Ags, overcome hAd5 PEI, and achieve antitumor effects (Peruzzi et al. 2009).

8.5.2.1 Prophylactic Adenoviral Cancer Vaccines

Prophylactic cancer vaccines utilize immunomodulatory mechanisms, leading to the development of adaptive immune responses against TAAs. Most important of those is the generation of TAA-specific $CD8^+$ T memory cells, which undergo rapid activation and acquisition of effector function should cancer cells expressing such TAAs emerge in the host. In addition, prophylactic cancer vaccines can elicit humoral immune responses characterized by production of TAA-specific Abs. The latter may play some role in the containment of primary tumor development as well as in preventing recurrence of previously eradicated tumors. However, a pivotal role in vaccine-based cancer prevention belongs to TAA-specific CTL-based cellular immunity (Finn and Forni 2002).

Adenoviral Vaccines Targeting Tumor-Associated "Self" Antigens

Many tumor Ags are simply normal, non-mutated tissue differentiation Ags, that are seen as "self" by the host immune system. Immunization with these "self" Ags could induce autoimmunity. Tumor prevention by stimulation of TAA-specific immunity and breaking the immunologic tolerance has been evidenced in numerous

transgenic mouse models overexpressing heterologous genes for TAAs, such as rat HER2/neu (Hutchinson and Muller 2000), human CEA (Kass et al. 1999; Mizobata et al. 2000), and human carcinoma-associated mucin (MUC1) (Taylor-Papadimitriou and Finn 1997; Rowse et al. 1998). The deliberate induction of self-reactivity using a recombinant viral vector has been shown to result in tumor destruction, and $CD4^+$ T lymphocytes were found to be an integral part of this process (Overwijk et al. 1999). Thus, vaccine strategies targeting tissue differentiation ("self") Ags may be valuable in the prevention/treatment of cancers arising from differentiated cells and tissues/organs, such as melanocytes, prostate, testis, breast, and ovary.

Studies in animal models have demonstrated that cancer vaccines are most effective in protection from tumor challenge or in prevention of tumor occurrence in genetically predisposed animals. In contrast, cancer vaccines have shown limited therapeutic efficacy against established tumors in animal models, which reflected their failure to demonstrate objective antitumor responses in most human clinical trials (Finn and Forni 2002). The superior effectiveness of cancer prevention versus therapy is not surprising, given the condition of the immune system prior to vaccination: neither impaired by tumor- and chemotherapy-induced suppression nor tolerant to TAAs in the established tumor environment.

A novel recombinant vector based on rAd40 encoding mouse mesothelin (Msln) has been described as an effective prophylactic cancer vaccine against the formation of metastatic lesions of pancreatic cancer in the corresponding mouse tumor model. Intravenous (IV) administration of rAd40 resulted in Msln delivery and expression in wider range of mouse organs as compared to conventional Ad5, distributed mainly to the liver, spleen, and lungs. Besides, rAd40 showed reduced levels of liver transduction or inflammatory responses, resulting in reduced liver toxicity relative to Ad5. Msln vaccination has been reported to enhance antitumor effects against Msln-expressing tumors via Msln peptide-specific $CD8^+$ T-cell-mediated immunity (Hung et al. 2007; Miyazawa et al. 2011). In line with this finding, a one-time IV administration of rAd40-Msln not only prevented growth of the primary tumors in the Ag-specific manner but also blocked metastases formation, indicating that rAd40-Msln vaccination is a promising approach to stimulate both transgene- and tumor-specific immunity (Yamasaki et al. 2013).

Guanylyl cyclase C (GUCY2C) is an autoantigen principally expressed by intestinal epithelium and universally by primary and metastatic colorectal tumors. Immunization with Ad expressing the structurally unique GUCY2C extracellular domain [GUCY2C(ECD)] produced prophylactic and therapeutic protection against GUCY2C-expressing colon cancer metastases in mice without collateral autoimmunity (Snook et al. 2007, 2008). The mechanism of this protection involves lineage-specific induction of Ag-targeted $CD8^+$ T cells, without $CD4^+$ T cells or B cells (Snook et al. 2012).

Recombinant Ad vaccine expressing a kinase-inactive mutant form of human HER2 used for immunization of BALB/c wild type (WT) or HER2 transgenic mice protected WT mice from the HER2-expressing mouse carcinoma D2F2/E2. Half of the HER2 transgenic mice were protected fully and long-term after preventive

one-time IP vaccination, whereas tumor growth in mice that eventually developed neoplastic lesions was delayed. Protection in WT and HER2 transgenic mice was associated with high or low levels of IgG2a antibodies, respectively. This study also evidenced that CTLs were induced in WT but not in HER2 transgenic mice and defined a critical requirement for NK cells in vaccine-induced protection against HER2-expressing tumors (Triulzi et al. 2010). A similar study demonstrated that vaccination with Ad vector Ad-HER2-ki encoding a full-length HER2, mutation-inactivated (non-oncogenic) for kinase function, resulted in robust polyclonal immune responses to HER2 in tolerant animal models and translated into strong and effective antitumor responses in vivo (Hartman et al. 2010).

Ad vector encoding human mucin (MUC1), a tumor antigen expressed in breast, pancreatic, and ovarian cancers, has been developed for transduction of DCs, which specifically stimulated autologous peripheral blood lymphocytes upon vector-mediated gene transfer, substantially improved by Ad relative to liposome transfection (Pecher et al. 2001). Ad vaccines encoding other TAAs are mentioned below (see Sect. 8.5.2.2).

Vaccines Against Virally Induced Cancers

The success in vaccine development against infectious diseases has been recently translated to prophylactic vaccines against virally induced neoplasms. The US Food and Drug Administration (FDA) has approved a preventive vaccine against HBV, causing 80 % of liver cancers, and two prophylactic vaccines against HPV: Gardasil® and Cervarix®, protecting against infection by the two types of HPV (type 16 and 18) closely associated with cervical and some head and neck cancers. While both HPV vaccines were derived from VLPs, composed of a single HPV protein, the alternate genetic vaccine approaches utilizing hAd vector for the delivery and expression of HPV or HBV Ags have been recently endeavored as well. Some of those studies are briefly described below followed by other examples of most recent vaccine strategies for cancer prevention.

Cervical cancer, whose causal association with genital HPV infection has been firmly established (Steller 2002; Walboomers et al. 1999), remains a leading cause of cancer-related mortality in women. The E6 and E7 genes of high-risk HPV types (HPV-16 and -18) encode oncoproteins capable of immortalizing human keratinocytes (Pecoraro et al. 1989) by altering cell growth regulation through inactivation of p53 and Rb tumor suppressors, respectively (Munger et al. 1992; Dyson et al. 1989). Oncoproteins E6 and E7, selectively retained and expressed in cervical tumors, are attractive targets for development of cancer vaccine for prevention of cervical cancer.

A recombinant Ad vector encoding mutant HPV-16 E6/E7 cassette has been described by Wieking and colleagues (Wieking et al. 2012) as a promising vaccine against HPV-positive head and neck cancers. Those engineered mutations render full-length forms of both E6 and E7 (E6Δ/E7Δ) non-oncogenic while preserving their antigenicity. At all dosages, mice inoculated with the Ad5 E6Δ/E7Δvector

completely cleared E6/E7-expressing HPV-positive head and neck squamous cell carcinoma tumors implanted 2 weeks after either intratracheal or submucosal Ad vaccine inoculation, with significant E6/E7-specific IFN-γ production. This result suggested that immunization with HPV-16 E6/E7 oncoproteins can be an effective method of protecting a host from E6/E7-expressing head and neck squamous cell carcinoma (Lee et al. 2008; Wieking et al. 2012). A non-oncogenic HPV-16 E6/E7 vaccine enhances treatment of HPV expressing tumors.

Calreticulin (CRT), an abundant 46 kDa Ca^{2+}-binding protein localized to the endoplasmic reticulum (ER), is considered to be related to the HSP family (Nash et al. 1994). The protein has been shown to aid in Ag presentation and can be complexed with peptides in vitro to elicit peptide-specific $CD8^+$ T-cell responses (Basu and Srivastava 1999). A replication-deficient Ad vector expressing a CRT/E7 fusion gene (Ad-CRT/E7) has been constructed to achieve higher efficiency of Ag delivery and improve Ag presentation of E7 protein through CRT. The ability of Ad-CRT/E7 to induce Ag-specific immunotherapeutic activity against E7-expressing tumors was explored in a mouse model. Vaccination with Ad-CRT/E7 induced stronger E7-specific immune responses (i.e., T-cell proliferation, IFN-γ production, and cytotoxicity) as compared to Ad vector expressing E7 alone. The Ad-CRT/E7 vector provided complete protection in mice challenged with E7-expressing TC-1 tumors and generated long-term immune memory that controlled recurrences. Most importantly, vaccination of tumor-bearing mice with Ad-CRT/E7 resulted in complete tumor regression in all tumor-bearing animals. Taken together, these results demonstrated that Ad-CRT/E7 is an effective vaccine to prevent E7-expressing cervical tumors and could potentially be clinically effective for the treatment of already established disease (Gomez-Gutierrez et al. 2007).

Antitumor immunity has also been reported with an Ad-based vaccine encoding the HPV E7 or ectodomain (ecd) of human epithelial self-Ag hMUC-1 genetically fused to 209-amino acid ecd of the CD40L. This vector also contained the human growth hormone signal sequence (sig) at the N-terminus of the ecdhMUC1-ecdCD40L fusion. MUC-1 is expressed from birth in normal epithelial cells but diffusely upregulated on epithelial surfaces in 90 % of cancers of the breast, ovary, colon, and lung. SC injection of the Ad-sig-E7/ecdCD40L vector into C57BL/6 mice generated a $CD8^+$ T-cell-dependent immune resistance to the growth of TC-1 tumors for up to 1 year. A robust growth suppression of hMUC-1-positive syngeneic tumors was also observed upon SC administration of the Ad-sig-ecdhMUC-1/ecdCD40L vector in a 100 % of hMUC-1-transgenic mice, initially immunologically unresponsive to the hMUC-1-positive syngeneic cancer cells. Thus, immunization with the sig-ecdhMUC-1/ecdCD40L fusion overcomes anergy to syngeneic tumors in this transgenic mouse model (Zhang et al. 2003).

Yet another protein fusion strategy was used for the enhancement of HPV E7 Ag immunogenicity. A fusion of the HPV16 E7 oncoprotein (either full or truncated) to the C-terminus of HBsAg was delivered in the context of non-replicative Ad5 vector using low immunization doses (10^6 infectious units per dose). The HBsAg/E7 fusion protein assembled efficiently into VLPs, which stimulated Ab responses against both the carrier and the foreign Ags and induced an E7-specific $CD8^+$

cytotoxic T-cell response. The Ab and the T-cell responses in this case were significantly stronger than those induced by a control Ad vector expressing wild-type E7 and were not affected by preexisting immunity against either HBsAg or Ad5. Mice vaccinated with Ad5 vectors encoding HBsAg/E7 developed Ab and CTL responses against both E7 and HBsAg. However, the Ab (IgG) titers were higher for E7 than for HBsAg (Baez-Astua et al. 2005).

In summary, Ad vectors show great potential as prophylactic cancer vaccines not only for immunization against virally induced neoplasms (HPV and HBV) but also against self-TAAs, due to the robust adjuvant properties of Ads and their ability to break immunologic tolerance to "self" Ags. However, the resulting induction of autoimmunity may present a substantial challenge to treatment of some patients. Besides, PEI to hAd2/5 in humans and efficient liver uptake can become major obstacles in hAd-based vaccination due to rapid clearance of the vector and possible toxicities. The above factors, however, can be circumvented by using vectors based on alternate Ad serotypes or nonhuman Ad species, implementation of genetic and/or chemical Ad shielding technologies, and utilization of alternate (mucosal) routes of vaccine delivery.

8.5.2.2 Therapeutic Cancer Vaccines

A broad variety of delivery vehicles, including hAd vectors, are currently being evaluated as therapeutic vaccines for treatment of established tumors. Although many of these approaches have failed to generate significant therapeutic benefits in clinical settings, the efficacy of therapeutic vaccines has been gradually improving, which in most cases is a result of expanding diversity of vaccination strategies and treatment combinations. Therapeutic anticancer vaccines have evolved from immunotherapy that can be used to prime–boost tumor-specific immune responses. Vaccines are typically used in combination with other cancer therapy strategies, such as immunomodulation, oncolytic virotherapy, DC-targeting/activation, and anti-angiogenesis to achieve a clinical response. This is because immune response to TAAs in the established tumor microenvironment is counterbalanced by various immune evasion mechanisms of which $CD4^+/CD25^+/FoxP3^+$ T regulatory cells (Tregs) play a central role. Tregs are critical for tolerance to self-Ags and play a major role in determining the effectiveness of anticancer vaccines. Therefore, strategies that target or overcome various immune evasion mechanisms may work in synergy with therapeutic vaccines for an effective clinical response (Yaddanapudi et al. 2013). For example, recombinant antibodies (αCTLA-4, αCD137, αCD3), cyto/chemokines (IL-15, LIGHT, mda-7), or costimulatory ligands (CD80) delivered through Ad-mediated gene transfer to tumors cannot overcome the immune evasion mechanisms (resistance) in both breast and cervical cancer models, as none of the Ad vectors displayed any significant therapeutic effect. However, the combination of Ad.αCTLA4 vector with systemic depletion of Tregs, using anti-CD25 Ab (breast cancer model) or low-dose cyclophosphamide (cervical cancer model), resulted in a significant tumor growth delay in vivo mediated by NKT cells and $CD8^+$ T cells (Liu et al. 2011).

Recently, another advance in improving efficacy of therapeutic vaccines has been made by specifically targeting intracellularly expressed TAAs to exosomes taking advantage of the ability of the factor V like C1C2 domain of lactadherin to specifically address proteins to exosomes. Adenoviral vectors expressing the extracellular domains of CEA and HER2 coupled to the C1C2 domain demonstrated significant improvement in Ag-specific immune responses to each of these Ags in naïve and tolerant transgenic animal models and significantly enhanced therapeutic antitumor effects in a human $HER2^+$ transgenic tumor model in mice (Hartman et al. 2011), suggesting that exosomal targeting could improve future antitumor vaccination.

Cancer Vaccines Using Immunomodulatory Factors

Ad-based cancer vaccines often combine delivery and expression of TAAs along with various immunostimulatory molecules, such as IL-2, IL-6, IL-12, IL-18, GM-CSF, TNF-α, IFN-α, IFN-γ (Waldmann 2006; Windbichler et al. 2000; Porta et al. 2007; Robertson and Ritz 1996; Tanaka et al. 1997; Hwang et al. 2004; Wright et al. 1999; Santodonato et al. 2001; Odaka et al. 2002; Khorana et al. 2003), and various chemokines (Lapteva et al. 2009; Namkoong et al. 2014). Apart from cytokines and chemokines, certain membrane-bound receptors are also required for efficient activation of immune response. In particular, T cells following recognition of foreign Ags in context of major histocompatibility complex (MHC) molecules ("signal 1") require another signal ("signal 2") delivered by costimulatory receptors of the CD28 and the TNFR superfamilies for activation. These two signals are then accompanied by a third signal ("signal 3"), in the form of cytokines. T cells receiving all three signals undergo proliferation, acquisition of effector function, and establishment of long-term memory (Lanzavecchia and Sallusto 2005). The ligands for CD28, CD80 and CD86, and TNFRα, such as 4-1BBL, OX-40L, are expressed by APCs (Choi et al. 2006; Greenfield et al. 1998; Iwakami et al. 2001; Yoshida et al. 2003; Loskog et al. 2004). Tumors, particularly those of non-hematopoietic origin, do not express costimulatory ligands and as such exploit the lack of costimulation as an effective means of immune evasion (Allison and Krummel 1995). Therefore, the use of cancer vaccines in combination with the aforementioned immune modulators has the potential to augment effector immune responses while overcoming various tumor immune evasion mechanisms for a pronounced antitumor immune response with clinical benefits (Putzer et al. 1997; Choi et al. 2006; Habib-Agahi et al. 2007).

Oncolytic Adenovirus-Based Cancer Vaccines

Therapeutic cancer vaccines in conjunction with oncolytic viral vectors (see Sects. 8.3.2 and 8.5.1) represent another attractive cancer treatment modality. Conditionally replicating Ad (CRAd) vectors proved to be a powerful tool for

tumor regression not only due to their direct lytic effect on infected cancer cells but also to the immune responses against TAAs, released upon CRAd-mediated oncolysis and naturally presented to immune system by macrophages and DCs at the site of tumor destruction. Thus, replicative Ad vectors, especially oncolytic vectors "armed" with transgenes encoding immunomodulatory factors, play an important role as adjuvant components of therapeutic cancer vaccines. It should be noted, however, that expressing TAAs in the context of highly potent oncolytic Ads may not be always an optimal vaccination strategy because of potentially short duration of transgene expression in the CRAd-infected cells due to the rapidly developing cytopathic effect leading to oncolytic cell destruction. Furthermore, attempts have been made to augment the immunotherapeutic potential of oncolytic Ads by vector incorporation of heat shock proteins as transgenes. Since heat shock proteins act as chaperones facilitating protein folding and translocation, their overexpression in cancer cells augments adaptive antitumor T-cell immunity by stimulating the processing and presentation of TAAs to T cells via APCs (Wang et al. 2001, 2006).

Dendritic Cell-Based Cancer Vaccines

Ad-based vaccines can be used in conjunction with APCs, particularly with DCs. DCs are highly efficient "professional" APCs that process and load intracellularly expressed TAAs onto both MHC I and MHC II molecules, thus allowing appropriate and relatively persistent induction of Ag-specific $CD8^+$ as well as $CD4^+$ T-cell responses. Ad vectors have been extensively used to transduce and modify DCs, either for effective TAA delivery and presentation to effector T cells or for their activation, i.e., induction of DC-mediated Ag-specific immune responses. Upon activation DCs migrate to the draining lymph nodes (LNs) and simultaneously upregulate costimulatory molecules and cytokines to prime both T helpers (Th) and CTLs in paracortical areas of LNs (Fig. 8.4). DCs thus provide a link between innate and adaptive immunity. A number of studies have demonstrated that ex vivo transduction of DCs with Ad vectors encoding TAAs and their expression in DCs results in induction of antitumor immunity upon DC inoculation back in tumor-bearing animals (Xia et al. 2006). Immune activation of DCs can be induced by delivering immunostimulatory factors or by genetic modification of the Ad5 capsid fiber protein (Worgall et al. 2004), for instance, with CD40 ligand, which binds to CD40 receptor, highly expressed on DCs (Fig. 8.4) (Belousova et al. 2003). Besides, Ad5 vectors exhibit adjuvant effect on their own and are capable of inducing DC activation and maturation upon transduction (Geutskens et al. 2000; Kanagawa et al. 2008).

Another important advantage of using DC-based vaccines is their susceptibility for ex vivo treatment with Ad vectors, circumventing the problem of Ad5-associated PEI encountered in vivo in human clinical trials. However, this treatment approach is laborious and costly. For this reason, specific targeting of

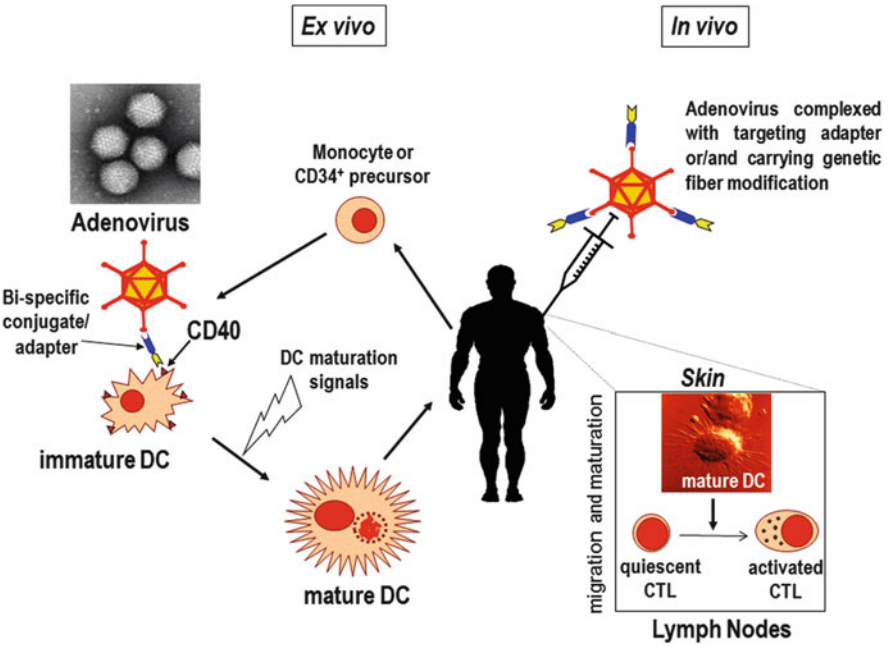

Fig. 8.4 Ex vivo and in vivo strategies for DC-based vaccines. Ad-based vaccines can be targeted to CD40 receptor expressed on DCs via an Ad5 fiber genetic modification or bi-specific conjugate/adapter molecules or combination thereof. In the ex vivo approach, patient-isolated DCs are transduced with Ad vaccines in a clinical laboratory settings and treated with maturation factors before infusing back into the patient. For in vivo vaccination, retargeted Ad-vectored vaccines with genetically modified fiber and/or complexed with adapter is injected intradermally into the patient to target DCs in their natural environment. This leads to antigen uptake by DCs and maturation and migration to lymph nodes where DCs activate T cells for the generation of CTL responses

DCs in vivo (mucosal tissues or skin) may serve a better treatment alternative (Fig. 8.4).

The major route of the receptor-dependent cell attachment of human Ads involves CAR, whose expression on immune cells, including DCs, is typically not detectable or actively downregulated. Various receptors expressed on the DC surface, such as CD40, TLR4, and DC-SIGN, can be utilized to achieve transductional retargeting of Ad vectors. Ad retargeting to these receptors would potentially allow effectively and specifically transduce DCs not only ex vivo but also in vivo (Geijtenbeek et al. 2000; Korokhov et al. 2005).

In order to infectivity-enhance/retarget Ad vectors for/to DCs, a number of fiber-modification strategies have been developed. These can be classified into genetic, nongenetic (bi-specific adapter based), and a new strategy combining the two. The genetic strategies (Table 8.2) introduce modifications in the Ad5 fiber protein C-terminal knob domain by: (1) incorporation of short peptide ligands (RGD4C, pK7), (2) knob-swap between different Ad serotypes (Ad5/3, Ad5/35 fiber

chimeras), or (3) whole knob replacement by artificial fusion molecules embodying the essential fiber trimerization motif and a receptor-binding motif (see also Table 8.2). This motif could be in the form of: (1) a targeting ligand (CD40L) (Belousova et al. 2003), (2) an Fc-binding domain of the *Staphylococcus aureus* protein A (Korokhov et al. 2003), (3) a single-chain Ab (scFv) (Hedley et al. 2006a), or (4) an affibody molecule with a given Ag-binding specificity (Belousova et al. 2008). Fiber incorporation of the Ad3 or Ad35 knobs or the CD40L has been shown to improve DC transduction efficiency, allowing selective delivery of Ags in vitro and in vivo using a human skin explant model (van de Ven et al. 2009; de Gruijl et al. 2006). The adapter-based strategies utilize engineered bi-specific fusion molecules bridging Ad capsid protein fiber and the targeted receptor on DCs (see Fig. 8.4). There are two types of engineered bivalent adapters for targeting CD40 receptor. The first type is represented by a chemically conjugated bi-specific Ab consisting of a Fab fragment of the Ad5 fiber knob-specific Ab "1D6.14" and anti-CD40 monoclonal Ab (mAb) "G28-5" (Tillman et al. 1999). The second type is a recombinant fusion between a soluble CAR (sCAR) and the CD40-specific, G28-5 mAb-derived single-chain Ab (scFv) (Pereboev et al. 2002; Hangalapura et al. 2012). Subsequently the latter adapter was improved by fusing CAR with CD40L via a trimerization motif-containing linker (Pereboev et al. 2004). The "combined" strategy utilizes a genetically modified fiber, fused to the *S. aureus* protein A Fc-binding domain (see above) in conjunction with a protein-fusion adapter, composed of Fc-binding domain portion of the human IgG1 fused to a CD40-specific scFv, derived from the G28-5 anti-CD40 Ab. This bi-specific adapter can bridge the fiber-modified Ad particles with the CD40 receptors on DCs (Korokhov et al. 2003). A similar strategy was used to target Ad5 to DCs via a DC-specific receptor DC-SIGN (Korokhov et al. 2005).

A very recent study assessed the ability of DC-based vaccines to induce a long-lasting tumor-specific CTL response in either prophylactic or therapeutic applications in spontaneous syngeneic murine tumor models (Ricupito et al. 2013). The authors found that priming with a DC-based vaccine induced a long-lasting CTL response in wt mice, while homologous boosting better sustained the pool of central memory T cells associated with potent protection against B16F1 melanoma challenge. Appropriate timing of booster vaccination was also critical. Conversely, prime–boost vaccination proved to be of no advantage or even detrimental in therapeutic settings in B16F1 and transgenic adenocarcinoma of the mouse prostate (TRAMP) models, respectively. These results indicate that booster vaccinations impact antitumor immunity to different extents, depending on their application (prophylactic or therapeutic), and suggest evaluating the need for boosting in any given cancer patient depending on the state of the disease (Ricupito et al. 2013).

The target genes transferred into the DCs by viral vectors, including Ads, fall into two categories: TAAs and immunomodulatory proteins, such as cytokines or costimulatory molecules. A variety of TAAs delivered to DCs via an Ad vector have been shown to boost immune responses to tumor cells (Gallo et al. 2005; Xia et al. 2006). Replication-deficient recombinant Ads, encoding human gp100 or MART-1 melanoma Ag, were used to transduce human DCs ex vivo in model

systems for cancer vaccine therapy. Vector-transduced DCs stimulated MART-$1_{(27-35)}$- or gp100-specific tumor-infiltrating T lymphocytes to produce IFN-γ and induced the peptides-specific, MHC class I-restricted CTLs within PBL from normal donors in vitro (Butterfield et al. 1998; Linette et al. 2000). Furthermore, a protective response to a lethal tumor challenge of unmodified murine B16 melanoma cells was observed upon vaccination of immunocompetent mice with bone marrow-derived murine DCs transduced with AdVMART-1 vector (Ribas et al. 2000). Genetic immunization using DCs transduced ex vivo with an hAd5 expressing the HER2/neu gene has also been demonstrated to induce $CD4^+$ and $CD8^+$ T-cell-based immunity against a breast tumor cell line overexpressing HER2/neu (Chen et al. 2001).

To augment the ability of DCs to present TAAs, a strategy of DC modification allowing them to constitutively express immunomodulatory proteins such as cytokines and chemokines has been developed. DCs genetically modified to express T-cell stimulatory factors/cytokines, such as GM-CSF, TNF-α, IL-12, SLC, lymphotactin, and CD40L, could possess adjuvant-like properties useful in the treatment of tumors as long as sources of TAA are available (Kirk and Mule 2000). A combined immunotherapy including gene therapy and DC vaccines would have some advantages over each modality administered as a monotherapy. Such combined cancer immunotherapy may include DC-based vaccines and Ad-mediated cytokine gene therapy with TNF-α or CD40 (Liu et al. 2004).

Combining an Ag-specific immunization with immunostimulation, using interleukins as vaccine adjuvants, is a widely used strategy in therapeutic vaccine development. A combined injection of mice with adenoviral vectors carrying either IL-12 or HPV-16 E7 oncogene (TAA) induced significant antitumor immunity. IL-12 is known to induce cellular immune responses, suppressing tumor growth and expression of E7. The utility of E7- (AdE7) and/or IL-12-encoding (AdIL-12) Ads for protection against TC-1 tumors was assessed using an animal tumor model. The protective effect of AdIL-12/AdE7 combination was significantly more profound than that of each vaccine alone and also resulted in regression of 9 mm-sized tumors in approximately 80 % of the experimental animals as compared to the "no vector" control group. Serum levels of E7-specific Ab and INF-γ production as well as T helper cell-proliferative responses were found to be significantly higher in mice coinfected with AdIL-12 and AdE7 than in experimental groups receiving injection of either AdIL-12 or AdE7 vaccines. CTL responses were only exhibited by the AdIL-12/AdE7 co-injected group, suggesting that the tumor suppression effect was mediated primarily by $CD8^+$ and, to a lesser extent, by $CD4^+$ T cells (Jin et al. 2005).

In another study, DCs from hepatocellular carcinoma patients were transduced ex vivo with Ad vector encoding α-fetoprotein (AFP) as TAA and human IL-2 genes and showed expression of both proteins. The DCs expressing AFP and IL-2 (AFP/IL-2-DCs) enhanced cytotoxicity of CTLs and significantly increased the production of IL-2 and IFN-γ compared with AFP-DC or plain (untransduced) DC controls. In vivo data from a mouse model suggested that immunization with AFP/IL-2-DCs enhances Ag-specific antitumor efficacy more potently than

immunization with IL-2-DCs or AFP-DCs individually. These findings provide a potential strategy to improve the efficacy of DC-based tumor vaccines (Yang et al. 2012).

A new cancer vaccine based on a truncated form of survivin (devoid of its anti-apoptosis function) as tumor Ag, combined with IL-2, was built and tested in DNA prime–rAd boost regimen for prophylaxis of melanoma cancer. While immunization with the DNA vaccine alone resulted in a weak immune response and modest antitumor effect, the tumor inhibition ratio upon DNA vaccine combination with IL-2 increased to 89 % and further increased to nearly 100 % by rAd–survivin boosting. Complete tumor rejection was observed in 5 out of 15 mice. Efficacy of the vaccine administered therapeutically was enhanced by nearly 300 % when combined with carboplatin. These results indicate that vaccination with a truncated survivin vaccine using DNA prime–rAd boost combined with IL-2 as adjuvant and carboplatin represents an attractive strategy for overcoming immune tolerance to tumors and holds potential therapeutic benefits for melanoma therapy (Zhang et al. 2012).

Vaccine Strategies Targeting Tumor Angiogenesis

Zhao and colleagues employed a conventional Ag immunization approach to induce angiogenic effect in tumors. These authors used a recombinant hAd encoding VE-cad, a transmembrane glycoprotein located at junctions between endothelial cells. VE-cad (VEc) is the most endothelial cell-specific cadherin that plays a critical role in regulating the process of normal and pathological angiogenesis (Vestweber 2008; Hendrix et al. 2001; Liao et al. 2002). To enhance Ag delivery to DC and macrophages, the recombinant AdVEc virus was coupled ex vivo with mannan under oxidizing conditions. Mannose receptors (MRs, CD206), a new family of multilectin receptor proteins, are the most ubiquitous receptors expressed on APCs, including DCs and macrophages. In addition to its role as a DC-targeting ligand, oxidized mannan coupled with Ad particles could act as an immunologic adjuvant, contributing to the ability of mature DCs to generate primary T-cell responses (Zhao et al. 2011). Vaccination with AdVEc–mannan complex (AdVEc-m) suppressed tumor angiogenesis and reduced density of tumor vasculature, resulting in both prophylactic and therapeutic effects on tumor growth and prolongation of mouse survival. Animals injected with AdVEc-m developed VE-cad-specific Ab as well as CTL responses. Depletion of $CD4^+$ or $CD8^+$ T lymphocytes in vivo impaired tumor suppressive activity of the AdVEc-m vaccine, suggesting important role of $CD8^+$ cytotoxic T cells in the tumor regression mechanism (Zhao et al. 2011).

Heterologous Prime–Boost Cancer Vaccination Strategies

A large number of Ad vectors encoding various TAAs (both tissue differentiation Ags and viral Ags/oncoproteins) have been developed to date for cancer vaccine

applications alone or in combination with adjuvants (immunomodulatory molecules) and/or other vaccine types (DNA, other viral vectors, proteins, DC vaccines) in a variety of heterologous prime–boost vaccination regimens. Examples of such TAA immunizations include: HER2/neu protein against breast cancer (Ren et al. 2012; Chen et al. 2011); Epstein–Barr virus (EBV) nuclear Ag-1 (EBNA1) (Smith et al. 2012) or LMP1/LMP2 (Chia et al. 2012) against nasopharyngeal carcinoma tightly associated with EBV infections; guanylyl cyclase C, a cancer mucosa Ag for prevention of primary and metastatic colorectal tumors (Snook et al. 2012); a prostate-specific Ag (PSA) (Lubaroff et al. 2009) or a combination (protein fusion) of PSA and prostate stem cell Ag for treatment of prostate cancer (Karan et al. 2011); a CEA for treatment of patients with advanced colorectal cancer (Mori et al. 2009; Morse et al. 2013) or its combination with HER2/neu (Diaz et al. 2013); a human telomerase reverse transcriptase (hTERT) (Liao et al. 2012; Chen et al. 2009a); and many others.

Therapeutic efficacy of homologous and heterologous prime–boost vaccine strategies against the 5T4 oncofetal antigen was evaluated by Ali and colleagues in an elegant study using 5T4 Ag-expressing replication-defective Ad (Ad5T4) and retrovirally transduced DC lines (DCh5T4) in a subcutaneous B16 melanoma model. All vaccine combinations tested could provide significant tumor growth delay. While DCh5T4 prime–Adh5T4 boost regimen was the best for tumor prophylaxis, it did not demonstrate any therapeutic efficacy in mice with established tumors. In contrast, Adh5T4 prime–DCh5T4 boost vaccination was the best regimen for tumor therapy. The authors concluded that prior immunization with Adh5T4 can condition the mice to induce 5T4-specific Th1 immune responses, which can be sustained and subsequently boosted with DCh5T4. In contrast, prior immunization with DCh5T4 augments Th2 immune responses (already induced by B16h5T4 tumor growth itself), such that a subsequent vaccination with Adh5T4 cannot elicit tumor regression, whose success is dependent on altering the polarizing immune responses from Th2 to Th1. Interestingly, depletion of CD25+ Tregs after tumor challenge, but before immunization, restored therapeutic efficacy (Ali et al. 2007).

A novel strategy of therapeutic immunization against prostate cancer using delivery of human prostate-specific TAA (hPSMA) to DCs by a CD40-targeted Ad vector was recently proposed by Williams and colleagues as an efficient means for DC activation and hPSMA presentation to T cells. A mouse model of prostate cancer was developed by the authors through generating clonal derivatives of the mouse RM-1 prostate cancer cell line expressing human PSMA. To maximize Ag presentation by DCs, expression of both MHC class I and TAP proteins was induced in RM-1 cells by transduction with another Ad vector encoding IFN-γ (Ad5-IFNγ). Administering hPSMA-expressing DCs as well as direct intraperitoneal injection of the Ad5-hPSMA vector resulted in high levels of tumor-specific CTL responses against RM-1-PSMA cells pretreated with Ad5-IFNγ. The CD40 targeting significantly improved the therapeutic antitumor efficacy of the PSMA-encoding Ad vector when combined with Ad5-IFNγ in the mouse model of human prostate cancer (RM-1-PSMA) (Williams et al. 2012). Some of those strategies have been evaluated in phase I/II clinical trials summarized in Table 8.3.

Table 8.3 Clinical trials with adenovirus-based cancer vaccines

Vaccine type/name; targeted antigen(s)	Vaccination regimen (prime–boost)	Trial phase (patients involved)	Immune response to vaccine	Clinical outcome	References
DCs transduced with Ad5 (Δ*E1*) vector expressing Gp100 and MART-1 melanoma Ags	Ex vivo transduction of autologous DCs with the Ad vector and patient infusion every 2–3 weeks	Phase I, 12 melanoma patients	Immunologic end points for this phase 1 trial are not available	Three patients developed leukoderma but experienced disease progression (no tumor eradication)	Tsao et al. (2002)
AdV-MART1/DC melanoma vaccine: DCs transduced with Ad5 (Δ*E1*) vector expressing MART-1 melanoma Ag	Ex vivo transduction of autologous DCs with the Ad vector followed by intradermal infusion	Phase I/II trial, 23 patients; 14 patients received all three scheduled DC vaccines	Activated both Th and CTL in vivo. Significant $CD8^+$ and/or $CD4^+$ MART-1-specific T-cell responses seen in 6/11 and 2/4 patients, respectively; $CD8^+$ and $CD4^+$ T-cell responses to additional Ags noted in two patients	No systemic grade II–IV toxicities. Worsening vitiligo in one patient. No hypersensitivity to 10^2–10^4 vp dose. This DC vaccine was safe and immunogenic	Butterfield et al. (2008)
Ad5(Δ*E1*) vector expressing PSA (Ad/PSA) for hormone-refractory metastatic prostate cancer	Single SC vaccine injection at one of three dose levels as an aqueous solution or suspended in Gelfoam matrix	Phase I trial, 32 patients with hormone-refractory metastatic prostate cancer	Anti-PSA T-cell responses in 68 % patients; PSA Ab produced in 34 % patients	55 % patients survived longer; decrease in anti-PSA Ab but increase in T-cell responses; no serious adverse events	Lubaroff et al. (2009)
INGN-225, a DC-based vaccine; transduced with wt p53-encoding Ad5 (Adp53)	Three doses of INGN-225 injected intradermally every 2 weeks	Phase I/II trial, 54 patients with SCLC	Specific anti-p53 immune response seen in 41.8 % with overall post INGN-225 response in 51.5 % patients; immune response data available in 29 patients (14 positive, 15 negative)	INGN-225 was well tolerated; toxicity grade ≤ 2, appears to sensitize SCLC for chemotherapy	Chiappori et al. (2010)

DCs transduced with Ad5/F35 (Ad35 fiber) encoding a truncated LMP1 (ΔLMP1) and full-length LMP2 (Ad-ΔLMP1-LMP2) Ags of EBV-positive NPC	Ex vivo transduction of DCs with the Ad vector with subsequent infusion into the patient; IL-2 immunomodulation	Phase II trial, 16 patients with metastatic NPC	Positive reactions to transduced DCs in 9 (75 %) out of 12 patients at the time of third vaccination; positive DTH reactions in 4/8 patients after fifth vaccination with smaller magnitude; 3/8 patients had positive reactions	No significant toxicity; three patients had clinical responses including one with partial response (for 7½ months) and two with stable disease (for 6½ and 7½ months	Chia et al. (2012)
T-cell-based vaccine (LMP1/EBNA1-specific T-cell lines) obtained by ex vivo transduction with Ad*E1*-LMPpoly vector encoding EBNA1 fused to multiple $CD8^+$ T-cell epitopes from EBV LMP1 and LMP2	Adoptive transfer with EBV-specific T cells using 3–8 infusions of 2.3×10^7 cells (median) in vitro; T-cell cultures with IL-2-supplemented medium	Phase I trial, 24 NPC patients with (16) or without/minimal (6) - EBV-specific T-cell expansion	Transient increase in the frequencies of LMP1- and 2- and EBNA1-specific T-cell responses after adoptive transfer to be associated with grade I flu-like symptoms and malaise; time to progression from 38 to 420 days	Adoptive immunotherapy with Ad*E1*-LMPpoly vaccine is safe and well tolerated and may offer clinical benefit to patients with NPC	Smith et al. (2012)
1. DNA vaccine (V930): HER2-ECDTM + CEA-LTB; 2. Bicistronic Ad vaccine (V932): Ad6 (Δ*E1*) HER2-ECDTM/CEA-LTB. (Targeting HER2 and CEA)	*Study 1*: V930 alone using electroporation or *Study 2*: in combination with V932. V930 prime–V932 boost	Phase 1, *study 1*: 28 stage II–IV patients with solid HER2 and/or CEA tumors; *Study 2*: 11 patients, 6 from study 1 and 5 new	No CMI augmentation to HER2/CEA, measurable Ab and CMI to LTB	Well tolerated without any serious adverse events. Prime–boost strategy did not augment any detectable CMI to HER2 or CEA	Diaz et al. (2013)

DCs dendritic cells, *CMI* cell-mediated immune response, *HER2-ECDTM* extracellular and transmembrane domains of human HER2, *CEA* carcinoembryonic antigen, *LTB* B subunit of *Escherichia coli* heat-labile toxin, *CEA-LTB* CEA fused to LTB, *IHC* immunohistochemistry, *CLL* chronic lymphocytic leukemia, *PFS* progression-free survival, *MART* melanoma antigen recognized by T cells, *DTH* delayed-type hypersensitivity, *SCLC* small cell lung cancer, *NPC* nasopharyngeal carcinoma, *SC* subcutaneous, *LMP* latent membrane proteins, *IL*-2 interleukin-2, *Ag* antigen

Finally, to assure high level of clinical efficacy, the field of cancer vaccine development has recently moved towards personalized, i.e., patient-specific approach. An example of such contemporary cancer vaccine-based therapy is the recently FDA-approved first ever vaccine for therapy of cancer called sipuleucel-T also known under commercial name of Provenge® (Dendreon, Inc.). Although formulation of this vaccine does not involve Ad-based gene delivery, it is important to mention here with regard to the underlying approach the clinical outcomes and historical significance of the treatment approval. This cell-based vaccine is created by isolating APCs from individual patient's peripheral blood mononuclear cells and culturing them with a fusion protein called PA2024, consisting of PAP prostatic acid phosphatase (PAP; an Ag that is expressed on the majority of prostate cancer cells but not on non-prostate tissues), linked to cytokine GM-CSF (PAP-GM-CSF). APCs cultured in the presence of PAP-GM-CSF display increased amounts of costimulatory molecules on their surface and constitute the active component of sipuleucel-T. These activated APCs are eventually infused back into the APC donor patient for treatment. In a multicenter, randomized, placebo-controlled clinical trial, sipuleucel-T increased the overall survival of metastatic castration-resistant prostate cancer patients by 4 months, however failed to induce tumor regression. Although the exact therapeutic mechanism remains elusive, the treatment appears to favor older patients. On the downside, the underlying personalized approach makes the treatment laborious, technically complex, and extremely expensive (Small et al. 2006; Gulley and Drake 2011).

While there are some obstacles that have been identified in the use of Ads as cancer vaccine carriers, extensive knowledge of Ad biology has enabled researchers to develop methods to effectively overcome these obstacles. As a result, Ad vectors have moved to the forefront of tumor vaccinology and are showing substantial promise as vehicles for cancer Ag delivery in conjunction with oncolytic therapy, immunotherapy, cell-based therapy, and other approaches.

8.6 Concluding Remarks

Ad-based vectors have exceptional ability to stimulate cellular immune responses against the protein product of the transgene, a feature that served the main impetus for the preferential use of these vectors for the development of therapeutic vaccines. In the past 20 years, recombinant Ad vectors expressing a variety of Ags have been constructed and tested for immunization in various settings. However, Ad infection induces pathogenic inflammatory responses in immunocompetent hosts, including humans, even if the viral vector does not replicate, as early Ad gene expression alone is responsible for the pathogenic reaction. The inflammation consists of an early phase, in which TNF-α plays a major role and a late phase consisting of an extensive T-cell response (Ginsberg 1996; Imler 1995). In addition, PEI to Ads is a major impediment for vectors derived from common human serotypes, such as 5 or 2. Preexisting NAbs against these serotypes can be found in up to 90 % of human

adults depending on age and geographic region (Chen et al. 2010). Preexisting NAbs limit Ad-delivered transgene expression, thereby reducing the immune efficacy of the vaccine to the target Ag. A series of approaches have been entertained to circumvent the negative impact of preexisting NAbs, which include genetic modifications of Ad vectors or the use of those isolated from different species, such as chimpanzees (Fitzgerald et al. 2003). Another strategy to avoid vector PEI is to genetically alter the natural tropism of the vector to improve its selective targeting to CD40 receptor on DCs in situ, thus making generation of autologous DCs more efficient and inexpensive (Hangalapura et al. 2012).

One of the important features of Ad-based vectors is the induction of strong $CD8^+$ T- and B-cell responses. The T-cell response is long lasting with minimal contraction and activated cells remaining in effector and effector memory stages for months (Tatsis et al. 2007). Although the basis of this observation is not known, it may reflect that Ad vectors, similar to wt viruses, persist mainly in T cells and remain transcriptionally active.

Ad vectors also allow the genetic incorporation of foreign sequences into the viral capsid proteins that may allow the expression of gene products on the surface in a repetitive and orderly fashion, thereby generating high-affinity B-cell responses (Matthews et al. 2008; Pichla-Gollon et al. 2009; Wu et al. 2005). The noninvasive mucosal vaccination of Ad vectors minimizes systemic inflammation, because preexisting Ad5 immunity does not interfere appreciably with the potency of an Ad-vectored nasal vaccine. Nasal administration of Ad vectors encoding Ags derived from selected pathogens not only provides a painless and practical means of immunization but also generates rapid and sustained protection against the pathogens. Importantly, Ad particles alone without transgene expression were shown to induce an anti-influenza state in the airway. Ad-vectored vaccines can also be used for mass immunization of animals with important application to veterinary medicine (Zhang et al. 2011).

Over the past two decades, virus chimera technologies have boosted the development of novel therapeutic Ads for applications in gene therapy and oncolysis. These strategies of developing virus chimerism address the most relevant drawbacks to clinical implementation of virus-based therapies, including those that have been identified very recently such as blood coagulation factor-mediated liver tropism of Ads (Waddington et al. 2008; Kalyuzhniy et al. 2008). Safety and increased therapeutic efficiency in clinical settings have already been demonstrated by several chimeric Ads (Koski et al. 2010; Pesonen et al. 2010). All these new insights and developments led to a high number of possible combinatorial treatment modalities and delivery schemes. This calls for careful preclinical evaluation in relevant ex vivo human models and transitional in vivo tumor models, before clinical translation. To bring optimized combinations of retargeted Ad configurations and adjuvants in optimized heterologous prime–boost schedules to the clinic will be a major effort both in terms of time and cost (de Gruijl and van de Ven 2012). We consider that the most promising approaches to Ad-based immune response are (1) incorporation of heterologous Ag into Ad capsid, (2) a DC-targeted Ad vaccines, and (3) an oncolytic Ads armed with both Ag and

immunomodulatory transgenes. Therefore, it is reasonable to view certain properties and components of the immune system as allies, rather than enemies, taking advantage of them for improving efficacy of cancer and other disease-targeted vaccines. In this regard, by making a part of the solution what has previously been viewed as a problem, we expect to observe more Ad-based cancer vaccines achieving clinical efficacy in the years to come.

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Chapter 9
Radiovirotherapy for the Treatment of Cancer

Kevin J. Harrington

Abstract This chapter focuses on the relatively narrow definition of radiovirotherapy as "the use of viral vectors engineered to express genes that drive uptake of radioisotopes into cancer cells for both detection and antitumor efficacy". Having said that, it also addresses issues relating to combinations of virotherapy and standard anticancer modalities (such as external beam radiotherapy and cytotoxic chemotherapy) and novel agents (such as molecular radiosensitisers). However, within the context of radiovirotherapy, those discussions are limited solely to approaches that aim to improve the efficacy of the radioisotopic treatment.

The introductory sections describe the biology of the sodium–iodide symporter (NIS)—the gene therapy approach that has been most widely exploited for radioisotope uptake. In subsequent sections, progress from initial work with replication-defective vectors onto replication-competent oncolytic vectors is described, and this recognises the increasing acceptance of replicating viruses as the vectors of choice for cytotoxic gene therapy strategies. The section on oncolytic NIS-expressing viruses deals mainly with vectors based on adenovirus and measles virus. The final sections describe the use of NIS-expressing vectors in multi-agent regimens and highlight the potential benefits of combinatorial therapeutic regimens. The limited number of clinical translational studies that have been performed to date are also reviewed, and opportunities for future development of NIS-based therapeutic strategies are discussed.

9.1 Introduction

Radiovirotherapy is a term that is used to describe virally directed radioisotope therapy (Hingorani et al. 2010a). Essentially, this approach involves the use of a virus, which may be either replication defective or replication competent, as a means of delivering an exogenous gene that encodes a protein that is capable of

K.J. Harrington (✉)
Division of Cancer Biology, Chester Beatty Laboratories, The Institute of Cancer Research, 237 Fulham Road, London SW3 6JB, UK
e-mail: Kevin.Harrington@icr.ac.uk

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mediating selective uptake of a specific radioisotope into a cancer cell. Most of the published literature on radiovirotherapy deals with the use of the sodium–iodide symporter (NIS) to mediate radioisotope uptake by virally infected cells [reviewed in Hingorani et al. (2010a)]. Indeed, this approach has a very strong basis in the extensive use of iodide-131 radioisotope therapy in patients with thyroid cancer. In this setting, iodide-131 has established a role as an adjuvant (radioablative) therapy post-thyroidectomy or as a definitive anticancer treatment in patients with metastatic differentiated (papillary and follicular) thyroid cancers. Therefore, in this chapter, we will focus on the use of NIS-mediated radiovirotherapy. Although this approach was initially conceived to be used with replication-defective viral vectors, in its most recent incarnation, the concept of radiovirotherapy has been modified to exploit the twin benefits of oncolytic cell killing by the virus and "bystander" killing of uninfected neighbouring cells by the emission of beta (or alpha) particles from accumulated radioisotopes.

9.2 Sodium–Iodide Symporter

NIS is a member of the sodium/solute symporter family (SSF) that includes more than 60 members of both prokaryotic and eukaryotic origin, many of which exhibit a high similarity of sequence and function. SSF members actively transport anions into cells by using a Na^+/K^+ electrochemical gradient that is generated by Na^+/K^+ ATPase (Jung 2002). NIS is expressed mainly on the basolateral membrane of thyroid follicular cells and cotransports sodium and iodide into cells (Fig. 9.1). Following uptake into thyroid cells, iodide is then transported to the follicular lumen and oxidised by the thyroid peroxidase in a reaction that promotes the iodination of tyrosyl residues of the thyroid hormones tri- and tetra-iodothyronine (T3 and T4) (Massart and Corbineau 2006). NIS is also expressed in a number of extrathyroidal tissues including gastric mucosa, salivary glands, digestive tract and lactating mammary glands. The expression of messenger RNA for NIS has also been documented in other tissues (e.g. pituitary gland, pancreas, thymus, lungs, prostate, testis, ovaries, kidneys, adrenal gland) by reverse-transcriptase polymerase chain reaction RT-PCR, although the functional significance of this observation is, as yet, unclear (Kogai et al. 2006; Vayre et al. 1999; Spitzweg et al. 1998, 2001a).

Human NIS (hNIS) was cloned in 1996. The human NIS gene is located on chromosome 19p12–13.2, with an open reading frame of 1929 nucleotides comprising of 15 exons and 14 introns that encodes a 643-amino acid protein. Human NIS exhibits 84 % identity and 93 % similarity to rat NIS (rNIS), with the main differences accounted for by a 5-amino acid insertion between the last two hydrophobic domains and a 20-amino acid insertion in the C-terminus (Smanik et al. 1996). The protein has 13 putative transmembrane domains, an intracellular C-terminus and an extracellular N-terminus. Membrane localisation of NIS is a prerequisite for its function. NIS gene transcription in thyroid tissue is mainly

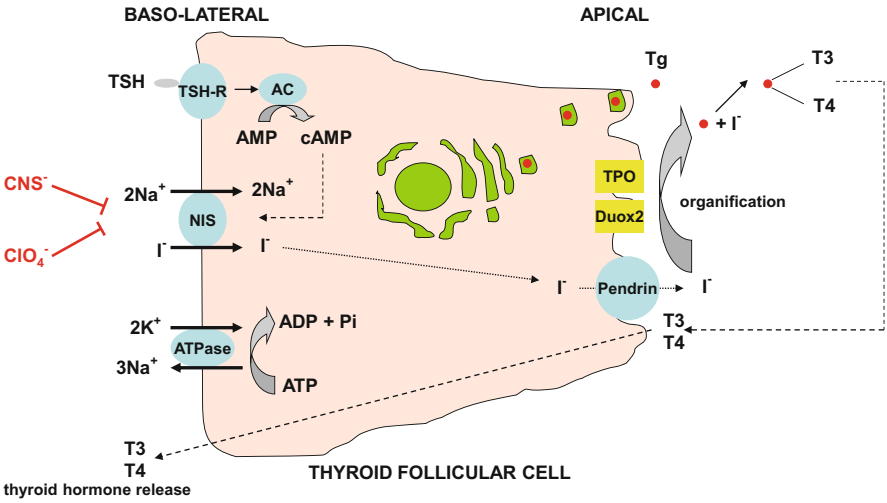

Fig. 9.1 Diagrammatic representation of the function of NIS in iodide transport in normal thyroid follicular cells. Thyroid stimulating hormone (TSH) binding to TSH receptor (TSH-R) activates adenylate cyclase (AC) which generates cyclic AMP (cAMP) from AMP. This drives NIS-mediated co-transport of two Na^+ ions along with one I^- ion, with the transmembrane sodium gradient serving as the driving force for iodide uptake. Thiocyanate (CNS^-) and perchlorate ($ClO4^-$) are competitive inhibitors of thyroidal iodide accumulation. The efflux of iodide from the apical membrane to the follicular lumen is driven by pendrin (Pendred syndrome gene product). Iodide organification within the thyroid follicular lumen [mediated by thyroperoxidase (TPO) and dual oxidase 2 (Duox2)] generates iodinated tyrosine residues within the thyroglobulin (Tg) backbone. These are ultimately released as active thyroid hormone (T3 and T4) (Modified from Spitzweg et al. *J. Clin. Endocrinol. Metab.* **2001**, 86, 3327–35)

regulated by thyroid-stimulating hormone (TSH), via the stimulation of the human NIS upstream enhancer (hNUE) (Taki et al. 2002). Interestingly, TSH can also modulate NIS phosphorylation and, thus, act as a post-transcriptional regulator of NIS (Riedel et al. 2001; Kogai et al. 2000). In the absence of TSH, NIS is redistributed to intracellular compartments and, therefore, functionally inactive (Riedel et al. 2001).

Other factors are also implicated in the regulation of NIS. The pituitary tumour transforming gene (PTGG), a proto-oncogene overexpressed in follicular thyroid cancers, and the PTGG-binding factor (PBF) inhibit NIS gene transcription through the inactivation of hNUE (Boelaert et al. 2007). Overexpression of these factors in thyroid cancers may have a negative impact on prognosis by reducing the potential efficacy of radioiodide therapy (Boelaert et al. 2007; Saez et al. 2006). TSH-induced NIS stimulation is also inhibited by a number of cytokines, including interleukin-1, TNF-α, interferon-α, interferon-β and interferon-γ in thyroid cells (Caraccio et al. 2005; Ajjan et al. 1998). In contrast, all-trans retinoic acid (atRA), dexamethasone and carbamazepine have each been shown to have a positive effect on NIS-mediated iodide uptake (Spitzweg et al. 2003; Willhauck et al. 2008a, 2011;

Unterholzner et al. 2006), although that has not yet translated to clinical use or benefit.

Radioiodide has been used for decades to treat hyperthyroidism (Ross 2011) and differentiated thyroid cancers. Currently, it is recommended as an adjuvant treatment following total thyroidectomy in patients with unifocal or multifocal primary tumours measuring more than 1 cm in greatest dimension (pT1b, pT2, pT3), with nodal extension (N1a and N1b) or with extrathyroidal extension (pT3). It is also a standard of care in patients with disseminated metastatic well-differentiated cancers, where its use can be curative (Cooper et al. 2009). The successful treatment of thyroid tumours by NIS-mediated radioiodide therapy has sparked significant interest in virus-mediated NIS delivery as a therapeutic option in a wide range of tumour types.

9.3 Preclinical Studies of NIS Gene Transfer by Replication-Defective Viruses

A schematic representation of NIS-mediated radiovirotherapy is shown in Fig. 9.2. Shimura et al. (1997) successfully transfected rNIS cDNA into malignant rat thyroid cells (FRTL-Tc) by electroporation, restoring iodide transport activity in the process. In vitro, FRTL-Tc cells stably expressing rNIS accumulated ^{125}I 60-fold and in vivo xenografts trapped up to 27.3 % of the total ^{125}I dose with an effective half-life of approximately 6 h. However, a therapeutic dose of 37 megabecquerels (MBq) of ^{131}I did not cause statistically significant tumour shrinkage (Shimura et al. 1997). Subsequently, several studies have confirmed the feasibility of inducing functional NIS expression in non-thyroidal tumours (Riesco-Eizaguirre and Santisteban 2006). Cho et al. (2000) showed that the delivery of exogenous hNIS by recombinant replication-defective adenovirus (Ad-CMV-hNIS) resulted in more than a 120-fold increase in iodide uptake in U1240 cells in vitro. Intratumoral injection of Ad-CMV-hNIS into subcutaneous U251 human glioma xenografts caused a 25-fold increase in ^{125}I accumulation compared to spleen or saline-injected tumours (Cho et al. 2000). Mandell et al. (1999) demonstrated in vitro and in vivo iodide accumulation in melanoma, liver, colon and ovarian carcinoma cells after retrovirus-mediated transfection with rNIS. NIS-transduced melanoma xenografts accumulated significantly more ^{123}I (6.9-fold increase) than non-transduced tumours (Mandell et al. 1999). Similarly Boland et al. (2000) used adenoviral-mediated NIS (CMV promoter) gene delivery to demonstrate functional levels of NIS expression in several tumour cell lines. Fold increases in iodide uptake of 125–225 times were observed in vitro, and 11 % of the total ^{125}I dose could be recovered per gram of adenovirus-infected tumour tissue. Although an in vitro cytotoxic effect was observed, the study failed to show in vivo therapy (Boland et al. 2000). Spitzweg et al. (1999) were the first to demonstrate tissue-specific expression of hNIS cDNA in androgen-sensitive human prostate

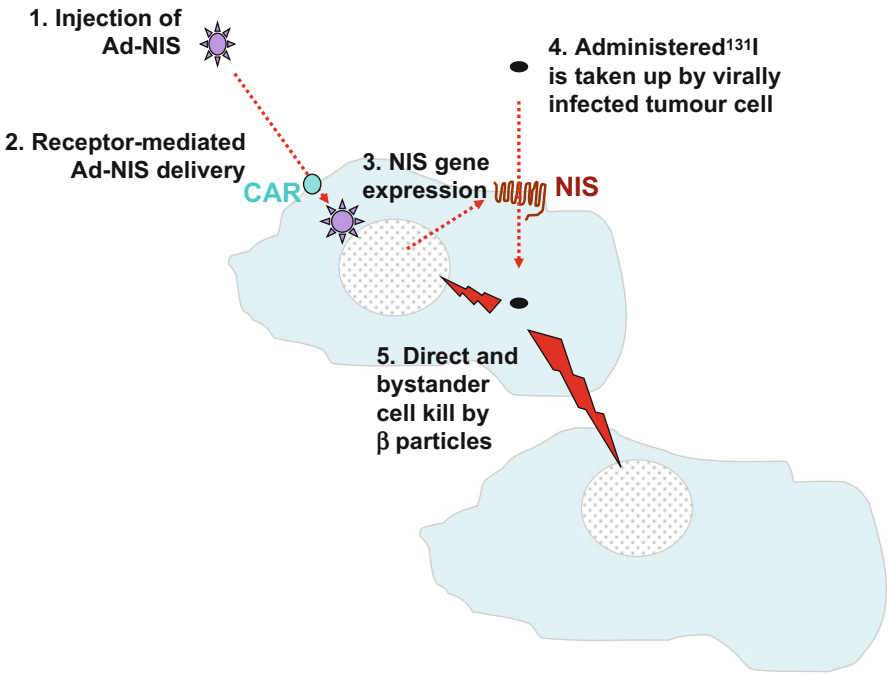

Fig. 9.2 Diagrammatic representation of NIS-mediated radiovirotherapy by replication-defective adenovirus: (1) Ad-NIS is injected by the intratumoural, locoregional or systemic route. (2) Ad-NIS infects target cells through the cognate coxsackie and adenovirus receptor (CAR). (3) Ad-NIS drives NIS gene expression and protein is displayed on the cell membrane in infected cells. (4) Radioiodide is administered systemically and is taken up in NIS-expressing tumour cells. (5) β-particulate radiation mediates both direct and bystander killing of cells

adenocarcinoma (LNCaP) following transfection with a eukaryotic expression vector in which the full-length hNIS cDNA was coupled to a prostate-specific antigen (PSA) promoter. Prostate cells transfected with PSA-NIS showed perchlorate-sensitive, androgen-dependent iodide uptake (Spitzweg et al. 1999). The same groups were also the first to demonstrate the feasibility and effectiveness of NIS-mediated cytotoxicity in vivo and, in doing, assuaged concerns that NIS-mediated therapy was unlikely to succeed due to lack of iodide-organification in non-thyroidal tumour tissues. LNCaP cell lines stably transfected with hNIS cDNA (NP-1) under the control of a PSA promoter (NP-1 cells) showed perchlorate-sensitive, androgen-dependent iodide uptake and killing in clonogenic assays in vitro. Xenograft NP-1 tumours in athymic nude mice accumulated 25–30 % of the total ^{123}I administered with a biological half-life of 45 h. In addition, NIS protein expression in LNCaP xenografts was confirmed by Western analysis and immunohistochemistry. Critically, a single therapeutic dose of 111 MBq ^{131}I yielded a dramatic therapeutic response in NIS-transfected LNCaP xenografts (Spitzweg et al. 2000). The same group subsequently developed a replication-deficient human adenovirus incorporating the hNIS gene driven by a CMV

promoter (Ad5-CMV-NIS) for in vivo NIS gene transfer into LNCaP tumours. Following intraperitoneal injection of a single therapeutic dose of 111 MBq ^{131}I 4 days after adenovirus-mediated intratumoral NIS gene delivery, LNCaP xenografts showed an average volume reduction of >80 % (Spitzweg et al. 2001b).

Kakinuma et al. (2003) evaluated adenoviral-mediated NIS expression regulated by the tissue-specific probasin promoter in prostate and various other tumour cell lines. Interestingly, androgen-dependent and perchlorate-sensitive iodide uptake was demonstrated in LNCaP cells that was 3.2-fold higher than an Ad-CMV/hNIS vector. Furthermore, iodide uptake in a panel of non-prostate tumour cell lines infected with Ad-ARR(2)PB/hNIS was significantly lower, confirming tissue specificity with this construct (Kakinuma et al. 2003). Sieger et al. (2003) investigated radioiodide uptake in a hepatoma cell line in vitro and in vivo following the transfer of hNIS under the control of a tumour-specific regulatory element, the promoter of the glucose transporter 1 (GT1) gene. NIS-expressing stable cell lines [rat hepatoma (MH3924A)] demonstrated perchlorate-sensitive increased iodide uptake (30-fold increase in vitro and 22-fold increase in vivo) compared to the wild-type cell line. Similarly, the mean radiation dose delivered to MH3924A xenografts was 10-fold higher after the administration of 18.5 MBq of ^{131}I (Sieger et al. 2003). Faivre et al. (2004) reported on an in vivo kinetic study of NIS-related iodide uptake in an aggressive chemically induced model of hepatocarcinoma in immunocompetent Wistar rats. An adenoviral vector expressing rNIS controlled by the CMV promoter (Ad-CMV-rNIS) was injected into the portal vein of 5 healthy and 25 hepatocarcinoma-bearing rats. This resulted in impressive (from 20 % to 30 % of the injected dose) and durable (>11 days) iodide uptake that contrasted with the rapid iodide efflux observed in vitro. Prolonged retention of iodide observed in vivo was not attributed to an active retention mechanism but to permanent recycling of the effluent radioiodide via the high hepatic blood flow. Radioiodide therapy in these circumstances was associated with strong inhibition of tumour growth, complete regression of small nodules and prolonged survival of hepatocarcinoma-bearing rats (Faivre et al. 2004).

Other preclinical studies have evaluated NIS-mediated radioiodide therapy in head and neck (FaDu, SCC-1, SCC-5) (Gaut et al. 2004), pancreatic neuroendocrine (Bon1, QGP) (Schipper et al. 2003), medullary thyroid (Cengic et al. 2005), colorectal (HCT116) (Scholz et al. 2005), breast (Dwyer et al. 2005a), ovarian (Dwyer et al. 2006a) and hepatocellular (HepG2) (Willhauck et al. 2008b) cancer models. Most of these studies confirmed that replication-defective viral vectors expressing NIS from a tissue-specific promoter were capable of mediating tumour-selective iodide uptake that translated into in vitro and in vivo therapeutic efficacy.

In addition to the rodent-based in vivo studies discussed above, a preclinical dosimetric study of NIS-mediated radioisotope therapy was performed in adult male beagle dogs. This was a safety and feasibility study in preparation for a phase I clinical trial in patients with locally recurrent prostate cancer. Animals received direct intraprostatic injections of 1×10^{12} viral particles of NIS-expressing replication-defective adenoviral vectors regulated by the CMV promoter. This was followed by intravenous injection of 111 MBq ^{123}I and serial image acquisition

using single photon emission computed tomography. Clear images of the prostate were obtained in all dogs that received Ad5-CMV-NIS, but none in the group that was injected with control virus. The average absorbed radiation dose to the prostate was estimated to be 23 ± 42 cGy/37 MBq ^{131}I. This indicated that a 3.15 GBq dose of ^{131}I would deliver a target dose of 20 Gy to the prostate. Following a therapeutic dose of ^{131}I (4.3 GBq/m^2), preceded by T3 supplementation for 8 days, the estimated mean doses delivered to the prostate, thyroid, stomach and liver were 12.5 ± 0.28, 0.12 ± 0.01, 0.23 ± 0.02 and 0.009 ± 0.006 Gy/37 MBq of ^{131}I. No major toxicities or changes in blood biochemistries were noted (Dwyer et al. 2005b).

9.4 Preclinical Studies and Clinical Translation of NIS Gene Transfer by Replication-Competent Viruses

As reviewed above, the initial development of virus-mediated NIS delivery was based on the notion that the virus would act purely as a vehicle for delivering the therapeutic gene. However, within the last decade, there has been a fundamental re-evaluation of strategy such that much greater emphasis is now placed on the use of replication-competent or oncolytic virus vectors (Fig. 9.3). There are a number of potential advantages to using oncolytic viruses, including their greater potency due to their intrinsic cytotoxicity and ability to self-amplify and, thus, increase levels of transgene expression and their impressive safety record. Initial concerns regarding the possible toxic consequences of using replication-competent viruses in immunocompromised cancer patients and the threat of environmental release and contamination have been largely assuaged by data from a series of phase I and II clinical trials (see Harrington et al. (2010) for an account of this process).

A number of NIS-expressing oncolytic agents have been assessed. We recently reported in vivo SPECT imaging of the time course of gene expression from two oncolytic adenoviruses expressing NIS in tumour-bearing mice (Merron et al. 2007). In these viruses, the hNIS cDNA was positioned in the E3 region either in a wild-type adenovirus type 5 (AdIP1) or one in which a promoter from the human telomerase gene (RNA component) was driving E1 expression (AdAM6). Viruses showed functional hNIS expression and replication in vitro, and the kinetics of spread of the two viruses in tumour xenografts were visualised in vivo using a small animal nano-SPECT/CT camera. The time required to reach maximal spread was 48 h for AdIP1 and 72 h for AdAM6 suggesting that genetic engineering of adenoviruses can affect the kinetics of their dissemination in tumours. More recently, we have shown that a Wnt-targeted oncolytic adenovirus expressing NIS can mediate a specific oncolytic effect that is augmented by the additional delivery of ^{131}I (Peerlinck et al. 2009). Importantly, SPECT imaging studies allowed careful evaluation of the optimal therapeutic schedule for virus administration and

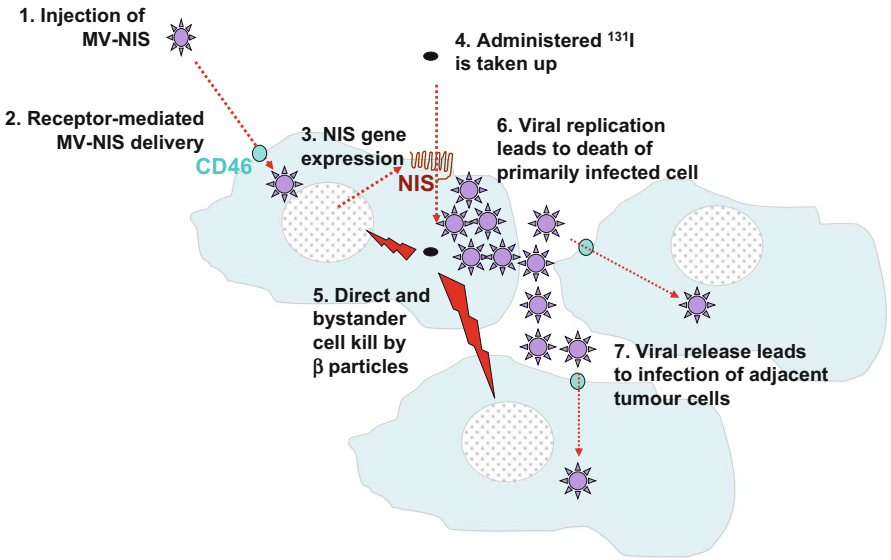

Fig. 9.3 Diagrammatic representation of NIS-mediated radiovirotherapy by replication-competent oncolytic measles virus: (1) MV-NIS is injected by the intratumoural, locoregional or systemic route. (2) MV-NIS infects target cells through the cognate CD46 receptor. (3) MV-NIS drives NIS gene expression and protein is displayed on the cell membrane in infected cells. (4) Radioiodide is administered systemically and is taken up in NIS-expressing tumour cells. (5) β-particulate radiation mediates both direct and bystander killing of cells. (6) Viral replication leads to death of tumour cell that was initially infected. (7) Viral oncolysis of primarily infected cell leads to release of progeny virions and second-wave infection of adjacent tumour cells

subsequent radioisotope delivery. Thus, a single radioisotope dose delivered 48 h after virus administration resulted in very significant antitumor efficacy.

This same approach has been taken in to the clinic in a phase I study of a replication-competent Ad5 adenovirus armed with two suicide genes (yeast cytosine deaminase, mutant thymidine kinase) and the human NIS gene (Ad5-yCD/TK-hNIS) (Barton et al. 2008). Men with clinically localised prostate cancer received an intraprostatic injection of Ad5-yCD/TK-hNIS, armed with two suicide genes and the NIS gene. Expression of the NIS gene was monitored non-invasively using single photon emission computed tomography (SPECT) after injection of $^{99m}TcO_4^-$. Ad5-yCD/TK-hNIS was well tolerated and 98 % of the adverse events were grade 1 or 2. Importantly, NIS gene expression was detected in the prostate of seven of the nine patients who received a dose of 1×10^{12} virus particles (vp), but not at 1×10^{11} vp. The authors reported on the time course and the geographical extent of NIS gene expression within and without the prostate following injection of 1×10^{12} vp in 1 cm^3. NIS gene expression peaked at 24–48 h after injection, but was still detectable in the prostate for up to 7 days. The volume of prostate in which gene expression was seen averaged 6.6 cm^3, representing 18 % of the total prostate volume. There was no evidence of extraprostatic dissemination of the adenovirus by SPECT imaging (Barton et al. 2008).

The same group conducted further studies in which they attempted to define the dose of radiotherapy that might be delivered to the entire prostate gland if ^{131}I-based Ad5-yCD/TK-hNIS radiovirotherapy were used as a definitive therapy (Barton et al. 2011). They recruited six patients with clinically localised prostate adenocarcinoma (5 T1c, 1 T2; Gleason range from 3 + 4 to 4 + 5) that was restricted to one sextant in four patients and bilateral in the other two patients. A standard injection protocol was used to optimise Ad5-yCD/TK-hNIS delivery across the entire prostate gland, such that a total of 5×10^{12} viral particles were injected in 12 separate deposits in a volume of 5 mL. The patients then underwent serial SPECT imaging following administration of $^{99m}TcO_4^-$. NIS gene expression was detected in the prostate of all six patients, and as with the previous study (Barton et al. 2008), there was no evidence of extraprostatic gene expression. The mean gene expression volume was 14.6 cm^3 with a range of 6.6–28.9 cm^3, and this represented 18–83 % (mean 45 %) of the total prostate volume. The authors then went on to estimate the radiation doses that would have been achieved had the patients received a dose of radioactive ^{131}I equivalent to 7.4 GBq (200 mCi). These data revealed that the mean absorbed dose to the prostate would have been 7.2 ± 4.8 Gy (based on a gene expression volume of 45 %) and 15.4 ± 3.3 Gy (had the entire prostate been transduced to the same extent). It is important to realise that, in order to make these estimates, a number of assumptions were necessary and that their general effect would have resulted in an overestimation of radiation dose delivery. First, the deposition of $^{99m}TcO_4^-$ within the prostate was calculated by relating the mean image pixel intensity to that in the iliac artery. Standard values for $^{99m}TcO_4^-$ blood clearance and estimation of tissue attenuation of the signals detected by SPECT were then applied to yield the radioisotope activity in the volume of the prostate gland transduced with Ad5-yCD/TK-hNIS. Thereafter, the dose in Gy that would be delivered following the administration of 7.4 GBq of radioiodide was calculated by assuming that the expression of hNIS remained constant and maximal throughout the entire in vivo life of the administered ^{131}I. The data revealed that neither of these assumptions held true (i.e. hNIS expression was not constant and maximal, but rather peaked at 2 days and declined rapidly). These findings are extremely important since they give a clear indication that, at least in the context of prostate cancer, current state-of-the-art radiovirotherapy will fail to achieve complete tumour responses because it does not deliver sufficient radiation dose (a curative course of external beam radiotherapy for prostate cancer involves the delivery of 70–84 Gy). Nonetheless, if this relatively modest virally delivered radiation dose (7–15 Gy) were to be used as a therapeutic boost in the context of a highly active oncolytic virus delivered during standard external beam irradiation with radiosensitising drug therapy, it might make a significant contribution to the overall treatment effect. This approach is discussed in greater detail in the section on combining radiovirotherapy with conventional therapeutics (see below).

In a follow-up publication, Rajecki et al. treated a single patient with chemotherapy refractory cervical cancer with direct intralesional injection of oncolytic Ad5/3-Δ24-hNIS adenovirus (Rajecki et al. 2011). Despite attempting to image

with ^{123}I and $^{99m}TcO_4^-$, the authors were unable to detect gene expression and hypothesised that this might have been due to differences in vector design between Ad5-yCD/TK-hNIS and Ad5/3-Δ24-hNIS. They suggested that NIS gene expression might be optimised by using viruses of relatively low oncolytic potency with slower replication kinetics in which the NIS transgene is under the control of a ubiquitous exogenous promoter (e.g. CMV promoter) rather than an endogenous adenoviral promoter.

A recombinant NIS-expressing Edmonston strain measles virus (MV-NIS) has also been shown to have significant potential both as an in vivo imaging tool and as a therapeutic agent in combination with ^{131}I (Dingli et al. 2004). An initial preclinical model of multiple myeloma demonstrated that MV-NIS was capable of mediating concentration of ^{123}I such that intratumoral spread of the virus could be imaged by serial gamma camera imaging. In therapy experiments, when MV-NIS was combined with ^{131}I, it was able to cause complete regressions of MM1 xenografts (which were resistant to virus therapy in the absence of radioisotope) (Dingli et al. 2004). Subsequent studies demonstrated that MV-NIS was active in mice bearing intraperitoneal SKOV3 ovarian cancer (Hasegawa et al. 2006) and subcutaneous BxPC-3 pancreatic cancer xenograft tumours (Carlson et al. 2006, 2009). In the first of these studies, the authors engineered measles virus to express carcinoembryonic antigen (CEA) and were able to use CEA levels in the blood as a surrogate marker of viral propagation. Further studies in prostate cancer models confirmed the ability of MV-NIS to mediate gene expression and therapeutic activity both in vitro and in vivo. MV-NIS showed single-agent activity which was enhanced by the addition of therapeutic radioisotope (^{131}I). Importantly, systemically administered virus showed activity in these models (Msaouel et al. 2009). Additional studies have shown that MV-NIS may also be a candidate for development in the context of malignant glioma, medulloblastoma, anaplastic thyroid cancer and head and neck cancer (Allen et al. 2013; Hutzen et al. 2012; Reddi et al. 2012; Li et al. 2012). The promising nature of these studies has led to instigation of phase I clinical trials of MV-NIS in patients with a range of tumour types (Penheiter et al. 2010).

9.5 Choice of Radioisotope for Radiovirotherapy

Adenoviruses, MV, vesicular stomatitis viruses (VSV), vaccinia viruses and baculoviruses have all been engineered to encode the NIS gene. Different research teams have evaluated therapies combining viral and iodide-131 injections. Most studies have confirmed that combinations of NIS-expressing virotherapy and radioisotope delivery yield better antitumoral effects than viral injections alone in a range of models (Faivre et al. 2004; Peerlinck et al. 2009; Penheiter et al. 2010; Chen et al. 2007; Dwyer et al. 2006b; Herve et al. 2008; Hakkarainen et al. 2009).

Iodine-131 is the most commonly used radioisotope in preclinical studies, but other isotopes have also been investigated. Rhenium-188 is a high-energy β emitter

with a 4 mm path length. Its half-life of 17 h, compared with 8 days for iodide-131, allows the delivery of high doses of radiation in relatively short periods of time. In a model of glioblastoma encoding NIS, rhenium-188 had a better effect on survival than iodide-131 therapy. In an in vivo model of prostatic cancer (LNCaP cells expressing NIS), rhenium-188 and iodide-131 therapies had similar therapeutic effects on small tumours, but rhenium-188 was superior in large tumours measuring more than 200 mm^3 (Willhauck et al. 2007). Astatine-211 is an α-particle emitter that delivers high-energy α-particles (6.8 MeV) over a very short distance (less than 70 mm) (Supiot et al. 2007). Astatine-211 may, therefore, be particularly advantageous for the treatment of small tumours. In a subcutaneous model of K1-NIS tumours (thyroid cells expressing NIS), intraperitoneal injections of astatine-211 led to a complete tumour regressions (Petrich et al. 2006). An antitumoral effect has also been demonstrated in NIS-expressing prostate cancer models (Willhauck et al. 2008c). However, the use of astatine-211 is currently limited by its low availability for clinical use.

9.6 Combining Radiovirotherapy with Conventional Therapeutics

Conventional cancer treatments may improve the efficacy of radiovirotherapy (and vice versa). Combinations of viral therapies and external beam radiotherapy have been tested in several clinical trials (Touchefeu et al. 2011). Many studies have reported that radiotherapy and viruses can exert synergistic antitumoral effects through a variety of different mechanisms. Viral therapies can enhance the cytotoxic effects of radiation and, in effect, act as radiosensitising agents. On the other hand, tumour irradiation can change the biological effects of virotherapy. Thus, depending on the specific virus and tumour models selected, radiotherapy can increase viral infection/uptake, replication, gene expression and cytotoxicity (Touchefeu et al. 2011). In the specific case of NIS gene therapy, radiotherapy was shown to increase gene expression in a range of cell lines infected with a replication-deficient adenovirus encoding NIS (Hingorani et al. 2008a). For tumours that are treated by radiotherapy with curative intent, radiovirotherapy could represent a means of selectively increasing the total radiation dose that can be delivered. The radiobiological relationship between radiation dose and tumour control is well established for many solid cancers (Harrington and Nutting 2002), but attempts to improve clinical outcomes by escalating the doses of radiotherapy delivered by conventional means have been limited by the occurrence of radiation-induced toxicity in normal tissues. As an example, in the treatment of prostate cancer, a dose escalation from 64 Gy to 74 Gy significantly increased the biochemical progression-free survival, but was associated with increased toxicity (Dearnaley et al. 2007; Syndikus et al. 2010). In the studies reported by Barton et al. (2008, 2011) in which patients with prostate cancer received intraprostatic

injections of NIS-expressing replication-competent oncolytic adenovirus, had the patients been treated with iodide-131, the estimated additional radiation dose that would have been delivered would have been 7.2 ± 4.8 Gy. Although this dose would be insufficient to exert single-agent efficacy, if delivered in the context of standard external beam radiotherapy, it might result in significant improvements in tumour control.

An alternative approach could involve the integration of existing or novel radiosensitising agents, such as DNA repair inhibitors, into combination regimens (Hingorani et al. 2008b, 2010b). Combinations of viral therapies with chemotherapy agents have also been investigated in vitro and in vivo and in a number of clinical trials. Alkylating agents (cyclophosphamide, cisplatin, temozolomide), microtubule stabilisers (paclitaxel and docetaxel), intercalating agents (doxorubicin) and mitomycin C have all shown synergistic antitumoral effects in different tumour models (Ottolino-Perry et al. 2010). More recently, the role of DNA repair inhibitors in the context of both replication-defective NIS-expressing adenovirus and oncolytic MV-NIS has been tested. In the latter study, the effect of combining a checkpoint kinase 1 inhibitor (SAR-020106) with MV-NIS, ^{131}I and external beam radiotherapy was assessed (Touchefeu et al. 2013). Combining MV-NIS-driven radioiodide therapy with external beam radiotherapy and targeted inhibition of the DNA damage response was shown to be a promising therapeutic approach in models of head and neck and colorectal cancer. By adopting a stepwise, iterative approach to preclinical in vitro testing, it was shown that single-agent MV-NIS therapy mediated a potent antitumoral effect, especially at relatively high MOIs or later time points. Combining MV-NIS with EBRT achieved an additive or synergistic effect in short-term cytotoxicity and longer-term clonogenic assays. Other doublet combinations also exerted synergistic (or statistically significant) activities. Thus, the combination of MV-NIS and Chk1 inhibition was superior to MV-NIS or Chk1 inhibition alone in head and neck (HN5) and colorectal (HCT116) cells. As expected, combined Chk1 inhibition and external beam radiotherapy were synergistic or additive at all radiation dose levels in all cell lines. Similarly, combined MV-NIS and ^{131}I were active in all cancer cell lines. It was subsequently shown that the triplet combination of MV-NIS, radioiodide and Chk1 inhibition was active in HCT116 cells. However, detailed analysis of the effects of the entire combination regimen—MV-NIS, ^{131}I, EBRT and Chk1 inhibition—required in vivo analyses due to very high levels of cytotoxicity in in vitro analyses. Initially, studies confirmed the in vivo activity of the triplet combination of virus, Chk1 inhibition and EBRT in HN5 xenografts—indeed, the level of activity precluded using this model to test the quadruplet combination. In contrast, in HCT116 xenografts, the triplet combination was active, but with scope for improvement by adding radioiodide. Subsequently, it was confirmed that the addition of ^{131}I to the combination regimen significantly increased its efficacy such that survival rates at study termination exceeded 80 %. Therefore, it would appear that this might represent a promising approach for clinical translation.

An alternative, or potentially complementary, approach may involve the use of targeted agents that exert direct anticancer effects and which also have the potential

to modulate the expression and biological activity of hNIS. This approach has been assessed in the context of thyroid cancer that has become refractory to radioiodide therapy (through downregulation of NIS expression/activity) and has largely focused on the use of lithium or retinoic acid derivatives (Coelho et al. 2004; Handkiewicz-Junak et al. 2009; Simon et al. 2002; Liu et al. 2006). In general, these attempts to restore radioiodide uptake to potentially therapeutic levels have been unsuccessful and have not, thus far, yielded therapeutic benefits for patients with thyroid cancer. However, a recent report using a targeted agent that inhibits signalling in the mitogen-activated protein kinase (MAPK) pathway has provided clear evidence that this approach may be worth pursuing (Ho et al. 2013). The scientific rationale of using MAPK inhibition to increase NIS expression/activity is based on data showing that MAPK signalling exerts an inhibitory effect on NIS expression. In that study, a selective, allosteric MEK1 and MEK2 inhibitor selumetinib (AZD6244, ARRY-142886) was given to patients with radioiodide-refractory differentiated thyroid cancer, and the effect on NIS activity was measured by repeated ^{124}I positron-emission tomography. A total of 20 patients were evaluable, and selumetinib was shown to increase ^{124}I radioiodide uptake in 12 patients. Importantly, a link to the MAPK pathway was confirmed by the fact that selumetinib was effective in four of nine patients with BRAF mutations and five of five patients with NRAS mutations. As yet, this approach has not been tested as an adjuvant to virally mediated NIS gene expression, but it would appear that this may present a promising approach to optimising NIS expression, radioiodide uptake and, ultimately, therapeutic gain.

9.7 Conclusions

Radiovirotherapy is an innovative approach that aims to deliver a radioisotopic treatment selectively to tumours. Initial attempts to exploit replication-defective viral vectors to deliver NIS have more recently given way to the development and clinical translation of replication-competent oncolytic viruses that can serve this function. This field is rapidly evolving with a number of potential avenues for improving therapeutic efficacy. These include the use of imaging to guide the timing of treatment, the application of non-iodide radioisotopes (rhenium-188, astatine-211), innovations in viral vector design and the combination of NIS radioisotopic therapy with standard anti-cancer treatments. Given this breadth of opportunities, it is to be hoped that effective clinical translation of NIS-mediated radiovirotherapy will soon be achieved.

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Chapter 10
TRICOM Poxviral-Based Vaccines for the Treatment of Cancer

Caroline Jochems, Jeffrey Schlom, and James L. Gulley

Abstract TRICOM poxviral-based vaccines are novel "off-the-shelf" agents designed to generate a robust immune response against tumor-associated antigens, which are commonly expressed on tumor cells. In this chapter, we elucidate the science behind the development and use of these vaccines. We also highlight the results of preclinical and clinical trials of these vaccines and the potential benefits of combining them with standard-of-care chemotherapy and other immunotherapies.

We compare the immune-stimulating effects of replication-competent versus replication-defective poxviral vectors and the rationale for using a diversified prime-boost regimen. We review current knowledge of tumor-associated antigens, including agonist epitopes and antigen spreading (or antigen cascade), as well as the use of immunostimulatory agents and T-cell costimulatory molecules. We also provide descriptions of completed and ongoing clinical trials of various TRICOM poxviral-based vaccines, including safety studies, phase II trials with vaccine alone, vaccine plus standard-of-care regimens, and an ongoing multicenter phase III trial.

We discuss tumor growth kinetics, an important emerging predictive factor for survival, in the context of immunotherapy for prostate cancer and, finally, propose potential strategies for maximizing the clinical impact of TRICOM poxviral-based vaccines.

Abbreviations

APCs	Antigen-presenting cells
CEA	Carcinoembryonic antigen
CEA-Tg	CEA-transgenic
Con A	Concanavalin A

C. Jochems • J. Schlom
Laboratory of Tumor Immunology and Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA
e-mail: jochemscm@mail.nih.gov; js141c@nih.gov

J.L. Gulley (✉)
Genitourinary Malignancies Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA
e-mail: gulleyj@mail.nih.gov

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CRPC	Castration-resistant prostate cancer
CTL	Cytolytic T cell
CTLA-4	Cytotoxic T-lymphocyte-associated antigen-4
DCs	Dendritic cells
EMT	Epithelial-to-mesenchymal transition
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HLA-A2	Human leukocyte antigen-A2 allele
HR	Hazard ratio
IFN-γ	Interferon gamma
irAEs	Immune-related adverse events
mCRPC	Metastatic castration-resistant prostate cancer
MHC	Major histocompatibility complex
MUC1	Mucin 1
MUC1-C	C-terminal region of MUC1
MUC1-N	N-terminal region of MUC1
MVA	Modified vaccinia Ankara
NSCLC	Non-small cell lung cancer
OS	Overall survival
PBMCs	Peripheral blood mononuclear cells
PSA	Prostate-specific antigen
rF	Recombinant fowlpox
rV	Recombinant vaccinia
TAAs	Tumor-associated antigens
Tregs	Regulatory T cells
TRICOM	TRIad of COstimulatory Molecules
VNTR	Variable number of tandem repeats
V-WT	Wild-type vaccinia

10.1 Introduction

Approval of the first therapeutic vaccine for cancer (sipuleucel-T, Provenge®, Dendreon), along with rapidly growing enthusiasm for immune checkpoint inhibitors, has sparked unprecedented interest in the field of cancer immunotherapy. While immune checkpoint inhibitors can nonspecifically magnify an underlying immune response, therapeutic vaccines are designed to mount a specific antitumor immune response. However, approaches that rely on obtaining patients' own cells to generate a vaccine are logistically complicated and may delay the start of treatment. In this chapter, we discuss a promising off-the-shelf vaccine platform utilizing poxviral vectors that is in the late stages of clinical development. We present the preclinical background as well as findings from multiple clinical trials suggesting that this off-the-shelf vaccine platform, while logistically simple, is immunologically advanced and safe and has shown promising early signs of efficacy both alone and in combination with other agents.

10.2 Viral Vector Vaccines

Tumor-associated antigens (TAAs) are by definition either weakly immunogenic or functionally nonimmunogenic. Vaccine strategies must be developed in which the presentation of these TAAs to the immune system results in far greater activation of T cells than is being achieved naturally in the host. One approach to triggering a robust immune response to TAAs involves the use of recombinant viral vectors to deliver the desired genetic material. The advantages of using a viral vector include (a) the ability to incorporate the entire tumor antigen gene, parts of that gene, or even multiple genes (including genes for costimulatory molecules and cytokines) into the vector; (b) the vector's ability to infect antigen-presenting cells (APCs), allowing the resulting foreign viral antigen to act as a danger signal that activates the immune system; and (c) the relatively low cost of producing viral vector vaccines compared to vaccines that require the preparation and purification of proteins.

10.2.1 Poxviral Vectors

Several properties of poxviruses have led to their extensive use as expression vectors, including their capacity for carrying large amounts of DNA and their wide range of hosts. These viruses have several advantages, including the ability to make stable recombinant vectors with accurate replication, efficient posttranslational processing of the transgene, and the fact that they do not integrate into eukaryotic DNA. These vectors can incorporate many genes for TAAs, cytokines, and costimulatory molecules, each under a different viral promoter within one vector. One of the most thoroughly investigated poxviral vectors is vaccinia, in use since 1796 when Jenner first demonstrated its ability to protect against subsequent variola inoculation and thus protect against smallpox. Jenner's technique has been employed in more than a billion smallpox vaccinations worldwide, leading to the eradication of this disease and providing highly accurate data on vaccinia's safety as a vector.

The *Poxviridae* family can largely be divided into those that are capable of replicating in mammalian species (e.g., *orthopoxviruses*) and those that can infect mammalian cells but cannot complete the replication process (e.g., *avipoxviruses*). There are advantages and disadvantages to the use of each as a vaccine vector. Replication-competent vectors can continue to infect cells and produce more TAAs until they are eradicated by the immune system. Vaccines based on such vectors are more immunogenic than those based on replication-defective vectors. However, replication-defective vectors are theoretically safer because they can only infect mammalian cells once and thus cannot cause the potentially life-threatening conditions in immunocompromised individuals that are rarely associated with vectors such as vaccinia.

Poxviral vectors are unique in that DNA replication and RNA transcription take place exclusively in the cytoplasm and can be demonstrated in enucleated cells. Because the vector's RNA is transcribed in the cytoplasm, the expression of any inserted transgenes requires poxvirus promoters. Ideally, an open reading frame of the TAA is juxtaposed to a viral promoter, resulting in gene expression levels typical for that promoter. Cytoplasmic replication abrogates many of the safety concerns inherent in the use of other recombinant viral vaccines, which replicate in the nuclear compartment in close proximity to cellular genes.

When infecting a cell, a poxvirus first undergoes endocytosis, and the viral core is released into the cytoplasm. The viral transcriptase is then activated, and viral mRNA is produced inside the cytoplasm within minutes after infection. This process also shuts down normal cellular transcription inside the nucleus. Among the first genes transcribed are those encoding for proteins that complete the uncoating of the viral core and other early genes that are transcribed prior to reproduction of the viral DNA. There are about 100 of these early genes, many with unique promoters, which can be replaced with transgenes encoding TAAs. In humans, viral DNA replication occurs within 1.5–6 h following infection with vaccinia. The processes of virion assembly and mature particle budding from the membrane complete the life cycle. Multiple virions budding simultaneously from the cell membrane cause cellular lysis.

With poxviral vectors that are replication defective in mammalian species (e.g., fowlpox), the early genes are transcribed within 30 min of infection, with expression of proteins peaking at about 6 h. However, viral DNA cannot be replicated, and the late viral genes, including those for the coat proteins, are also not translated. This decreases the potential for a host immune response to the vector. Cells infected with these virions also shut down cellular transcriptions, causing the cells to slowly die over 14–21 days, generally by apoptosis. These cells do not release infectious virions.

10.2.2 Replication-Competent Vectors

Vaccinia, the prototype poxvirus, has a complex dsDNA genome containing 186 kb encoding over 200 proteins enclosed in a bilipid envelope. Many of the viral genes can be deleted and replaced by TAA gene(s), except for the inverted terminal repeat sequences within the genome that are responsible for viral replication.

Recombinant vectors are produced by homologous recombination after transfection of vaccinia virus-infected cells with plasmid DNA constructs. This virus is replication competent and cytopathic, with a broad host range that includes both vertebrates and invertebrates. The advantages of using vaccinia viruses for gene transfer include their ability to accommodate inserts of at least 25 kb, allowing for large or multiple gene inserts (up to seven); their ability to infect cells regardless of their mitotic state; and their cytoplasmic replication, which avoids any potential for chromosomal mutation upon insertion. Additionally, mammalian proteins

expressed from transgenes inserted into vaccinia vectors are processed and modified in a manner comparable to that when expressed in their native environment. These foreign antigens are presented to the immune system with the large number of proteins produced by the vector itself, which likely is responsible for the significant inflammatory response. This inflammatory process, in turn, could lead to cytokine production and T-cell proliferation, which may further amplify the immune response to the foreign antigen. This process favors induction of both a cell-mediated immune response and humoral responses to the foreign antigen.

Multiple administrations of vaccinia virus, however, could result in an antivector host immune response caused by prior vaccination for smallpox (using vaccinia), which could impair the patient's immunity to the transgene. On the other hand, the potential disadvantage of intrinsic antigenicity can be exploited to elicit a robust immune response against TAAs to activate an antitumor immunotherapeutic response in vivo. In fact, one of the main advantages of using recombinant vaccinia viruses to develop cancer vaccines is that when a gene for a weakly immunogenic protein is inserted into recombinant vaccinia and used as an immunogen, the recombinant protein is much more immunogenic than if it were used with adjuvant. This was previously shown when the effects of injections of carcinoembryonic antigen (CEA) protein in adjuvant were compared to injections of recombinant vaccinia (rV) virus containing the CEA transgene (rV-CEA) in CEA-transgenic (CEA-Tg) mice (Kantor et al. 1992).

10.2.3 Replication-Defective Vectors

Modified vaccinia Ankara (MVA) is a replication-defective poxvirus derived from vaccinia following 500 passages in chicken embryo cells (Sutter and Moss 1995). It has been employed in many experimental studies and has been administered to more than 120,000 humans without apparent side effects. A recent molecular characterization found that MVA has lost several genes involved in host-range determination and possible immunosuppression. While MVA efficiently infects human cells and expresses both early and late genes, it is replication defective and thus incapable of producing infectious progeny in mammalian cells. MVA recombinant viruses have been shown to be highly immunogenic in both rodents and primates. An MVA vector expressing the genes for IL-2 and the pan-adenocarcinoma antigen mucin 1 (MUC1) has been tested in several clinical trials (Doehn and Jocham 2000; Scholl et al. 2000). Preliminary results suggested that the vector was well tolerated and produced immunologic responses in prostate and breast cancer patients.

Avipoxviruses are also potentially attractive vectors for use in cancer vaccines. While the immunogenicity of the inserted transgene may not be as potent as that of vaccinia virus, avipoxviruses such as fowlpox and ALVAC can be administered numerous times to enhance immunogenicity (Fries et al. 1996; Hodge et al. 1997; Tartaglia et al. 1998). Since they are replication defective, induction of any host

immune responses should be relatively inconsequential. Avipoxviruses are further distinguished from vaccinia in that the inserted transgene is expressed in infected cells for up to 3 weeks before cell death occurs. In a vaccinia-infected cell, the transgene is expressed for 1–2 days until cell lysis and for approximately 1 week in the host until viral replication is arrested by host immune responses.

10.2.4 Diverse Prime-Boost Regimens

Preclinical and clinical studies (Eder et al. 2000; Chatterjee et al. 1999; Kaufman 2005; Marshall et al. 2005) have demonstrated that optimal generation of specific T-cell responses can be achieved by employing a heterologous prime-boost strategy. Preclinical studies used a primary vaccination with an rV virus expressing human CEA (rV-CEA) and single or multiple boosts with recombinant avipoxvirus expressing CEA (avipox-CEA). In these studies (Grosenbach et al. 2001), mice were first immunized with wild-type vaccinia virus to simulate patients who had previously received vaccinia as a smallpox vaccine. An adequate initial dose (pfu) of rV-CEA induced an immune response to CEA, but a second vaccination of rV-CEA produced only a small increase in CEA-specific T-cell responses. However, when avipox-CEA was administered as a booster vaccination, a marked increase in CEA-specific T-cell responses was achieved, which was far superior to giving multiple injections of either rV-CEA or avipox-CEA. A subsequent study showed that recombinant avipoxviruses can be given multiple times with subsequent increases in immune response to the TAA transgene (Kass et al. 2001). This is due to the fact that the transgene is expressed via an early poxvirus promoter (Chakrabarti et al. 1997), while many structural proteins of the avipoxvirus that are normally expressed on late poxvirus promoters, including viral coat proteins targeted by a host neutralizing immune response, are not expressed in mammalian cells. Thus, even if there are some antiavipox immune responses, no neutralization of the avipoxvirus is evident even after multiple vaccinations (Kass et al. 2001). Furthermore, when mice were given rV-CEA as a prime and recombinant CEA protein as a boost, greater T-cell responses to CEA were achieved than with the use of either immunogen alone (Hodge et al. 1997). This concept of heterologous prime-boost immunization has been validated in other model systems employing vaccines for infectious agents and cancer.

A small randomized trial was conducted to determine the most effective heterologous prime-boost regimen in humans (Marshall et al. 2000). The study compared giving rV-CEA as the initial priming vaccination followed by avipox-CEA boosts (VAAA) ($n = 9$) versus giving three vaccinations with avipox-CEA followed by rV-CEA (AAAV) ($n = 9$) and found that immune responses were greater in the VAAA arm than in the AAAV arm. Continued follow-up of these patients suggested that patients in the VAAA arm had a higher 2-year survival rate, apparently related to a greater immune response. Patients who had a $\geq$2.5-fold increase in CEA-specific T cells lived longer ($P = 0.03$).

10.3 Tumor-Associated Antigens, Including Agonist Epitopes

10.3.1 Carcinoembryonic Antigen (CEA)

CEA is a 180-kDa immunoglobulin-like oncofetal glycoprotein that is expressed on the cell surface of normal colonic mucosa and primarily functions in cellular adhesion (Benchimol et al. 1989). CEA is also commonly overexpressed on adenocarcinomas arising from the breast, cervix, lung, and gastrointestinal tract (Robbins et al. 1993; Tendler et al. 2000). Increased expression of CEA may increase cellular adhesion, which has been implicated in the proliferation and metastasis of malignant cells. In colon cancer cell lines, the enhanced adhesion from increased CEA expression distorts normal cellular architecture, reducing cellular differentiation and inhibition of cellular proliferation (Ilantzis et al. 2002). The increase in cellular adhesion may also allow for cells that have broken off from the main tumor to establish metastatic sites of disease (Hostetter et al. 1990).

10.3.2 Mucin 1 (MUC1)

MUC1 (CD227) is a large transmembrane glycoprotein normally expressed at the apical surface of glandular epithelial cells (Gendler et al. 1990). In adenocarcinoma (i.e., breast, prostate, colorectal, ovarian, lung, bladder, and pancreatic), it is overexpressed and aberrantly glycosylated (Hollingsworth and Swanson 2004; Kufe et al. 1984). Loss of epithelial cell polarization also results in MUC1 expression throughout the cell surface. These characteristics make MUC1 a potential target for immunotherapy (Kufe 2009). MUC1 is also expressed in hematologic malignancies such as B-cell lymphoma, chronic myelogenous leukemia, and multiple myeloma (Kawano et al. 2007; Yin et al. 2010; Yin and Kufe 2011). The N-terminal (MUC1-N) is the large extracellular domain that consists of a variable number of tandem repeats (VNTR) region and a non-VNTR region. MUC1-N is shed from cells and is present in the circulation of patients with advanced cancer. It is used as a tumor marker (CA15-3) in breast cancer patients (Hayes et al. 1986). The C-terminal of MUC1 (MUC1-C) has been shown by several groups to be extremely important in the initiation and progression of a range of human neoplasms. Overexpression of MUC1-C makes it possible for malignant cells of epithelial or hematopoietic origin to exploit the physiologic stress response and thus stimulate their expansion and survival (Uchida et al. 2013). The MUC1-C oncoprotein has also been shown to induce tamoxifen and herceptin resistance in human breast tumor cells (Fessler et al. 2009; Kharbanda et al. 2013) and predict poor outcome in breast and lung cancer patients (Lacunza et al. 2010). The MUC1-C oncoprotein has also been shown to confer androgen-independent growth in human prostate cancer cells, regulate survival of pancreatic cancer cells (Banerjee

et al. 2012), and enhance invasiveness of pancreatic cancer cells by inducing epithelial-to-mesenchymal transition (EMT) (Roy et al. 2011).

10.3.3 Prostate-Specific Antigen (PSA)

PSA is a 34-kDa glycoprotein that is expressed in normal prostate tissue and prostate cancer (Balk et al. 2003). PSA is also expressed at very low levels in the paraurethral and perianal glands, placenta, breast (including breast cancer), and thyroid. However, except for breast cancer, these tissues do not secrete a significant amount of PSA into the serum. Normally, PSA is secreted into the prostatic ducts. However, in prostate cancer, the disordered glandular architecture causes increased amounts of PSA to diffuse into the serum, allowing PSA measurements to serve as screening and prognostic markers for prostate cancer. PSA is also the most sensitive and widely used marker of response to therapy in patients with prostate cancer. The immunogenicity of PSA has been demonstrated in multiple studies. Because PSA is secreted, it is not a good target for an antibody response. However, T cells can recognize any proteins made by cells once fragments of these proteins (peptides) are processed and presented on major histocompatibility complex (MHC) molecules. It has been demonstrated that human cytotoxic T cells specific for PSA can be generated in vitro (Correale et al. 1997) and that some patients with advanced prostate cancer have naturally occurring PSA-specific T-cell responses (McNeel et al. 2001). Furthermore, Gulley et al. demonstrated that in patients with prostate cancer, a PSA vaccine can generate PSA-specific T cells that secrete interferon gamma (IFN-γ) and lyse PSA-expressing tumor cells in an MHC-restricted manner (Gulley et al. 2005).

10.3.4 Agonist Peptide Epitopes

All known TAAs are weak antigens incapable of eliciting a strong T-cell response. One proven method for enhancing the effectiveness of a vaccine that incorporates TAAs is to make alterations in the amino acid sequence of putative T-cell epitopes, altering either the T-cell binding region or the MHC anchor regions of peptides, which can potentially enhance T-cell activation and specific T-cell killing of tumor cells (Grey et al. 1995; Terasawa et al. 2002). For instance, Terasawa et al. demonstrated that changing an isoleucine to a leucine at position 155 on PSA (within the MHC binding region) made the peptide bind with higher affinity to the MHC class I molecule and produced higher levels of IFN-γ in PSA-specific T cells. T-cell lines generated with this agonist epitope efficiently lysed tumor cells expressing native PSA in an MHC-restricted manner (Terasawa et al. 2002). In addition, Zaremba et al. demonstrated that an enhancer agonist epitope of CAP1, an immunogenic human leukocyte antigen-A2 allele (HLA-A2) binding peptide

derived from CEA, could generate specific T cells from previously non-immunized individuals, whereas the native epitope could not. These T cells recognized cells expressing both the native and agonist CEA sequence and lysed tumor cells endogenously expressing native CEA (Zaremba et al. 1997). Seven novel CTL epitopes in the MUC1-C region of MUC1 have been identified recently along with enhancer agonists for each of these epitopes (Jochems et al. 2013). This was demonstrated by the greater ability of the agonist peptides, compared to their corresponding native peptides, to generate MUC1-C-specific T-cell lines, enhance IFN-γ production by T cells, and lyse human tumor cell targets endogenously expressing the native epitopes in an MHC-restricted manner. The MUC1-C agonist epitopes span class I MHC HLA-A2, -A3, and -A24, which encompass the majority of the population. The studies provide the rationale for clinical immunotherapy studies employing a range of vaccines that target the C-terminus of MUC1 and thus target the biologically relevant processes of cancer cell progression and drug resistance.

10.4 Clinical Safety Trials Employing Viral Vector Vaccines

Numerous studies have demonstrated the safety of administering a live recombinant viral vector to patients with advanced cancer. In a clinical trial in advanced colon carcinoma ($n = 26$), rV-CEA vaccine given monthly for 3 months was well tolerated (Tsang et al. 1995). While no clinical responses were observed, this study demonstrated for the first time the generation of a human cytolytic T-cell (CTL) response to specific epitopes of CEA. The CTL lines generated lysed CEA peptide-pulsed targets and tumor cells endogenously expressing native CEA. Other trials with rV-CEA have shown lack of toxicity (McAneny et al. 1996), antibody responses to a TAA (Conry et al. 2000), and the apparent equivalence of subcutaneous and intradermal vaccinations (Conry et al. 1999).

The first phase I trial of any recombinant avipox vector in cancer patients defined the safety of recombinant avipox-CEA in patients with advanced CEA-expressing carcinoma (Marshall et al. 1999). Generation of statistically significant increases in CEA-specific CTL precursors from peripheral blood mononuclear cells (PBMCs) were seen in 7/9 HLA-$A2^+$ patients after vaccination (Marshall et al. 1999; Zhu et al. 2000).

Early trials of an rV-PSA vaccine showed that it was well tolerated and could elicit specific cellular immunity (Eder et al. 2000; Gulley et al. 2002; Sanda et al. 1999). These trials also added to evidence from preclinical models that levels of antigen-specific immune responses declined after the second and third vaccinations, presumably due to a vigorous immune response to vaccinia proteins. For this reason, avipox vectors, which do not generate a significant immune response to their viral proteins, were also developed to encode PSA.

10.5 Immunostimulatory Agents

10.5.1 Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)

GM-CSF is a small, 127-amino acid protein with a molecular weight of 18 kDa and a half-life in blood of approximately 3 h. GM-CSF acts as an immune stimulant by enhancing antigen processing and presentation by dendritic cells (DCs), increasing expression of MHC class II molecules, augmenting the primary antibody response, and inducing localized inflammation when administered by injection (Warren and Weiner 2000; Chang et al. 2004). When administered intradermally with vaccine, GM-CSF elicits strong delayed-type hypersensitivity reactions to peptide antigens (Disis et al. 1996). In preclinical studies, both recombinant human GM-CSF and recombinant poxviruses expressing GM-CSF enhanced the immunological effects and antitumor activity of vaccines (Kass et al. 2001). Moreover, preclinical data suggested that optimal responses were achieved only with the use of GM-CSF. A number of small, uncontrolled clinical vaccine trials that either used GM-CSF as an immune stimulant or engineered tumor cells to secrete GM-CSF showed encouraging preliminary results in the treatment of solid tumors, including melanoma, breast carcinoma, pancreatic cancer, renal cell carcinoma, non-small cell lung cancer (NSCLC), and prostate cancer (Schmidt et al. 1997; Simons et al. 1997; Hung et al. 1998; Soiffer et al. 1998; Disis et al. 1999; Gaudernack and Gjertsen 1999; Leong et al. 1999). Other reports, however, challenge the benefits of GM-CSF and suggest that it may induce a weaker immune response (Parmiani et al. 2007). Recently, a multicenter, randomized phase II trial in 119 patients with resected stage IIB to IV melanoma was conducted with 12 MHC class I-restricted melanoma peptides given alone or in combination with GM-CSF (Slingluff et al. 2009). The multipeptide vaccine achieved high immune response rates, but $CD8^+$ and $CD4^+$ T-cell responses were lower when vaccine was administered with GM-CSF, a finding that directly challenges the utility of GM-CSF as an adjuvant. There is also some evidence that systemic use of GM-CSF can increase tumor-mediated immunosuppression by acting as a growth factor for myeloid-derived suppressor cells (Talmadge 2007). It is not known if the addition of GM-CSF can strengthen the antitumor response induced by poxviral vectors, and it is possible that the addition of TRICOM (see Sect. 10.7) to these vectors is enough to elicit a strong immune response. This possibility will be evaluated in the ongoing multinational phase III trial employing PROSTVAC with and without GM-CSF (Gulley and Kantoff 2011).

10.5.2 Cytokine Expression Driven by Poxviral Vectors

Both orthopox (vaccinia) and avipox (fowlpox and canarypox) vectors have been engineered to express GM-CSF. Direct injection of rV-GM-CSF into tumors

enhanced antitumor effects both in experimental studies (Chatterjee et al. 1999) and in clinical studies of melanoma (Mastrangelo et al. 1999; Mastrangelo et al. 2000). Fowlpox expresses transgenes for 14–21 days and does not replicate in human tissues, providing a possible safety advantage over vaccinia. A study in mice compared the biological activity of recombinant GM-CSF with that of fowlpox expressing GM-CSF (Kass et al. 2001). Compared to four daily injections of GM-CSF, a single injection of rF-GM-CSF produced significantly higher numbers of APCs, particularly DCs, in regional lymph nodes. In addition, 9 days after the last of the four daily injections, there was no evidence of increased expression of APCs in regional lymph nodes, yet increased APCs in regional lymph nodes were still observed 28 days after administration of recombinant fowlpox (rF)-GM-CSF. Of equal significance is the fact that rF-GM-CSF can be given repeatedly with the same level of biologic effect, despite the formation of antifowlpox antibodies—further evidence that antivector responses against avipoxviruses are usually not neutralizing. No anti-GM-CSF antibodies were detected in mice after three vaccinations.

10.6 T-Cell Costimulatory Molecules

The induction of a vigorous T-cell immune response requires at least two signals. Signal 1 is mediated through the peptide MHC complex on the surface of the APC that interacts with the T-cell receptor, and signal 2 is mediated through the interaction of T-cell costimulatory molecules on the surface of the APC with their ligands on the T-cell surface. Genes for costimulatory molecules can be inserted into poxviral vectors to provide additional costimulatory molecules for the APCs. T-cell costimulation is especially required in the presence of a weak signal 1, such as a TAA. To date, numerous T-cell costimulatory molecules have been identified. Preclinical studies in murine models have revealed significantly improved specific T-cell activation and tumor killing with the addition of costimulatory molecules encoded by a poxviral vector (Grosenbach et al. 2001). The majority of these studies have been conducted with the T-cell costimulatory molecules B7-1 and B7-2, using either retroviral vector constructs or antibodies directed against the T-cell ligand CTLA-4 (Chakraborty et al. 2007). B7-1 and B7-2 act in a similar fashion to enhance T-cell responses and antitumor immunity (Hodge et al. 1994). Studies were therefore conducted in which three other T-cell costimulatory molecules were evaluated for their ability to enhance antigen-specific and antitumor T-cell responses. ICAM-1, LFA-3, and CD70 were chosen for evaluation because each has an individual ligand on T cells. In a series of three publications, it was shown that rV-ICAM-1 (Uzendoski et al. 1997), rV-CD70 (Lorenz et al. 1999a), and rV-LFA-3 (Lorenz et al. 1999b) each had the ability to enhance T-cell responses and/or antitumor activity. These studies used either recombinant vector-infected irradiated tumor cells as a vaccine or costimulatory molecules admixed with rV-CEA as a vaccine.

10.6.1 Admixing Versus Dual-Gene Constructs of Recombinant Poxviral Vectors

When using vectors to deliver both signal 1 and signal 2 to APCs, it is essential that both be expressed on the same cell for effective T-cell activation. Recombinant avipoxviruses expressing both signal 1 and one or more costimulatory molecules have been constructed. Since vaccinia is a replication-competent virus, studies were conducted to determine if admixtures of various rV viruses could be used to efficiently and rapidly determine the effects of different T-cell costimulatory molecules. In a study by Kalus et al., an admixture of rV-CEA and rV-B7 was just as effective as the dual-gene construct rV-CEA/B7 in infecting APCs in vitro and generating CEA-specific T-cell responses in vivo (Kalus et al. 1999), as long as both recombinant viruses were administered at doses of 5×10^7 pfu or greater either subcutaneously or intradermally. The strategy of admixing also demonstrated a therapeutic antitumor response in a preclinical study (Akagi et al. 1997) of lung metastases expressing the MUC1 breast cancer-associated gene. Tumor-bearing mice primed with an admixture of rV-MUC1 and rV-B7 followed by two boosts with rV-MUC1 showed a significant reduction in pulmonary metastases ($P < 0.0001$), which correlated with 100 % survival compared to multiple rV-MUC1 vaccinations (Akagi et al. 1997). In several clinical trials, the addition of B7-1 into nonreplicating viruses has produced low-toxicity vaccines that generate specific immune responses (Horig et al. 2000; von Mehren et al. 2001).

10.7 TRICOM Vectors: Preclinical Models

10.7.1 In Vitro Experiments

The use of poxvirus vectors has provided a unique opportunity to evaluate the possibility that multiple costimulatory molecules can have either additive or synergistic effects in activating T cells. This is because the different costimulatory molecules must all be expressed on the same cell as the TAA to be effective and recombinant poxviruses can be constructed in which a different poxvirus promoter drives each transgene. B7-1, ICAM-1, and LFA-3 T-cell costimulatory molecules were chosen to comprise a triad of costimulatory molecules (designated TRICOM), since they have all been previously shown to bind different ligands on the T cell and signal through different pathways (Damle et al. 1992; Parra et al. 1993).

Recombinant vaccinia and avipox vectors have been designed and generated that contain transgenes for one, two, or all three of the TRICOM triad (B7-1, ICAM-1, and LFA-3), driven by different poxvirus promoters (Hodge et al. 1999). The MC38 cell line was employed as an APC in initial studies, since it lacks expression of any known costimulatory molecules. MC38 cells infected with TRICOM vectors expressed all three costimulatory molecules on their surface within 5 h, as determined by 3-color flow cytometry analysis. Moreover, levels of cell-surface expression of all

three costimulatory molecules, as determined by mean fluorescence intensity, were similar whether using TRICOM vectors or vectors expressing individual costimulatory molecules. Studies were first conducted (Hodge et al. 1999) to determine the level of activation of naive T cells in vitro using Concanavalin A (Con A) as a pharmacological signal 1. A >10-fold increase in T-cell proliferation was seen in cells infected with either rV- or avipox-TRICOM vectors compared to cells infected with vectors expressing any single costimulatory molecule.

To determine the effects of costimulation by single or multiple costimulatory molecules on cytokine production, purified $CD4^+$ and $CD8^+$ T cells were cocultured with various stimulator cells expressing either B7-1, ICAM-1, or LFA-3 singly or stimulator cells expressing TRICOM. Marked increases in the production of IL-2 by $CD4^+$ cells and IFN-γ and IL-2 by $CD8^+$ cells were observed with the TRICOM vectors (Hodge et al. 1999). Little if any increase in IL-4 production was observed. Thus, the TRICOM vectors appeared to preferentially stimulate production of T_H1 cytokines.

The pronounced levels of T-cell activation seen with the use of TRICOM vectors raised the possibility that overstimulated T cells would undergo programmed cell death (apoptosis). T cells were thus activated with Con A for signal 1 and cultured with MC38 cells that had been infected with wild-type vaccinia (V-WT), rV-B7, or rV-TRICOM. T cells activated with either MC38-B7 or MC38-TRICOM actually exhibited less apoptosis than T cells activated with either MC38 or MC38-V-WT (Hodge et al. 1999).

10.7.2 In Vivo Experiments

Transgenic mice expressing CEA (CEA-Tg) in normal gastrointestinal tissue were evaluated in vaccine strategy studies and antitumor therapy studies using vaccinia and fowlpox CEA-TRICOM vaccine (Grosenbach et al. 2001; Aarts et al. 2002). A single administration of rF-CEA-TRICOM more efficiently induced CEA-specific T-cell responses than four vaccinations with rF-CEA or two vaccinations with rF-CEA/B7-1. Furthermore, up to four vaccinations with rF-CEA-TRICOM induced greater CEA-specific T-cell responses with each subsequent vaccination. A diversified prime-boost strategy using a prime with rV-CEA-TRICOM and a boost with rF-CEA-TRICOM induced more potent CEA-specific T-cell responses than the repeated use of rF-CEA-TRICOM alone. The addition of GM-CSF to the rF-CEA or rF-CEA-TRICOM vaccinations via the simultaneous administration of an rF-GM-CSF vector enhanced CEA-specific T-cell responses. Antitumor effects were seen in CEA-Tg mice with liver metastases.

10.7.3 APC Vaccines with Poxviral Vectors

DCs' ability to induce antigen-specific T-cell responses has led many investigators to focus on the potential use of DCs in immunotherapy for cancer and infectious

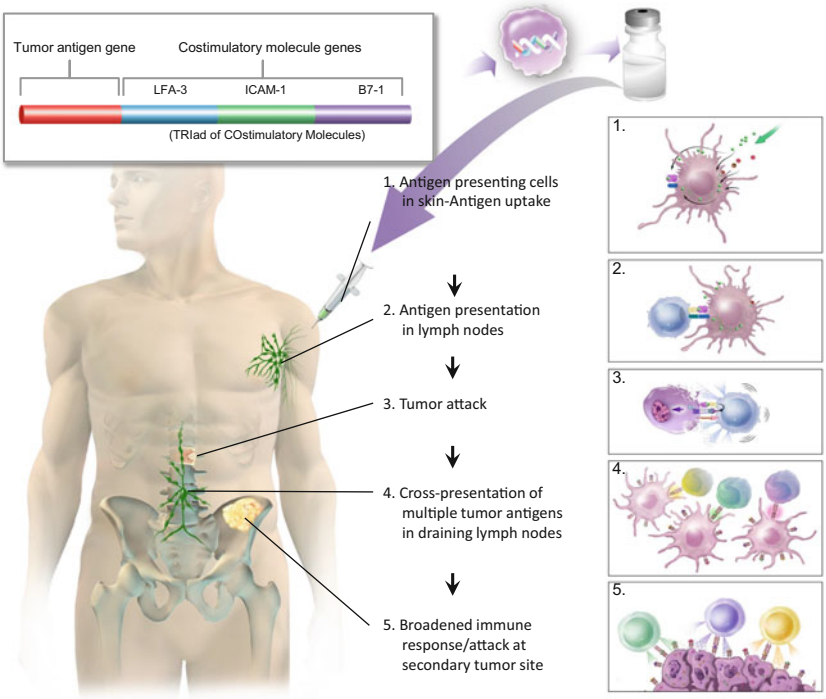

Fig. 10.1 Schematic overview of TRICOM vaccines showing the tumor antigen gene and the genes for the three costimulatory molecules LFA-3, ICAM-1, and B7-1 that are inserted within the virus. The vaccine is prepared and administered "off the shelf." 1: Subcutaneous administration leads to antigen uptake by antigen-presenting cells (APC) in the skin. 2: Antigen presentation occurs in the draining lymph nodes, activating antigen-specific T cells. 3: Tumor sites are attacked by antigen-specific cytotoxic ($CD8^+$) T cells. 4: Tumor cell lysis leads to cross-presentation of multiple tumor antigens in the draining lymph nodes (antigen spreading/antigen cascade). 5: Antigen cascade leads to activation of additional antigen-specific T cells, which increases the breadth and quite possibly the clinical activity of the antitumor response

diseases. However, the use of DCs in immunotherapy has been limited by their trace levels in the peripheral organs. Hodge et al. showed that infecting bone marrow progenitor cells (BMPCs) with vectors containing TRICOM (either avipox or vaccinia) significantly enhanced both naïve and effector $CD4^+$ and $CD8^+$ T-cell populations in mice (Rad et al. 2001). Thus, BMPCs can be made more potent APCs by the TRICOM vector-driven overexpression of the three TRICOM costimulatory molecules. This has direct implications for the use of such technology with human cells, using TRICOM vectors containing human B7-1, ICAM-1, and LFA-3 as transgenes. Human DCs for clinical trials have been prepared from $CD34^+$ progenitor cells mobilized from the bone marrow via treatment with Flt-3 ligand (Ratta et al. 1998). TRICOM vector-infected $CD34^+$ progenitor cells could potentially be made into more potent APCs. A schematic overview of TRICOM vaccines with their mode of action is given in Fig. 10.1.

10.8 TRICOM Vectors: Clinical Trials

10.8.1 Safety Studies

10.8.1.1 CEA-TRICOM

The first phase I clinical trial of the CEA-TRICOM vaccines (also including an enhancer agonist epitope (6D) within the CEA gene) was published in 2005 (Marshall et al. 2005). Patients with advanced CEA-expressing cancers ($n = 58$) were randomized to eight cohorts receiving vaccinations with different combinations of rF-CEA(6D)-TRICOM and rV-CEA(6D)-TRICOM plus GM-CSF. Vaccines were administered every 28 days for six doses and then every 3 months. Reverting to vaccination every 28 days was allowed if patients progressed on the 3-month schedule. No significant toxicity was observed. Of the 23 patients (40 %) who had stable disease for $\geq$4 months, 14 had prolonged stable disease (>6 months). Eleven patients had decreasing or stable serum CEA, and one had a pathologic complete response. Enhanced CEA-specific T-cell responses were observed in the majority of patients tested.

In another phase I study, 14 patients (11 with colorectal cancer and 3 with NSCLC) were treated with one or two cycles of four triweekly injections of ex vivo-generated DCs modified with rF-CEA(6D)-TRICOM (Morse et al. 2005). Controls consisted of immature DCs loaded with tetanus toxoid and an HLA-A2-restricted peptide derived from cytomegalovirus pp65 protein. Twelve patients completed at least one cycle of immunization. There were no grade 3/4 toxicities. One patient's CEA level went from 46 to 6.8, accompanied by a minor regression in adenopathy. Five other patients were stable through at least one cycle of immunization (3 months). Analysis of PBMCs by ELISPOT assay showed an increase in the frequency of CEA-specific T cells in 10/15 patients. Cytokine flow cytometry showed CEA-specific immune responses in both $CD4^+$ and $CD8^+$ T cells in all responders.

In a pilot safety trial, 12 patients with metastatic gastrointestinal malignancies were treated with a combination of radiotherapy and rV-CEA-TRICOM primary vaccination and rF-CEA-TRICOM boosts (Gulley et al. 2011b), and this combination was found to be safe.

10.8.1.2 CEA-MUC1-TRICOM

A pilot study of 25 patients with metastatic carcinoma demonstrated the safety of CEA-MUC1-TRICOM engineered into vaccinia (PANVAC-V) as a prime vaccination and CEA-MUC1-TRICOM engineered into fowlpox (PANVAC-F) as a boost (Gulley et al. 2008). The vaccine was well tolerated; apart from injection site reaction, no grade $\geq$2 toxicity was seen in >2 % of the cycles. Postvaccination immune responses to MUC1 and/or CEA were seen in 9/16 patients tested. One

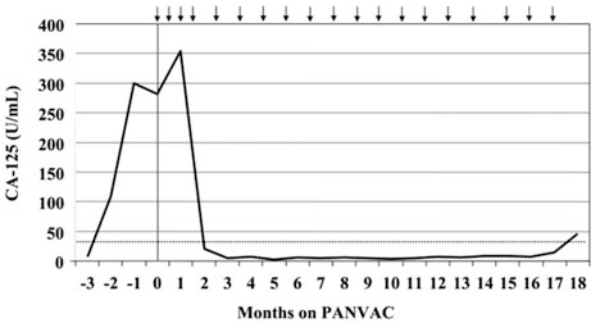

Fig. 10.2 Serum CA-125 levels from a 42-year-old patient (#22) with platinum-refractory clear cell ovarian cancer who received PANVAC-V on day 1, followed by multiple boosts with PANVAC-F (vaccinations designated by arrows). The CA-125 level decreased from a peak of 351 U/mL to less than 10 U/mL out to 18 months on study. Adapted from Gulley et al. Pilot study of vaccination with recombinant CEA-MUC-1-TRICOM poxviral-based vaccines in patients with metastatic carcinoma. Clin. Cancer Res. 14:3060–3069, 2008. American Association for Cancer Research

patient with clear cell ovarian cancer and symptomatic ascites had a durable (18-month) clinical response (Fig. 10.2), and one breast cancer patient had a confirmed decrease of >20 % in the size of large liver metastases.

10.8.1.3 PSA-TRICOM

The clinical safety of an rV prime and rF boost vaccine regimen (PROSTVAC-VF) was evaluated in a phase I trial in 10 patients with androgen-independent prostate cancer with or without metastatic disease (DiPaola et al. 2006). The vaccines contained transgenes for PSA whose amino acid sequence was modified to enhance its immunogenicity, as well as TRICOM. There were no serious adverse events. Four patients had stable disease (with a <25 % increase in PSA) during the 8-week study period. The treatment did not induce anti-PSA antibodies; however, antivaccinia titers increased in all patients.

The clinical safety of PROSTVAC-VF in combination with GM-CSF in patients with prostate cancer was evaluated in a concurrent phase I trial ($n = 15$) (Arlen et al. 2007). Some grade 2 toxicity was seen in patients who received a higher dose of rF-GM-CSF, but no toxicity exceeded grade 2. Viable vaccinia was detected after vaccination at the site swab of 1/4 patients analyzed. PSA-specific immune responses were seen with the ELISPOT assay in 4/6 patients who were HLA-$A2^+$, and decreases in serum PSA velocity were observed in 9/15 patients.

A phase I trial of intraprostatic PSA-TRICOM in men with locally recurrent or progressive prostate cancer was recently published (Gulley et al. 2013). Overall, 19/21 patients on trial had stable ($n = 10$) or improved ($n = 9$) PSA values. There was a marked increase in $CD4^+$ and $CD8^+$ T-cell tumor infiltrates in post- versus

pretreatment tumor biopsies. Four of nine patients evaluated had peripheral immune responses to PSA or NGEP (new gene expressed in prostate). Intraprostatic administration of PSA-TRICOM was found to be safe, feasible, and able to generate a significant immunologic response.

10.8.2 Phase II Trials with Vaccine Alone

10.8.2.1 PANVAC

In a phase II randomized, controlled study in patients ($n = 74$) with completely resected metastases from colorectal cancer who had received perioperative chemotherapy (Morse 2005; Morse et al. 2013), patients were randomized to receive four vaccinations with either PANVAC or DCs modified with PANVAC. There was no significant difference in 2-year recurrence-free survival among the DC arm (50 %), the PANVAC arm (56 %), and a contemporary control arm (55 %). However, at a median follow-up of 40 months, the survival rate for vaccinated patients was 81 %, which far exceeded that of the unvaccinated controls (Fig. 10.3). The results of this study warrant a randomized trial comparing poxviral vector vaccines with standard follow-up after metastasectomy. PANVAC-VF was also evaluated in a pilot study in patients ($n = 26$) with metastatic breast and ovarian carcinoma (Mohebtash et al. 2011). Patients were given monthly vaccinations, and some who had limited tumor burden and who had received minimal prior chemotherapy seemed to benefit from this treatment. One patient with breast cancer had a complete response by RECIST and remained on study for 37+ months. Another patient with metastatic disease confined to the mediastinum had a 17 % reduction in mediastinal mass and was on study for 10 months.

10.8.2.2 PSA-TRICOM

PSA-TRICOM (PROSTVAC®, Bavarian Nordic) has been evaluated in mCRPC. This off-the-shelf, vector-based vaccine consists of a prime-boost regimen (rV prime and 5–6 rF boosts). In a double-blind, placebo-controlled, 43-center, randomized phase IIB trial in mCRPC ($n = 125$), patients who received PROSTVAC had improved OS. At 3 years' post-study, 30 % of vaccinated patients were alive compared to 17 % of controls. Median OS was 8.5 months longer for vaccinated patients than for controls (25.1 vs. 16.6 months, $P = 0.0061$, hazard ratio [HR] 0.56) (Kantoff et al. 2010) (Fig. 10.4). A smaller study ($n = 32$) reported OS of 26.6 months, with a median improvement of 9.2 months over predicted survival (Gulley et al. 2010). This study also suggested that patients who mount the most vigorous immune response to vaccine may have improved OS (Gulley et al. 2010). A subsequent analysis of samples from these two studies suggested that a preexisting antibody to a glycoprotein antigen in the vector was also associated

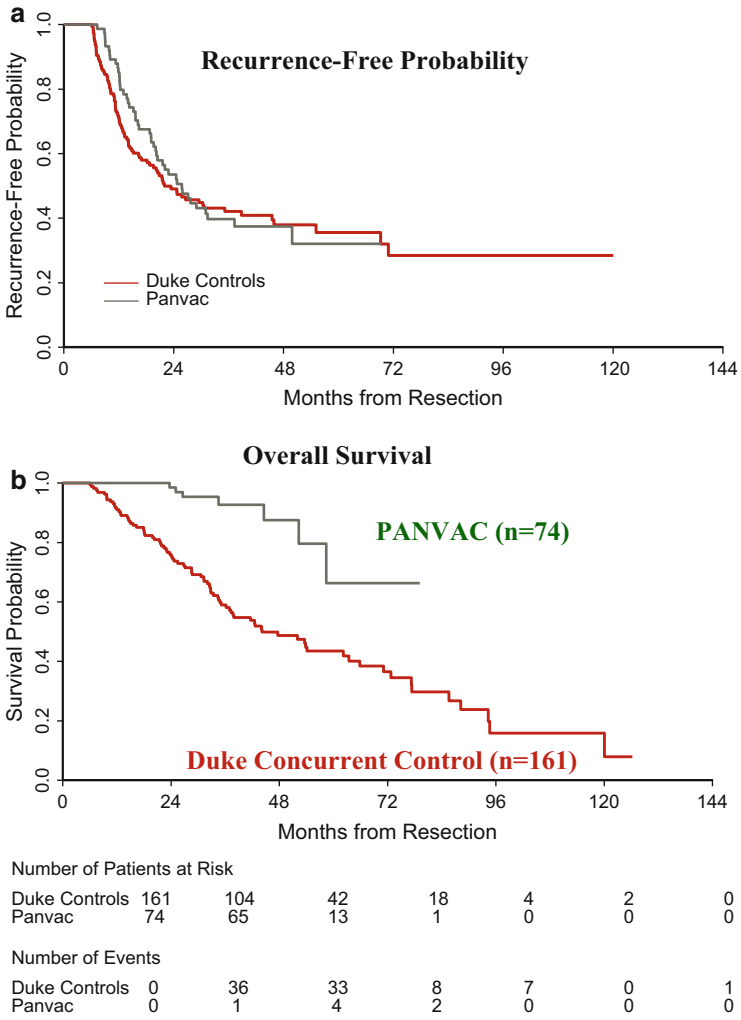

Fig. 10.3 (**a**) Recurrence-free survival (RFS) for vaccinated patients combined versus contemporary, unvaccinated controls. RFS was measured from the date of metastasectomy until documented disease recurrence at any site. Median RFS (95 % CI) was 21.9 (16.9–38.8) and 25.7 (20.0–37.2) (Fig. 10.3) months, respectively. (**b**) Overall survival (OS) for vaccinated patients combined compared with contemporary, unvaccinated controls. OS was measured from metastasectomy until death from any cause. Patients were censored on the date of last known follow-up. Median OS (95 % CI) was not reached and 44.1 (36.2–63.4) months, respectively. 95 % CI indicates 95 % confidence interval. Adapted from Morse et al. A randomized phase II study of immunization with dendritic cells modified with poxvectors encoding CEA and MUC1 compared with the same poxvectors plus GM-CSF for resected metastatic colorectal cancer. Ann. Surg. 2013. May 7 (Epub ahead of print). Wolters Kluwer Health/Lippincott Williams & Wilkins

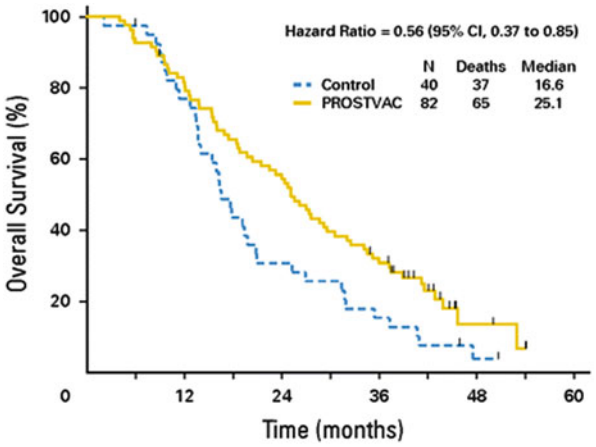

Fig. 10.4 Overall survival. Kaplan-Meier estimator for PROSTVAC (a vaccine containing two recombinant viral vectors [vaccinia and fowlpox] and three immune costimulatory molecules [B7-1, ICAM-1, and LFA-3]) arm is shown as a *solid gold line*, and estimator for the control arm is a *dashed blue line*. The small *vertical tick marks* show the censoring times. The estimated median overall survival is 25.1 months for the PROSTVAC arm and 16.6 months for the control arm. Adapted from Kantoff et al. Overall survival analysis of a Phase II randomized controlled trial of a poxviral-based PSA targeted immunotherapy in metastatic castration-resistant prostate cancer. J. Clin. Oncol. 28:1099–1105, 2010. American Society of Clinical Oncology

with improved outcome in patients treated with vaccine, but not with wild-type vector (Campbell et al. 2013).

10.8.3 Phase II Trials with Vaccine + Combination

A series of hypothesis-generating, randomized phase II trials at the National Cancer Institute are comparing standard-of-care hormonal therapy, radiation therapy, and chemotherapy, alone and in tandem with a poxviral TRICOM-based vaccine (Table 10.1). In patients with nonmetastatic CRPC, interval data favor patients receiving flutamide (an androgen receptor antagonist used as a second-line hormonal therapy) with PSA-TRICOM compared to flutamide alone. With about half of the patients enrolled on this 62-patient trial, median time to progression with flutamide alone was 85 days versus 233 days for flutamide with PSA-TRICOM (Bilusic et al. 2011). Final results of this study are expected in 2014. This trial has led to the opening of two ongoing studies evaluating the activity of enzalutamide with or without PSA-TRICOM in two different populations of prostate cancer patients (Madan 2013a, b).

Another combination study evaluated 44 patients with advanced mCRPC who had already progressed on docetaxel (Anderson and Nunez 2007). In this small phase II trial, patients were randomized to receive Quadramet® (chelated

Table 10.1 Ongoing clinical trials with TRICOM vector vaccines

Study	Trial design	Patient population	1° endpoint	NCT #
Enzalutamide ± PSA-TRICOM	Randomized phase II	Nonmetastatic CNPC	PSA kinetics	NCT01875250
Flutamide ± PSA-TRICOM	Randomized phase II	Nonmetastatic CRPC	PFS	NCT00450463
Enzalutamide ± PSA-TRICOM	Randomized phase II	Metastatic CRPC	PFS	NCT01867333
PSA-TRICOM ± GM-CSF vs. Placebo	Randomized phase III	Metastatic CRPC	OS	NCT01322490

CNPC castration-naïve prostate cancer, *CRPC* castration-resistant prostate cancer, *OS* overall survival, *PFS* progression-free survival

samarium-153, a radiopharmaceutical that delivers localized radiation to bone metastasis and is FDA approved for palliation) versus Quadramet plus PSA-TRICOM. This recently completed trial reported a statistically significant improvement in progression-free survival for Quadramet plus PSA-TRICOM (3.7 months) versus Quadramet alone (1.7 months) ($P = 0.034$, HR 0.48) (Heery et al. 2013). A follow-up study with radium-223, a recently approved radionuclide, is in the planning stages. Radium-223 appears to have less bone marrow toxicity than Quadramet and, unlike Quadramet, is associated with improved OS in men with prostate cancer.

The final results of a third combination study are revealing. This randomized phase II study of PANVAC in combination with chemotherapy (Gulley 2005) was recently reported in abstract form (Heery et al. 2012a). The study enrolled 48 patients with metastatic breast cancer and randomized them to weekly docetaxel with monthly PANVAC versus weekly docetaxel alone. Median progression-free survival (the primary endpoint) for patients randomized to the combination arm was 6.6 months vs. 3.8 months for the chemotherapy-alone arm. This randomized study suggests that the combination of PANVAC with docetaxel in metastatic breast cancer may provide greater clinical benefit than docetaxel alone. The authors suggested that the clear separation of the curves indicates potential benefit, which is not statistically significant, likely due to the small number of patients enrolled. This study, designed as a hypothesis-generating trial, may provide both rationale and statistical assumptions for a larger definitive randomized study.

10.8.4 Ongoing Phase III Trial of PSA-TRICOM

Based on the evidence of safety and immunogenicity and promising preliminary evidence of efficacy (46 % reduction in the risk of death associated with an 8.5-month improvement in OS in a randomized placebo-controlled study), a multinational phase III trial of PSA-TRICOM was initiated (Gulley and Kantoff 2011). The

study will randomize 1,200 patients in a double-blind fashion to three arms at a 1:1:1 ratio: PSA-TRICOM, PSA-TRICOM with GM-CSF, or vector control placebo. The 5-month treatment regimen will include a priming vaccination with PROSTVAC-V and six booster vaccinations with PROSTVAC-F. At the end of the treatment, other therapies for mCRPC may be initiated at the investigators' discretion. Eligible patients will be chemotherapy naïve and will have asymptomatic or minimally symptomatic mCRPC and progression despite androgen ablation. The projected trial size is 400 patients per arm for $\geq$85 % power. The primary endpoint is OS, and the final analysis will be event driven and will compare each active arm independently with placebo. Patients will be followed for 12 months after the projected number of events in each arm is realized. A secondary endpoint is the proportion of event-free (radiological progression, pain progression, chemotherapy initiation, or death) patients at 6 months after the start of treatment compared to placebo. A number of exploratory endpoints are planned, including immune response to the immunizing antigen, prostate antigens not contained in the vaccine, and TAAs, as well as changes in levels of baseline biomarkers and circulating tumor cells, and characterization of T-cell subpopulations.

10.9 Antigen Spreading

It has been suggested that a broader immune response caused by expansion of a T-cell response to epitopes not found in the vaccine may lead to a more clinically relevant antitumor immune response (Gulley 2013). This concept, known as epitope spreading, antigen spreading, or antigen cascade (Kudo-Saito et al. 2005), has been associated with both MHC class I- and II-restricted responses and reflects cross-presentation of tumor antigens. Thus, when tumor-specific T cells lyse tumor cells, the dead or dying tumor cells may be taken up by APCs, with the result that multiple, perhaps even more immunogenic, tumor antigens can be presented to immune cells, initiating a broader immune response.

As a consequence of antigen cascade, it is possible that the same vaccine may induce completely different immune responses in different patients with the same type of cancer. Furthermore, the immune response to antigens not present in the vaccine may continue over time, eventually broadening into an immune response that may be even more clinically relevant than the initial response to the epitope in the vaccine. This phenomenon has been described by many investigators, but a recently published preclinical study highlights the impact of antigen cascade (Hodge et al. 2012). In this study, mice implanted in the flank with a CEA-expressing tumor were vaccinated subcutaneously with a CEA-based vaccine. In the opposite flank, a parental tumor that did not contain CEA was implanted. An immune response to the CEA antigen in the vaccine could not directly affect the growth of the CEA^- tumor, yet vaccinated mice had a substantial decrease in the size of both the CEA^+ and CEA^- tumors compared to nonvaccinated mice. In fact, the antitumor immune response appeared to be due to T cells specific for gp70, an

endogenous murine retroviral antigen present in the tumors implanted in both flanks, but not included in the vaccine. In vaccinated mice, the IFN-γ response to gp70 was about 15 times greater than the response to the CEA present in the vaccine, suggesting that antigen cascade was critical to the activity of the vaccine in this model.

Many examples of T-cell antigen cascade have been reported in clinical trials of therapeutic vaccines in cancer patients (Madan et al. 2012; Gulley et al. 2005; Lechleider et al. 2008; Disis et al. 2009; Hardwick and Chain 2011; Walter et al. 2012), and several of these trials have suggested improved clinical outcomes for patients who demonstrated a broadened immune response (Disis et al. 2009; Hardwick and Chain 2011; Walter et al. 2012). The phenomenon of antigen cascade means that a patient treated with a therapeutic vaccine could potentially generate an immune response tailor-made to his or her individual tumor—a response that may be more clinically relevant than the response to the epitope found in the vaccine. Furthermore, unlike with traditional therapies, an ongoing, dynamic immune response can adapt to subsequent mutations within the tumor, continuing or expanding a therapeutic response. The built-in ability of an immune response to adjust to changes within the tumor, to target mutations (which may be much more immunogenic than TAAs), and to develop higher-avidity T-cell responses over time may very well provide the best opportunity for truly personalized medicine. A lack of significant side effects, along with our growing understanding of when to use this modality and what clinical outcomes to look for, indicates a bright future for therapeutic vaccines in our increasingly more sophisticated and, from a patient standpoint, better tolerated, fight against cancer.

10.10 Tumor Growth Kinetics

The clinical trials of sipuleucel-T and PROSTVAC have demonstrated a statistically significant and clinically meaningful improvement in OS in patients with mCRPC, with no associated improvement in time to progression, which may be a class effect of immunotherapies. In the context of traditional cytotoxic therapies, this may seem counterintuitive. However, it must be understood that therapeutic cancer vaccines differ from conventional therapies in several distinct ways. First, their primary target is not the tumor itself, but the immune system, which subsequently targets the tumor. It may take weeks to months to mount a clinically significant immune response following vaccine (Harty and Badovinac 2008). However, vaccines may induce the development of long-lived memory cells with the potential to provide continuous immunologic pressure that results in a slowing of the tumor's net growth rate. Within a tumor, new cells are constantly being produced, while other cells are dying. The rate of tumor growth is thus influenced by tumor biology (the intrinsic rate at which new daughter cells are formed) offset by host biology (the rate of tumor cell loss resulting from antitumor immune

response), combined with factors introduced into the tumor environment (e.g., killing of tumor cells by conventional therapies).

An effective anticancer immune response may reset the tumor growth equilibrium so that more tumor cells are killed by the immune system. This effect may not translate into objective responses or short-term improvements (within 3–4 months) in time to progression, but because this effect may be both long lasting and augmented by subsequent therapies (Schlom et al. 2007; Gulley et al. 2007), the end result may be eventual slowing of the tumor growth rate, leading to improved OS (Madan et al. 2010) (Fig. 10.5). Indeed, recently published data from prostate cancer vaccine trials at the National Cancer Institute support the concept of eventual decreased growth rate following treatment with a therapeutic vaccine (Stein et al. 2011).

This new understanding of the kinetics of the clinical response following treatment with a therapeutic vaccine, coupled with clinical experience showing that an endpoint of OS may be the only valid discriminator of activity in single-agent vaccine studies, poses a dilemma for accelerating proof-of-concept studies. Because trials with a survival endpoint typically take years to accrue and mature, identifying and validating intermediate endpoints is crucial to facilitating efficient life cycles for phase II studies in immunotherapy for prostate cancer.

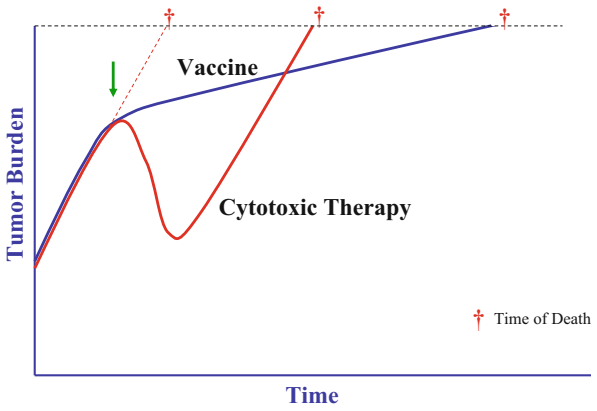

Fig. 10.5 Tumor growth is a dynamic biologic process that is the combined result of cells dividing and other cells dying. Intrinsic tumor biology, as well as extrinsic factors such as therapies, affect the tumor's growth rate. However, chemotherapy (*red line*) only affects the tumor growth rate while it is being administered, which may result in a dramatic but transient response. Following discontinuation of chemotherapy, the growth rate returns to its pretreatment slope, driven by the underlying biology of the tumor. Immunotherapy (*blue line*), on the other hand, can alter the biology of the host by inducing an active antitumor immune response including a memory response. This may not cause an immediate or dramatic change in tumor burden, but continued cumulative slowing pressure on tumor growth rate, especially if started early in the disease course, may lead to substantially longer overall survival. The *arrow* indicates the initiation of treatment; *cross* indicates time of death from cancer. Adapted from Madan et al. Therapeutic cancer vaccines in prostate cancer: the paradox of improved survival without changes in time to progression. Oncologist. 15:969–975, 2010. AlphaMed Press

10.11 Potential for Maximal Clinical Impact

10.11.1 Early Treatment and Optimal Patient Population

The recently published phase II trial in mCRPC (Gulley et al. 2010) suggests that treatment with vaccines may be more beneficial for patients with less aggressive disease. The median OS for patients in this trial was 26.6 months (nomogram-predicted median OS was 17.4 months). Patients with a predicted survival of <18 months (median predicted 12.3 months) had an actual improvement in median OS of 2.3 months, while those with a predicted survival of >18 months (median-predicted survival 20.9 months) had an actual improvement of 16.5 months, with 12/15 patients living longer than predicted ($P = 0.035$). This hypothesis-generating study thus provided evidence that patients with more indolent mCRPC (predicted survival >18 months) may benefit most from vaccine therapy. This is further supported by results from the IMPACT phase III trial of sipuleucel-T in 512 patients with prostate cancer (Schellhammer et al. 2013). In that trial, patients with lower serum PSA levels at baseline benefited significantly more from the treatment. The OS hazard ratio for patients in the lowest baseline PSA quartile (≤22.1 ng/mL) was 0.51 (95 % confidence interval, 0.31–0.85) compared with 0.84 (95 % confidence interval, 0.55–1.29) for patients in the highest PSA quartile (>134 ng/mL). Estimated improvement in median survival varied from 13.0 months in the lowest baseline PSA quartile to 2.8 months in the highest quartile. These findings suggest that patients with less advanced disease may benefit the most from sipuleucel-T while providing a rationale for immunotherapy as an early treatment strategy in sequencing algorithms for mCRPC (Schellhammer et al. 2013). One reason for these findings may be that immunotherapy slows the rate of tumor growth, which suggests that treating patients with immunotherapy earlier rather than later in the course of disease may result in far better outcomes. Patients treated with vaccines within the last 6–12 months of life (a typical phase I patient population) may not experience any clinically relevant improvement in survival from a slightly altered tumor growth rate, but those with a lower tumor burden, treated earlier in their disease course, may experience a significant improvement in OS with the same modest decrease in the rate of tumor growth (Gulley et al. 2011a) (Fig. 10.5).

10.11.2 Combination Strategies

10.11.2.1 Vaccine and Radiation

Combining vaccine therapy with other treatment modalities can enhance antitumor efficacy. Preclinical studies have shown that radiation therapy increases tumor cell expression of MHC class I, TAAs, Fas, and other surface molecules (Friedman 2002; Quarmby et al. 2000; Sheard et al. 1997; Chakraborty et al. 2003). As was

shown in CEA-Tg tumor-bearing mice, this upregulation of surface molecules can make tumor cells more susceptible to killing by antigen-specific $CD8^+$ T cells (Chakraborty et al. 2004). A phase II clinical trial in localized prostate cancer (Gulley et al. 2005) randomized patients to receive definitive radiation therapy with or without vaccine. Of 17 patients in the combination arm who completed all 8 vaccinations, 13 had $\geq$3-fold increases in circulating PSA-specific T cells, while no detectable increases in PSA-specific T cells were seen in the radiotherapy-only arm ($P < 0.0005$). Patients in the combination arm also showed evidence of de novo generation of T cells specific for prostate-associated antigens not present in the vaccine (antigen cascade), providing indirect evidence of immune-mediated tumor killing.

As described in Sect. 10.7.3, a trial of PSA-TRICOM in combination with samarium-153 (Quadramet) showed an improvement in progression-free survival for samarium-153 with PSA-TRICOM vaccine versus the radionuclide alone (Heery et al. 2012b), indicating synergy between PSA-TRICOM and bone-seeking radiopharmaceuticals.

10.11.2.2 Vaccine and Chemotherapy

Chemotherapy combined with vaccine may have a synergistic effect. Several chemotherapy drugs, including 5-fluorouracil, have been shown to upregulate MHC class I and TAAs on the surface of tumor cells, which can increase killing by $CD8^+$ T cells (AbdAlla et al. 1995; Aquino et al. 1998; Fisk and Ioannides 1998; Friesen et al. 1999; Maas et al. 1991; Matsuzaki et al. 2000; Micheau et al. 1997). In murine studies, the chemotherapy agents cyclophosphamide, doxorubicin, paclitaxel, and docetaxel enhanced antitumor immune response to a whole tumor cell vaccine (Chu et al. 2006; Machiels et al. 2001). Immune enhancement may occur through several possible mechanisms. For example, preclinical studies have suggested that doxorubicin increases macrophage antitumor activity (Maccubbin et al. 1992) and docetaxel treatment has been shown to increase cytokine production, which promotes inflammation (Chan and Yang 2000). Another important mechanism of immune enhancement is negative regulation of immunosuppressive entities such as regulatory T cells (Tregs). Cancer patients have increased levels of Tregs compared to healthy controls, and in cancer patients, Tregs appear to have increased suppressive function (Huen et al. 2013). Low-dose cyclophosphamide given prior to immunotherapy reduced both the level and function of Tregs in preclinical and clinical studies (Lutsiak et al. 2005; Ghiringhelli et al. 2007). Some chemotherapeutic agents may also kill tumor cells in a way that promotes their uptake by DCs and subsequent activation of cytotoxic $CD8^+$ T cells (Lake and van der Most 2006).

10.11.2.3 Vaccine and Immune Checkpoint Inhibitors

Ipilimumab (YERVOY®, Bristol-Myers Squibb) is a human IgG1κ monoclonal antibody that targets cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4). Ipilimumab was the first in a class of therapies targeting T-cell activation and regulation to be licensed in the broad category of agents known as immune checkpoint inhibitors, based on improved OS in patients with metastatic melanoma (Hodi et al. 2010). Ipilimumab has also previously been investigated for the treatment of prostate cancer in a pilot trial of patients with hormone-refractory prostate cancer (Small et al. 2007). They found a PSA decline of ≥50 % in 2/14 patients and concluded that further investigations were warranted. Ipilimumab alone or in combination with radiotherapy was also investigated in a recently reported phase I/II trial of 75 patients with mCRPC (Slovin et al. 2013). Both PSA decline and tumor response were observed, and 8/34 patients in the 10 mg/kg ± radiotherapy group had a confirmed PSA decline of ≥50 %. Of these, six had received prior chemotherapy, and two were chemotherapy naïve. One of the tumor-evaluable patients in the 10 mg/kg ± radiotherapy group achieved a confirmed complete response, and two patients achieved an unconfirmed partial response. Six patients had stable disease. The median OS was 17.4 months (Slovin et al. 2013). A unique set of toxicities referred to as immune-related adverse events (irAEs) has been seen with the use of anti-CTLA-4 antibodies, including infiltration of inflammatory cells into nonsterile epithelial surfaces (i.e., colon and skin, which likely have ongoing immune activity) and endocrine organs (i.e., thyroid, adrenals, and pituitary, which have been associated with autoimmune disease). In most instances, these irAEs can be readily managed medically.

A phase I dose-escalation trial of ipilimumab in combination with PSA-TRICOM in patients with mCRPC recently demonstrated encouraging activity (Madan et al. 2012). Patients ($n = 30$) were treated with escalating doses of ipilimumab and a fixed dose of vaccine. Of 24 chemotherapy-naïve patients, 58 % had a PSA decline. The median OS was 34.4 months, compared with a nomogram-estimated median OS of 18 months. Combination therapy did not exacerbate the irAEs associated with ipilimumab.

Further trials are needed to evaluate combination therapy with the immune checkpoint inhibitors anti-PD-1 and anti-PD-L1, which have recently shown promising antitumor activity (Topalian et al. 2012; Brahmer et al. 2012).

10.12 Vaccine Therapy Compared to Adoptive T-Cell Transfer

Different modes of immunotherapy that target the same tumor antigen can lead to contrasting results. This can be exemplified in a series of studies, both preclinical and clinical, involving vaccine therapy and adoptively transferred T cells targeting

CEA (Table 10.2). Both modalities used human CEA-Tg mice, where CEA is a self-antigen, for the preclinical studies.

In one study, an avipox vector (with no costimulatory molecules)- or DNA-based vaccine against CEA was incapable of rejecting CEA^+ tumors (Bos et al. 2007), and it was concluded that effective tumor targeting could only be achieved by adoptive transfer of T cells (Bos et al. 2008). However, this treatment resulted in severe intestinal autoimmune pathology associated with weight loss and mortality. In a clinical study (Parkhurst et al. 2011), autologous T cells were genetically engineered to express a T-cell receptor directed against a specific human CEA epitope and adoptively transferred to three patients with metastatic colorectal cancer. All patients had decreases in serum CEA levels (74–99 %), and one patient had an objective regression of metastases to the lung and liver. However, severe transient inflammatory colitis represented a dose-limiting toxicity that was induced in all three patients. The authors concluded that this study demonstrated the limitations of using CEA as a target for cancer immunotherapy.

These studies are contrasted with the use of the CEA-TRICOM vaccines. Tumor-bearing CEA-Tg mice received a prime vaccination with rV-CEA-TRICOM and multiple booster vaccinations with rF-CEA-TRICOM. Antitumor immunity led to cure in approximately 60 % of mice, in the absence of any autoimmunity (Hodge et al. 2003). Indeed, no evidence of toxicity was seen in clinical serum and urine assays and histopathologic evaluation of all tissues after one year. Similar results were also obtained in CEA-Tg mice crossed with mice bearing a mutation in the APC gene. These mice develop spontaneous intestinal tumors. Vaccination resulted in improved overall survival in the absence of autoimmunity (Greiner et al. 2002). A third study (Zeytin et al. 2004) demonstrated that CEA-TRICOM vaccination in combination with celecoxib elicits antitumor immunity and long-term survival in CEA-Tg/MIN mice in the absence of autoimmunity.

Table 10.2 Comparison of two modes of immunotherapy targeting CEA

Therapy	Preclinical or clinical	Therapeutic benefit	Toxicity	Reference
Avipox/DNA vaccine	Murine	No	No	Bos et al. (2007)
Adoptive T-cell transfer	Murine	Yes	Yes, autoimmunity	Bos et al. (2008)
rV-, rF-TRICOM	Murine	Yes, survival	No	Hodge et al. (2003)
rV-, rF-TRICOM	Murine	Yes, survival	No	Greiner et al. (2002)
Adoptive T-cell transfer	Phase I	Yes, RECIST	Yes, autoimmunity	Parkhurst et al. (2011)
rV-, rF-TRICOM	Phase II multicenter	Yes, survival	No	Morse et al. (2013)

RECIST Response Evaluation Criteria in Solid Tumors

Several clinical studies have now shown evidence of antitumor immunity in metastatic cancer patients employing CEA-TRICOM- and CEA-MUC1-TRICOM-based vaccines in the absence of autoimmunity (Marshall et al. 2005; Morse et al. 2013). In a phase II trial, patients with metastatic colorectal cancer to the liver and/or lung were vaccinated with PANVAC vaccine following metastasectomy (Morse et al. 2013). At 40 months' follow-up, 90 % of the vaccinated patients survived versus 47 % in the contemporary control group. Moreover, no evidence of autoimmunity was reported in the patients receiving PANVAC.

These studies demonstrate the balance that can be achieved between the induction of an antitumor immune response to a self-antigen and the absence of autoimmunity and illustrate the distinctions between different forms of immunotherapy targeting the same antigen.

10.13 Conclusions

Poxviral vectors generate a robust immune response to TAAs incorporated within the virus, and the use of multiple T-cell costimulatory molecules further enhances this therapeutic immune response. Preliminary clinical data show that this immunologically sophisticated platform is well tolerated and has early evidence of clinical effectiveness. The fact that these vaccines have minimal side effects allows us to test them in the early stages of disease, when patients are asymptomatic and thus may be reluctant to undergo treatment with agents that have significant toxicities.

Recent data from large clinical studies are increasing our understanding of the patient populations most likely to benefit from poxviral-based immunotherapies, as well as expected outcomes. Used alone, therapeutic vaccines are unlikely to induce initial tumor shrinkage but may nonetheless lead to improved OS by slowing the rate of tumor growth through continued immunologic pressure that may persist long after vaccination. Clinical data also suggest that vaccines are optimally effective in patients with low tumor volume. This new paradigm points toward more rational clinical trial design, the use of vaccines earlier in the disease course, and combinations with other therapies.

The combination of therapeutic vaccines with immune checkpoint inhibitors holds particular promise, since immune checkpoint inhibitors have led to long-term disease-free survival in a proportion of patients. If a modality designed to nonspecifically magnify an underlying immune response can produce this outcome, then its combination with a therapeutic vaccine designed to generate a specific antitumor immune response has the potential to substantially prolong long-term disease control for many patients. Initial studies with poxviral vectors have demonstrated the safety and feasibility of such an approach, and efficacy studies are currently being planned.

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Chapter 11
The Use of Oncolytic Herpesvirus for the Treatment of Cancer

Tasha Hughes and Howard L. Kaufman

Abstract Oncolytic immunotherapy is a new form of cancer treatment that utilizes native or genetically modified viruses to directly infect tumor cells. These viruses selectively replicate in tumor cells and may induce systemic antitumor immune responses. To date, the herpesviruses have been the most widely evaluated for clinical development as a cancer therapeutic. A modified herpesvirus encoding human granulocyte-macrophage colony-stimulating factor (GM-CSF) has been named talimogene laherparepvec (T-VEC) and has shown clinical benefits in a randomized Phase III clinical trial in patients with advanced melanoma. This chapter will review the basic mechanisms of oncolytic viruses, describe the basic biology of herpesviruses, and discuss the clinical results of trials with T-VEC. Future directions and priorities for clinical development of T-VEC and other oncolytic viruses will be discussed.

11.1 General Principles of Oncolytic Virotherapy

Viruses are potentially pathogenic agents that have several unique properties that can be exploited for the treatment of cancer. Native and genetically modified viruses can be used to selectively target and replicate in tumor cells providing a highly targeted strategy for identifying tumor cells and preventing injury to normal tissues. Viruses can be modified by viral gene deletion resulting in safer and more immunogenic vectors, while foreign transgenes can be engineered for viral expression resulting in highly immunogenic and more therapeutically effective agents. Viruses that selectively kill tumor cells are generally referred to as oncolytic viruses, and oncolytic immunotherapy is evolving as a new therapeutic paradigm for cancer therapy.

T. Hughes
195 Little Albany Street, New Brunswick, NJ 08901, USA

H.L. Kaufman (✉)
Rutgers Cancer Institute of New Jersey, 195 Little Albany Street, New Brunswick, NJ 08901, USA
e-mail: howard.kaufman@rutgers.edu

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Oncolytic viruses and immunotherapy are distinct from previous recombinant vaccine strategies because intra-tumoral injection of these vectors obviates the need for using defined tumor-associated antigens to prime an adaptive immune response. Although the specific mechanism(s) of tumor regression following oncolytic immunotherapy remains incompletely understood, several features of local viral delivery underscore the putative processes that promote tumor eradication with these agents. First, oncolytic viruses can infect and selectively replicate in tumor cells resulting in direct killing of tumor cells. Oncolytic viruses typically utilize a selective entry receptor and may naturally or through genetic modification, selectively replicate only in tumor cells resulting in a direct lytic effect. In some cases, the selective replication is dependent on tumor cell-expressed entry receptors, aberrant antiviral response elements in transformed neoplastic cells, and the availability of a larger nucleic acid pool in tumor cells that can be utilized for viral replication. Following cell lysis, mature viral particles are released and can enter nearby tumor cells leading to a replication cascade and amplification of cell death throughout a tumor mass. The spread of an oncolytic infection in this manner can have a profound impact even on large tumor volumes.

Secondly, oncolytic viruses have the capacity to induce local and systemic antitumor immune responses, which can further mediate tumor rejection of infected and possibly uninfected tumor cells. The local response develops when tumor-associated antigen-presenting cells, such as dendritic cells, engulf viral particles and tumor-associated antigens from dying cells that are subsequently recognized and taken up to prime helper and cytotoxic T lymphocytes. The priming of tumor-specific T cells can lead to rejection of tumors in an antigen- and HLA-specific manner. The presence of virus-specific and tumor-specific cytotoxic T cells within the tumor microenvironment results in local release of perforins and granzyme B, which may kill additional tumor cells through a bystander effect. Although less well understood, the local immune response can be amplified to generate broader tumor-specific immune cells that can induce distant antitumor immunity in some cases. The rejection of uninjected tumors has been seen in both murine tumor models and reported in clinical trials with oncolytic viruses, supporting the notion that oncolytic immunotherapy can induce systemic antitumor immunity.

While a variety of oncolytic viruses have been identified and indeed many have entered into clinical cancer trials, including adenovirus, Newcastle disease virus, vaccinia virus, Coxsackie A virus, and others, the herpesviruses are the furthest along in clinical development and will be the focus of this chapter. We will discuss the basic biology of herpesviruses, describe the development of the an oncolytic herpesvirus vector encoding the human granulocyte-macrophage colony-stimulating factor (GM-CSF) termed talimogene laherparepvec (or T-VEC) for oncolytic immunotherapy, report clinical trial results of T-VEC, and briefly characterize some of the other oncolytic herpesviruses in clinical development (Fig. 11.1).

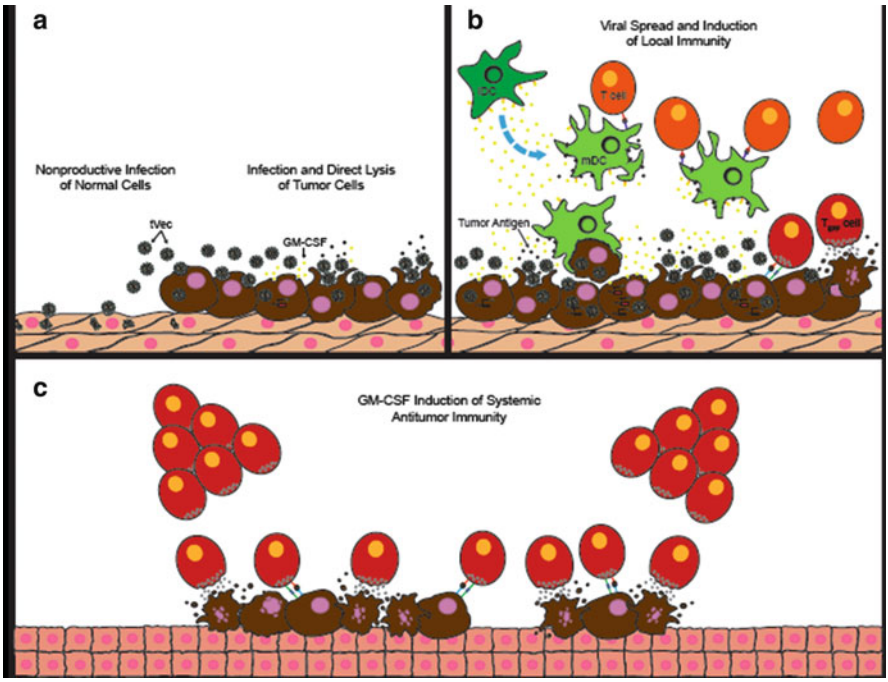

Fig. 11.1 Dual mechanism of action with oncolytic virus immunotherapy. T-VEC selectively infects and replicates in tumor cells, causing cell lysis and local release of GM-CSF, while the virus cannot replicate in normal cells (**a**). The virus causes lytic death of tumor cells and release of tumor-associated antigens which can be engulfed by immature dendritic cells (IDC), and the antigen-loaded IDC are matured in the presence of local GM-CSF (**b**). Mature DC can initiate a tumor-specific T-cell response, which can result in regression of injected tumor (**b**) and distant tumor cells (**c**)

11.2 Basic Biology of Herpesviruses

Herpes simplex virus type 1 (HSV-1) is the prototypical herpesvirus and has a large, double-stranded DNA genome with significant portions of the genome coding nonessential genes. The DNA genome is housed within a central core surrounded by a glycoprotein-rich envelope and a capsid. The tegument is a small area that exists between the envelope and the capsid. HSV-1 is a minor human pathogen that enters the human host through mucosal surfaces using several well-described receptors. The first of these receptors is the herpesvirus entry mediator (HVEM), a member of the TNF superfamily of proteins and widely expressed on natural killer (NK) cells and CD8+ T cells. To a lesser degree, HVEM is also expressed on CD4+ T cells and dendritic cells. A second set of receptors are from the nectin family, which is part of the IgG superfamily. These receptors are expressed on a wider variety of cell types than HVEM and occur on mucosal epithelial cells. A third herpes receptor is heparan sulfate, which can bind to gylcoproteins on the surface of

viral particles. The virus is able to replicate quickly in host cells and induce cell lysis. An additional feature of HSV-1 that makes it an ideal vector is the absence of insertional mutagenesis during viral replication.

In humans, HSV-1 infection is associated with relatively mild skin conditions, including the common cold sore or fever blister. Once the virus has entered through the mucosal surface, it then can travel by retrograde flow into axons of neurons and remain dormant there for many years. While the neurons lack the nucleic acid pool necessary for viral replication, there are some latency-associated protein products produced by the virus in this dormant phase that ensure viral survival and are utilized during reactivation of viral infection. Most commonly, reactivation occurs during periods of host stress. Once reactivated, viral particles travel anterograde down the axon and reenter epithelial cells and produce herpetic skin lesions. An important feature of HSV-1 is its immunogenicity and ability to induce both neutralizing antibody as well as host T-cell responses following infection.

11.3 The Development of Oncolytic Herpes Virotherapy

There are several strains of herpesvirus that have been used for oncolytic activity and clinical development. These strains and clinical results will be briefly characterized herein.

11.3.1 HSV17+ (HSV1716) and G207

The HSV1716 and G207 strains are derived from serially passaged copies of HSV-1 and have a deletion of the viral ICP34.5 neurovirulence factor. This deletion renders these viruses replication competent but nonpathogenic in non-tumor cells. To date, results are limited to Phase I trials in malignant glioma and melanoma. HSV 1716 has been studied in three Phase I trials of patients with glioma. The first study treated nine patients with intracerebral injections and found that no patients developed reactivation of HSV skin lesions and no patients developed HSV encephalitis (Rampling et al. 2007). A second trial confirmed the safety and tolerability of the injections and also confirmed survival and replication of the virus within injected tissue as well as at distant tumor sites (Papanastassiou et al. 2002). A third Phase I trial of 12 patients again confirmed tolerability in glioma patients. In this study, 10/12 patients had HSV DNA by PCR in the injected site and 4/12 patients had evidence of HSV at distant tumor sites. This study also reported 3/12 patients to be alive and clinically stable at 22, 18, and 15 months postinjection (Harrow et al. 2004). Phase I trials of strain G207 in recurrent malignant glioma found no toxicity associated with intracerebral injection in 21 patients treated with varying doses of virus. In a follow-up Phase Ib study of six patients, different doses and modes of injection were studied, and again, no viral encephalitis was noted in any of

the treated subjects. In addition, HSV was detected in all six patients by PCR, and in 50 % of patients, there was evidence of viral replication at the site of injection (Markert et al. 2009).

11.3.2 NV1020

The NV1020 strain is a wild-type HSV-1 with multiple mutations in the viral genome resulting in enhanced attenuation. NV1020 contains two deleted regions, an internal repeat of the UL56 gene and the gene-encoding viral thymidine kinase. The UL56 gene product is a type II membrane protein involved in axonal transport of viral envelope glycoproteins. While the viral thymidine kinase gene is deleted, a functional thymidine kinase gene was inserted into the UL56 position, rendering the virus replication competent but less pathogenic (Kemeny et al. 2006). In a Phase I dose-escalation trial of 12 patients with colorectal cancer metastatic to the liver, this strain was given intra-arterially through the hepatic artery in combination with cytotoxic chemotherapy. Long-term follow-up of these patients, although not the primary endpoint of the study, found that all patients had at least a partial response to therapy with tumor reduction between 39 and 81 % (Fong et al. 2009).

In a second Phase I/II trial, 13 patients with hepatic metastases from colon cancer were treated, and the optimum dose for hepatic artery infusion was determined to be 10^8 plaque-forming units (pfu)/ml. This dose was then administered, in conjunction with conventional chemotherapy, to 22 patients in the Phase II portion of the study. Eleven out of 22 patients had stable disease, and one patient had a partial response. The median time to progression was 6.4 months (Geevarghese et al. 2010).

In each of these studies, the most common adverse events were pyrexia, fatigue, and myalgia, and they were mild in most cases. In both trials, only one serious adverse event, a grade 4, transient elevation in GGT, was noted. Even in patients who had HSV-1-positive swabs of the skin or saliva, they did not manifest any symptoms of viral infection.

11.3.3 HF10

The HF10 HSV-1 strain has two deletions that result in two incomplete copies of the UL56 gene. The function of UL56 is described previously, and the lack of UL56 protein product significantly reduces the neurotoxic effects of HSV-1 without affecting viral replication. In a small pilot study of six patients with cutaneous or subcutaneous metastases from breast cancer, the HF10 virus was injected intratumorally (Kimata et al. 2006). One of six patients had a significant reduction in tumor size, and histopathologic analysis revealed a high degree of necrosis within

injected tumor sites. This therapy was well tolerated, and no adverse events were reported in the six patients treated.

11.3.4 rRp450

The rRp450 strain has a deletion of the herpesvirus UL39 gene, which codes for the large subunit of ribonucleotide reductase. This deletion allows selective viral replication in rapidly dividing cells. The deleted segment was replaced with the CYP2B1 gene, which encodes for an enzyme in the cytochrome p450 cascade that activates cyclophosphamide and related chemotherapeutic agents. In this way, the virus was engineered to help potentiate the antitumor effect of concomitant cytotoxic chemotherapy treatment (Pawlik et al. 2000). This herpesvirus strain is unique because it has its own direct lytic effects on tumor cells while also having the ability to enhance chemotherapy. A Phase I trial of rRp450 in patients with primary and metastatic liver malignancies is underway. The virus will be administered via the hepatic artery, similar to the NV1020 clinical trial. To date, no safety, tolerability, or efficacy data is available for this strain.

11.4 JS1 and Talimogene Laherparepvec

The JS1 strain of HSV-1 was originally isolated from a cold sore in a reactivated infection and was selected for clinical development based on in vitro data demonstrating superior cell lysis at lower doses than comparable herpesvirus strains (Liu et al. 2003). Additionally, JS1 was found to be less virulent compared to wild-type HSV-1 as demonstrated in both in vitro and in vivo models. The JS1 strain demonstrates limited growth in culture media known to foster growth of wild-type HSV-1. In animal models, JS1 exhibits limited or no pathogenicity, suggesting that the JS1 strain is less virulent than wild-type HSV-1. Further, the JS1 strain was unable to replicate in normal, non-tumor, eukaryotic cells adding to the advantageous features of this strain (Liu et al. 2003).

Talimogene laherparepvec (T-VEC) was generated from the JS1 strain with several additional modifications. First, T-VEC has a deletion of the viral ICP34.5 gene. ICP34.5 is a neurovirulence factor and interacts with proliferating cell nuclear antigen (PCNA) for DNA repair and replication. This deletion makes the virus less pathogenic as well as enhancing selective replication in tumor cells. In the setting of a large pool of nucleic acids (i.e., tumor cells), the virus is able to replicate despite this deletion, rendering the virus incompetent in normal eukaryotic cells but replication competent in tumor cells (Liu et al. 2003). In vitro studies directly comparing JS1 and HSV-1 17+ strains both deficient in this neurovirulence factor demonstrated improved tumor cell lysis with the JS1/ICP34.5− strain compared to the HSV-1 17+ strain (Table 11.1).

Table 11.1 Genetic alterations in talimogene laherparepvec (T-VEC)

Gene alteration	Function in normal HSV-1 infection	Antitumor effect in T-VEC
ICP34.5 deletion	Neurovirulence factor; DNA repair and replication due to interaction with proliferating cell nuclear antigen (PCNA)	Elimination of infectivity and virulence in non-tumor cells; selective replication in tumor cells with low PCNA expression
ICP47 deletion	Blocks antigen presentation, allowing virus to evade immune response	Enhanced antigen presentation, alteration of US11 promoter gene expression
Addition of coding gene for GM-CSF production	N/A	Production of human GM-CSF, which is thought to increase both local and systemic immune responses

T-VEC also harbors a second deletion of the viral ICP47 gene, whose gene product is responsible for immune evasion by blocking antigen presentation during HSV infection. The exact mechanism is through downregulation of MHC class I molecules on the surface of infected cells through interference with the transporter associated with antigen processing (TAP). Deletion of ICP47 therefore increases antigen presentation by infected cells and enhances immunogenicity. Deletion of ICP47 also alters the normal modulatory effect of ICP47 on the herpesvirus US11 promoter. ICP47 deletion results in immediate early expression of the US11 promoter, which then inhibits protein kinase R (PKR). Additionally, many tumor cells have weak or absent expression of PKR. Combined, the effects of decreased overall PKR results in promotion of viral replication. In animal models of nude Balb/c mice, there was enhanced therapeutic activity of JS1/ICP34.5−/ICP47− against multiple established tumor models, including HT-29 colon adenocarcinoma, FaDu (hypopharyngeal) carcinoma, and U87MG gliomas (Liu et al. 2003).

T-VEC is further modified by the insertion of the gene-encoding human GM-CSF. GM-CSF is thought to enhance the immunogenicity of the virus through recruitment, activation, and maturation of macrophages and dendritic cells in the local tumor environment. Although less well defined, the addition of GM-CSF may also contribute to the induction of a systemic immune response. The addition of GM-CSF was evaluated in a mouse tumor model using the A20 cell line. In this model, bilateral flank tumors were established and the right flank lesions were injected with JS1/ICP34.5−/ICP47− or JS1/ICP34.5−/ICP47−/GM-CSF+ viral strains. In both groups, the right flank injected lesions regressed comparably, but only in the GM-CSF+ group, was there significant regression of tumor in the contralateral left flank, supporting a systemic immune effect of the T-VEC strain. Splenocytes from T-VEC-injected mice had a higher concentration of IFN-γ-producing T cells, suggesting enhanced cellular immunity in these animals (Liu et al. 2003).

GM-CSF has been widely studied for its immunotherapy potential but still has an ill-defined role in its overall potential as a cancer therapeutic. Complete reviews of

GM-CSF in the setting of cancer therapy are available elsewhere (Grotz et al. 2014; Spitler et al. 2000, 2009). Although GM-CSF is classically understood to stimulate the immune system, there is emerging evidence that it may also have immunosuppressive effects, particularly when administered at high doses. Animal models have demonstrated this using a dose-escalation design in which tumor-bearing mice treated with low-dose GM-CSF had improved survival compared to untreated mice, while those mice treated with high-dose GM-CSF did not experience any therapeutic benefit (Serafini et al. 2004b). Evaluation of T-cell populations in this model revealed two important findings. First, tumor-specific CD4+ T cells did not expand in mice treated with high-dose GM-CSF. Second, transient expression of myeloid-derived suppressor cells (MDSC) were detected in mice treated with high-dose GM-CSF. These MDSC were further evaluated and a specific population of CD11b+GR1+ cells were identified and were able to suppress the function of tumor-specific T cells, which may have accounted for the impaired therapeutic responses seen in mice treated with high-dose GM-CSF. CD14+CD11b+MDSC were also identified in patients treated with a peptide vaccine and adjuvant GM-CSF (Serafini et al. 2004a). Others have also seen an expansion of CD14 +HLA-DR-lo MDSC following treatment with recombinant GM-CSF (Filipazzi et al. 2007). In a model of GM-CSF-secreting whole tumor cell vaccines, GM-CSF was shown to increase secretion of the milk fat globule protein EGF 8 (MFG-E8), and this factor was responsible for suppressing T-cell responses. Additionally, immunologic and therapeutic responses were improved by co-treatment with MFG-E8 blockade prior to GM-CSF administration (Jinushi et al. 2007).

Animal models have provided support for the role of GM-CSF as an adjuvant to tumor vaccines (Dranoff et al. 1993; Disis et al. 1996). Immunologic responses in patients treated with vaccines containing GM-CSF regimens have varied with conflicting outcomes on antigen-specific T-cell responses. In a Phase II trial, patients with melanoma were treated with a variety of MHC class I-associated peptide vaccines with or without GM-CSF, and peptide-specific T-cell responses were evaluated in both the peripheral blood and vaccine-draining lymph nodes. T-cell response was found to be greater in patients receiving vaccine with GM-CSF (Slingluff et al. 2003). In contrast, studies comparing whole tumor cell vaccines did not show convincing evidence of an enhanced immune response in patients receiving vaccine containing GM-CSF, compared to patients receiving vaccine alone (Carson 2005). In a randomized trial comparing a multipeptide vaccine regimen, consisting of 12 MHC Class I-restricted melanoma-associated peptides, with and without the addition of GM-CSF, CD8+ and CD4+ T-cell responses were lower in patients treated with GM-CSF compared to vaccine alone (Slingluff et al. 2009). GM-CSF has also been used as an adjuvant for prostate cancer vaccines with conflicting results. The FDA-approved sipuleucel-T is an autologous dendritic cell vaccine that is modulated ex vivo to express the prostate acid phosphatase antigen and GM-CSF, and the vaccine regimen has shown therapeutic benefits in patients with advanced castrate-resistant prostate cancer. Other prostate cancer vaccine trials, however, have not adequately demonstrated that GM-CSF improved outcomes for vaccination (Small et al. 2000, 2006). In addition to its use as a

vaccine adjuvant, GM-CSF has been evaluated as a single-agent therapy for patients with Stages III and IV melanoma. In early uncontrolled clinical trials, GM-CSF demonstrated prolonged overall and disease-free survival in treated patients compared to matched historical controls (Spitler et al. 2000, 2009). These data have not been replicated in a prospective, randomized clinical trial. The conflicting results may be due to the immune-modulating effects of GM-CSF where activation and suppression of T-cell immunity are related to the dose and context of GM-CSF expression within the local tumor microenvironment or tumor-draining lymph node basin. Thus, the current clinical data is not consistent and further research is needed to better define the role, optimum dose, and schedule for GM-CSF as a vaccine adjuvant. GM-CSF may be more helpful in oncolytic immunotherapy because viral-encoded GM-CSF levels may be optimal for promoting T-cell priming and the viral infection may alter the milieu in which T cells are being primed favoring activation of T-cell immunity.

11.5 Results of Clinical Trials of Oncolytic Herpesvirus Encoding GM-CSF

T-VEC has been the most widely tested oncolytic virus for human cancer and initial studies focused on melanoma because of the lytic efficiency of T-VEC against melanoma cells in vitro, the accessibility of melanoma tumors to injection, and the inherent immunogenicity of melanoma. An initial Phase I clinical trial enrolled 30 patients with melanoma, breast, gastrointestinal, or head and neck malignancies (Hu et al. 2006; Table 11.2). All patients had accessible lesions for intra-tumoral

Table 11.2 Summary of clinical trials of talimogene laherparepvec (T-VEC)

Phase (N)	Malignancy treated	Available results	PMID
Phase I (30)	Melanoma, breast, gastrointestinal, head and neck	3/26 evaluable patients had SD	17121894
		Established 10^8 pfu/ml as starting dose and 10^8 pfu/ml as maximum tolerated dose	
Phase II (50)	Melanoma	85 % of patients with toxicity limited to fever and local injection site reactions	19884534
		26 % ORR	
		58 % 1-year survival	
Phase I/II (17)	Squamous cell of the head and neck	23.5 % CR, 58.8 % PR	20670951
		100 % local control rate	
Phase III (436)	Melanoma	Primary endpoint was met with 16 % durable response rate	Not available
		26 % ORR	

PMID PubMed ID number, *SD* Stable Disease, *pfu/ml* plaque forming unit per milliliter, *ORR* objective response rate, *CR* complete response, *PR* partial response

injection. Safety, dosing, and objective response rates were evaluated. Thirteen patients received single-dose regimens (10^6, 10^7, or 10^8 plaque-forming units (pfu)/ml) and 17 patients received various multidose regimens. Pyrexia and local injection site erythema were the most common side effects and these were not dose-dependent. Based on major injection site reactions, 10^7 pfu/ml was determined to be the maximum tolerated dose among HSV seronegative patients. However, seronegative patients treated with an initial lower dose of 10^6 pfu/ml were then able to tolerate 10^8 pfu/ml following seroconversion. Based on these results, future studies employed this combined dosing regimen of an initial 10^6 pfu/ml dose for seroconversion with subsequent doses of 10^8 pfu/ml. This study also took viral swabs of injection sites to evaluate viral clearance. At 2 weeks postinjection, virus was still present at the injection site, suggesting 2–3 weeks to be an adequate dosing schedule for implementation in multidose cohorts as well as future studies. In this study, a subset of 19 patients had posttreatment biopsies. Of these, 14/19 had convincing pathologic evidence of tumor necrosis or apoptosis. All tumors were also stained for the presence of HSV. The same 14 biopsies demonstrating significant tumor cell death stained positive for HSV, while biopsy specimens without evidence of either residual tumor or cell death did not have evidence of HSV. Clinical response data was available in 26 patients. Stable disease was reported in 3/26 patients, and no patients were determined to have partial or complete responses.

A subsequent multi-institutional Phase II trial enrolled 50 patients with unresectable (Stage IIIc or IV) melanoma. Based on the results of the previous trial, patients received a single dose of 10^6 pfu/ml followed 3 weeks later by 10^8 pfu/ml intra-tumorally, followed by serial injections of 10^8 pfu/ml every 2 weeks for up to 24 total treatments (Senzer et al. 2009). Safety, clinical activity, and survival were reported after a median 18 months of follow-up. Consistent with Phase I results, toxicity was common (reported in 85 % of patients) but was limited to low-grade fever and injection site reactions. Eight patients (16 %) had progressive disease and were removed from the study. Among the remaining 42 evaluable patients, the objective response rate in injected and non-injected lesions was 26 %. Two additional patients were considered complete responders after they were rendered free of disease surgically (Figs. 11.2 and 11.3). Survival rates were reported, with median survival greater than 16 months and a 1-year survival of 58 %. Importantly, among patients with an objective response to therapy (partial, complete, or surgical complete responders), the 1-year survival was 93 %. As a secondary outcome, viral swabs of the injection sites were conducted on 19 different patients. Viral shedding was present at only a low level in one patient. To evaluate systemic dissemination of the virus, blood and urine samples were evaluated with no HSV detected by PCR assay in any body fluids.

A Phase I/II dose-escalation trial enrolled 17 patients with advanced squamous cell carcinoma of the head and neck (SCCHN) to evaluate the combination of T-VEC with standard chemoradiation therapy (Harrington et al. 2010). All patients received an initial seroconversion dose of 10^6 pfu/ml followed by one of three doses (10^6, 10^7, or 10^8 pfu/ml). Patients received 70 Gy/35 fractions and cisplatin

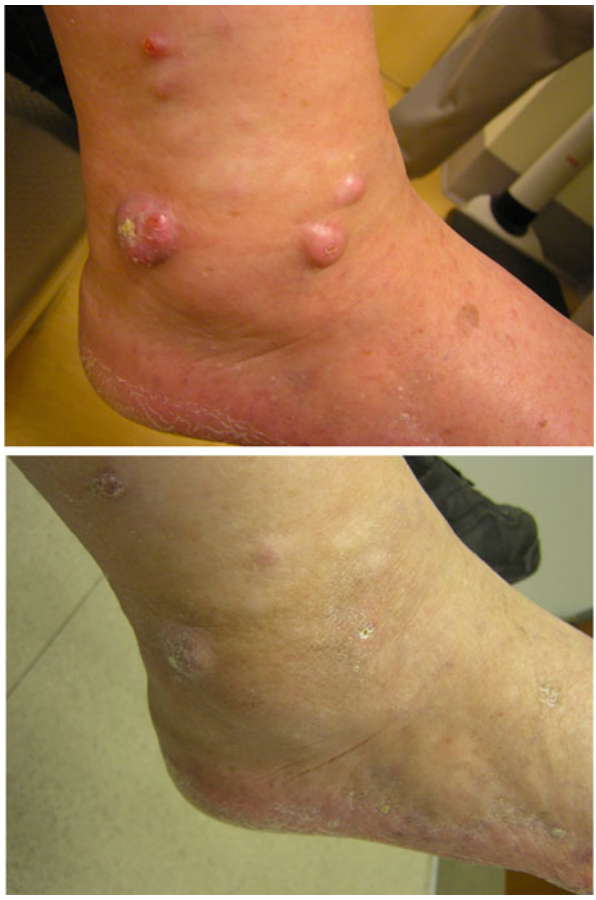

Fig. 11.2 Clinical response in a patient following treatment with talimogene laherparepvec (T-VEC). Several melanoma tumors are seen on the lateral malleolus (*top*), and the largest tumor was injected with T-VEC in the Phase II clinical trial (Senzer et al. 2009). Following six injections of T-VEC, the tumors have flattened and a slight area of induration is present (*bottom*)

100 mg/m^2 dosed at three time points during the study period. Safety, response and relapse rates and survival were reported. There was no dose-limiting toxicity and, like previous studies, adverse events were common but were all low-grade. At all doses, the locoregional control rate was 100 % and 4/17 patients had a complete pathologic response, while another 10 patients had a partial response. At a median follow-up of 29 months, the overall survival was 70.6 %.

T-VEC was recently evaluated in a large, multi-institutional, prospective, randomized Phase III clinical trial in which 439 patients with unresectable Stages IIIB, IIIC, or IV melanoma were eligible (Kaufman and Bines 2010). Patients were randomized in a 2:1 fashion to receive either T-VEC or recombinant GM-CSF alone. All patients had at least one lesion measuring 1 cm and accessible for intratumoral injection. Utilizing dosing studies conducted previously, patients in the treatment arm received a single dose of 10^6 pfu/ml followed 3 weeks later by10^8 pfu/ml every 2 weeks for up to 24 doses. Patients randomized to the control arm received GM-CSF 125 μg/ml subcutaneously for 14 days of every 28-day

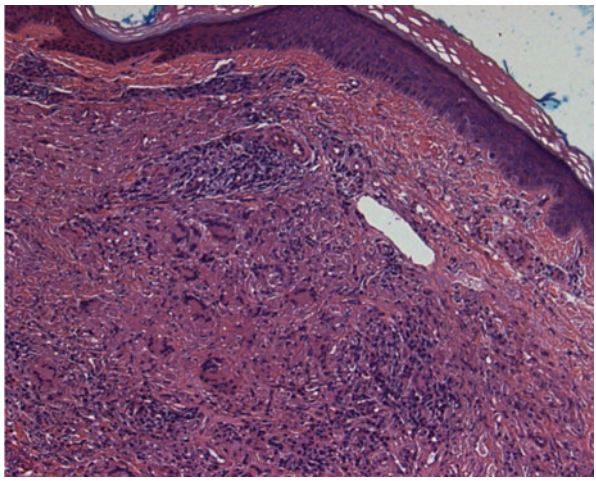

Fig. 11.3 Complete pathologic response following treatment with talimogene laherparepvec (T-VEC). A biopsy of the melanoma site seen in Fig. 11.2 reveals lymphocyte infiltration and the presence of melanophages, but no viable tumor cells are seen

cycle. Patients were removed from study for a documented complete response, prespecified high-grade toxicity, or clinically significant disease progression. The primary endpoint was durable disease response (complete response + partial response) lasting 6 months or more and initiating within 12 months of starting treatment. Secondary endpoints were safety, disease-free, and overall survival as well as quality-of-life parameters.

Recruitment and accrual to the study was completed in June 2011 with ongoing follow-up at the time of this publication. 295 patients received T-VEC, while 141 patients were randomized to GM-CSF alone. The median age was 63 years, and 57 % of patients enrolled were male. Among patients randomized, 30 % had Stage III disease, 37 % had Stage IVM1a disease (metastases limited to the skin, soft tissue, or lymph nodes), 21 % had Stage IVM1b disease (metastases limited to the lung), and 22 % had metastatic disease involving the other viscera or the central nervous system (Stage IVM1c). The durable response rate (DRR) was 16 % in the treatment arm compared to 2 % in the control arm ($p < 0.0001$). This difference was apparent across all stages of disease, although the benefit was most pronounced in Stage III patients. Among Stage III patients, the DRR was 33 % among patients treated with talimogene laherparepvec compared to 0 % in the GM-CSF group. The overall objective response rate among treated patients was 26 %, compared to 6 % among patients receiving GM-CSF alone. While the follow-up data is not yet mature, the survival curves are demonstrating a trend favoring treatment with T-VEC (HR 0.79, 95 % CI: 0.61,1.02) (Andtbacka et al. 2013). The treatment was well tolerated with fever, fatigue, and injection site reactions comprising the majority of adverse events. The only serious adverse event occurring in more than 2 % of all patients was cellulitis in patients treated with T-VEC. Final results are anticipated in the near future.

11.6 Induction of Antitumor Immunity by Talimogene Laherparepvec

There is limited data on the role of T-VEC in priming tumor-specific T-cell responses and understanding how the oncolytic virus mediates local and systemic antitumor immunity. Eleven patients enrolled in the Phase II melanoma clinical trial underwent tumor biopsy after six injections to evaluate local tumor effects of the treatment. A cohort of subjects who had undergone metastasectomy of melanoma and were not enrolled in an oncolytic virus trial were used as controls. In addition to tumor specimens, peripheral blood mononuclear cells (PBMC) were collected for analysis (Kaufman et al. 2010).

Tissue specimens from T-VEC-injected tumors had significant necrosis and lymphocyte infiltration compared to untreated tumors. The tumor-infiltrating T cells present in T-VEC treated lesions revealed a high frequency of activated (CD45RO+) and antigen-specific (MART-1) T cells at the tumor site compared to T cells evaluated in PBMC samples from the same patient or in non-injected control tumors. Additional studies of the MART-1-specific tumor-infiltrating lymphocytes suggested that these cells have an activated CD8+ T-cell phenotype as evidenced by expression of CD25, HLA-DR, PD-1 perforin, and granzyme B. In addition to the markedly increased numbers of activated CD8+ T cells, there was an associated decrease in regulatory CD4+FoxP3+ T cells, CD8+FoxP3+ suppressor T cells, and myeloid-derived suppressor cells (MDSC). These data suggested that T-VEC could induce local CD8+ effector T cells and reduce the number of regulatory and suppressor T cells and MDSC.

In selected subjects, tissue samples from injected and distant, non-injected lesions from the same patient were available for analysis. Both injected and non-injected lesions had a predominant MART-1-specific CD8+ T-cell response. However, there were quantitatively a greater number of T cells in the injected lesions compared to non-injected lesions. Non-injected lesions also had a greater number of regulatory CD4+FoxP3+ T cells compared to corresponding injected lesions, but the level was lower than that seen in uninjected control patients. This data indirectly supports the generation of systemic immunity and suggests a qualitatively similar response at distant uninjected tumor lesions, but it is quantitatively less robust than the response observed within injected tumors. This might suggest the need to combine T-VEC with other immune-potentiating agents, and a clinical trial combining T-VEC and the anti-CTLA-4 monoclonal antibody ipilimumab is already underway. Further immune evaluation in a larger series of subjects is needed to better define the true impact of T-VEC on local and systemic antitumor immunity.

11.7 Future Applications of Oncolytic Herpesvirus for the Treatment of Cancer

Talimogene laherparepvec (T-VEC) is an oncolytic herpesvirus based on the JS1 HSV-1 strain in which the viral ICP34.5 and ICP47 genes have been deleted and in which the human GM-CSF gene has been inserted. T-VEC has been evaluated in Phases I, II, and III clinical trials in patients with unresectable and metastatic melanoma. The Phase III clinical trial met the primary endpoint demonstrating an improvement in durable response rate for patients treated with T-VEC compared to patients treated with recombinant GM-CSF, and overall survival data is expected shortly. Although there is limited data on the mechanisms of antitumor activity with T-VEC in cancer patients, there is some evidence that T-VEC induces local infiltration of antigen-specific CD8+ T cells and inhibits the infiltration of regulatory and suppressor T cells and myeloid cells. The final clinical trial results will be critical to delineating the impact of T-VEC for the treatment of melanoma. Further studies of T-VEC with other immune-potentiating agents, such as ipilimumab, interleukin-2 (IL-2), and anti-programmed death 1 (PD1) or anti-PD ligand 1 (PDL1) monoclonal antibodies, would be important avenues to pursue. It may also be prudent to consider combining T-VEC with other standard therapeutic strategies, such as cytotoxic chemotherapy or radiation therapy.

The subset analysis of the Phase III clinical trial suggested that patients with unresectable Stage III disease had a particularly strong response to T-VEC. This suggests that patients with melanoma in-transit metastases may be especially likely to respond, and future studies may focus on these indications. In addition to melanoma, other tumors may be amenable to treatment with T-VEC, including head and neck cancers and possibly others that may be accessible to injection. While current studies have required clinically palpable or ultrasound identified lesions, it may also be possible to access visceral tumors through CT-guided needle access which could increase the number of directly injectable lesions and the types of cancer that can be tested.

Among the most desirable features of this mode of therapy is its excellent patient tolerance and mild toxicities. While short-term evaluation of these patients suggests that systemic and latent infection does not occur with the current dosing regimen, long-term follow-up is needed to assure that latent infections with HSV-1 do not develop. To this end, a registry of all patients treated with the current strain of oncolytic herpesvirus has already been initiated to monitor this and other long-term effects of therapy. Issues of biosafety with this type of medication are also important as it is uniquely different from standard methods of treatment. However, the data currently available suggests that viral shedding at tumor injection sites is minimal, making the virus safe to administer in the outpatient setting with dedicated training of healthcare personnel.

11.8 Conclusions

Oncolytic viruses have been shown to selectively infect and replicate in tumor cells and induce tumor-specific immunity in preclinical models. The herpesviruses have been among the most widely studied viruses for oncolytic immunotherapy development, and talimogene laherparepvec (T-VEC) is a modified oncolytic HSV-1 virus encoding GM-CSF and is the first oncolytic virus demonstrating a therapeutic benefit against advanced cancer in patients. The success of the T-VEC clinical trials supports further research with oncolytic viruses in other cancers, in combination with other standard antineoplastic and immunotherapy agents, and in visceral locations. Further research should focus on better understanding how these agents promote tumor rejection through direct lytic effects and induction of local and systemic antitumor immunity. Oncolytic herpesviruses provide a new class of therapeutic agent for the treatment of human cancer.

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Chapter 12
SA-4-1BBL: A Novel Form of the 4-1BB Costimulatory Ligand as an Adjuvant Platform for the Development of Subunit Cancer Vaccines

Rajesh K. Sharma, Abhishek K. Srivastava, Hong Zhao, Esma S. Yolcu, and Haval Shirwan

Abstract Protein-based therapeutic subunit vaccines against cancer have proven efficacy in various preclinical models. The translation of their efficacy into the clinic, however, has been challenging. Although there are many factors impacting the efficacy of vaccines in humans, the most important ones are the prolonged tumor development and progression, altered immune responses due to extensive exposure to environmental pathogens, stage of cancer, standard treatments to control cancer, and effect of such treatments on the patient's immune system prior to vaccine administration. It is a common consensus that the presence of cancer is an indication of effective immune evasion responses initiated and perpetuated by tumor. Immune evasion involves well-orchestrated cellular and molecular

R.K. Sharma
James Brown Cancer Center, University of Louisville, Louisville, KY, USA

A.K. Srivastava
National Institutes of Health, Bethesda, Maryland, USA

Surgery Branch, Center for Cancer Research, National Cancer Institute, 10 Center Drive, Building 10-Hatfield CRC, Rm 3-5888, Bethesda, MD 20892-1201, USA

H. Zhao
Institute for Cellular Therapeutics, University of Louisville, Louisville, KY, USA

E.S. Yolcu
Institute for Cellular Therapeutics and Department of Microbiology and Immunology, University of Louisville, Louisville, KY, USA

H. Shirwan (✉)
Department of Microbiology and Immunology, University of Louisville, Louisville, KY, USA

Institute for Cellular Therapeutics, University of Louisville, 570 S. Preston St., Donald Baxter Biomedical Bldg, Suite 404E, Louisville, KY 40202, USA
e-mail: haval.shirwan@louisville.edu

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mechanisms that control tumor-specific effector immune responses in favor of tumor progression. Therefore, protein-based subunit vaccine formulations will require immune adjuvants that not only generate the desired effector immune responses, particularly those driven by $CD8^+$ T cells, but also reverse the regulatory immune evasion network in place to combat tumor. Costimulation through tumor necrosis factor receptor (TNFR) superfamily is critical for T-cell activation, expansion, acquisition of effector function, and establishment of long-term memory required for tumor eradication and control of recurrences. As such, agonists of TNFRs have great potential as immune adjuvants. We recently generated a novel form of the 4-1BB ligand, SA-4-1BBL, a member of TNF family, and demonstrated its robust pleiotropic effects on the cells of innate, adaptive, and regulatory immunity. Importantly, as the adjuvant component of tumor-associated antigen (TAA)-based vaccine formulations, SA-4-1BBL demonstrated therapeutic efficacy in various preclinical tumor models in the absence of detectable toxicity. This chapter will discuss SA-4-1BBL as a novel adjuvant with demonstrated desired mechanisms of action for tumor eradication and its prospect for human use as monotherapy or in combination with other immune modulators with synergistic mechanisms of action.

List of Abbreviations

Abs	Antibodies
Ags	Antigens
APCs	Antigen-presenting cells
bFGF	Basic fibroblast growth factor
CD	Cluster of differentiation
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CTLs	Cytotoxic T lymphocytes
DC	Dendritic cells
DNA	Deoxyribonucleic acid
FasL	Fas ligand
FDA	Food and Drug Administration
GITR	Glucocorticoid-induced tumor necrosis factor receptor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBsAg	Hepatitis B virus surface antigen
HER-2	Human epidermal growth factor receptor 2
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HSV	Herpes simplex virus
HVB	Hepatitis B virus
ICOS	Inducible T-cell costimulator
IDO	Indoleamine 2,3-dioxygenase
IFNγ	Interferon γ
IgG	Immunoglobulin G
IL-10	Interleukin-10

LCV	Lymphocytic choriomeningitis virus
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCMV	Mouse cytomegalovirus
MDSCs	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
MPL	Monophosphoryl lipid
NK cells	Natural killer cells
NKT cells	Natural killer T cells
NLRs	NOD-like receptors
NSCLC	Non-small-cell lung carcinoma
ODN	Oligodeoxynucleotides
PAMPs	Pathogen-associated molecular patterns
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death protein ligand 1
PI3K	Phosphatidylinositol-3-kinase
PRRs	Pattern recognition receptors
PSMA	Prostate-specific membrane antigen
Rag2−/− mice	Recombination-activating 2-deficient mice
RLRs	RIG-I-like receptors
SA	Streptavidin
SIV	Simian immunodeficiency viruses
TAA	Tumor-associated antigen
TGF-β	Transforming growth factor beta
Th1	T helper 1
TLRs	Toll-like receptors
TNFR	Tumor necrosis factor receptor
TRAFs	TNFR-associated factors
Treg cells	T regulatory cells
5-FU	5-fluorouracil

12.1 Introduction

The concept of therapeutic cancer vaccines dates back to 1893 when William B. Coley observed regression of tumor in some cancer patients with acute infections and attempted to use bacteria or bacterial products for the treatment of cancer patients (Coley 1891, 1910). Regression of tumors in some of Coley's vaccinated patients was believed to be due to infection-induced excessive inflammation. Most importantly, Coley's studies demonstrated that the immune system of cancer patients can be activated to combat tumor without major adverse effects on nonmalignant cells, a clear indication of the exquisite specificity and efficacy of the immune response. Although these initial observations were extremely exciting and marked the origin of modern cancer immunotherapy, the concept of cancer

vaccines faced significant skepticism due to various setbacks in achieving the desired therapeutic efficacy. As such, the role of immune system in fighting tumors was called into question. However, increased tumor incidences in immunodeficient mice and in patients on immunosuppressive regimen, such as transplant recipients, provided undisputable evidence that the immune system plays a critical role in controlling tumor progression. This so-called immunosurveillance theory was further supported by findings that genetically modified mice lacking key immune effector molecules, such as IFNγ, granzyme B, and perforin, develop spontaneous tumors with significantly increased frequencies as compared to immunocompetent mice (Smyth et al. 2000; Kaplan et al. 1998).

The advances in molecular techniques and recombinant DNA technology led to a better understanding of the immune system, tumor development and progression, and most importantly the extensive and complex nature of interactions/regulation between the immune system and the tumor, dictating tumor elimination versus progression. This accumulated knowledge led to a better design of vaccines that yielded consistent and reproducible therapeutic responses in various preclinical models (Lesterhuis et al. 2011). However, the translation of preclinical success of cancer vaccines to the clinic became a far-reaching goal. It is unclear as to why vaccine formulations that work so effectively in rodents have minimal to no clinical benefits in humans. The complex nature of the human immune system, its altered state due to continuous exposure to various environmental antigens, spontaneously arising tumor with protracted progression before diagnosis, coevolution of tumor and immune systems during progression, and most importantly, the stage of tumor in patients and standard treatment history to control the tumor prior to vaccination represent some of the contributing factors. The failure of numerous vaccine concepts in the clinic does not invalidate the potential of this therapy, but it certainly indicates that our understanding of the human immune system and tumor progression has not elevated to the level that will allow for the design of effective vaccine formulations. Irrespectively, the approval of the first ever therapeutic vaccine, DC-based Provenge®, for the treatment of prostate cancer by FDA in 2010 was a major milestone, instilled faith, and renewed interest in cancer vaccines.

In this chapter, we will discuss some of the difficulties in translating the efficacy of vaccines from preclinical settings to the clinic and argue that the use of adjuvants that boost immune effector responses for tumor eradication will be key to the clinical success of vaccines. SA-4-1BBL will be presented as adjuvant with such potential. We will then make a case in favor of combinatorial approaches involving adjuvants and selected immune modulators for the design of cancer vaccines with the potential to overcome various immune setbacks and achieve maximal efficacy in the clinic.

12.2 Immune System and Cancer: A Love/Hate Relationship

The requisite role of the immune system against infections has been well recognized and confirmed by the development of various prophylactic vaccines that save millions of lives worldwide annually. In marked contrast, the role of the immune system in tumor development, progression, and control has been the subject of significant controversy over the past several decades. The initial report of Coley that acute infections may cause spontaneous tumor remission in patients, presumably because of infection-induced inflammation, provided evidence for the role of the immune system in eradicating cancer (Coley 1891, 1910). The concept that immune system is important in controlling tumors under normal physiological conditions was conceived by Paul Erlich in 1909 and formulized by Lewis Thomas and Macfarlane Burnet under the hypothesis of "tumor immune surveillance" in 1957. However, the lack of direct evidence for this hypothesis resulted in significant controversy. Further fueling this debate were observations that nude mice lacking adaptive immunity and their syngeneic wild-type counterparts develop similar incidences of spontaneously arising, non-virus-driven tumors (Stutman 1974; Rygaard and Povlsen 1974). However, subsequent studies over the years demonstrated that nude mice are not totally immune incompetent as they do generate extrathymically developed T cells and innate immune cells (Ikehara et al. 1984), and irrespective of partial immune competency, such mice have higher incidences of tumor as compared with wild-type mice (Engel et al. 1996).

Technological progresses in biomedical sciences allowed the design of sophisticated studies to delineate mechanistic basis of immune responses, which elevated our understanding of the immune system against cancer. In particular, targeted alteration of the immune system in mice via transgenic technology presented the opportunity for rigorous testing of the immune surveillance hypothesis. Mice lacking selected immune cells, such as T and B cells ($Rag^{-/-}$); or effector molecules, such as perforin (Smyth et al. 2000); or IFNγ (Shankaran et al. 2001) provided unequivocal evidence that the immune system is critical for the control of tumors. The higher incidences of tumors in immunosuppressed individuals, particularly transplant recipients (Engels et al. 2011), as compared with normal population provided clinical evidence for the role of immune surveillance hypothesis. The accumulated knowledge of the immune system combined with the identification of TAAs resulted in the design of immune therapies, particularly cancer vaccines that showed efficacy in various preclinical models (Lesterhuis et al. 2011; Schlom 2012). The efficacy of various immune therapies in eliminating established tumors not only provided direct evidence for the importance of the immune response in controlling tumor but also set the stage for harnessing the power of the immune system for the eradication of tumor (Lesterhuis et al. 2011; Schlom 2012), renewing faith in the early observations of Coley and generating confidence in the promise of cancer vaccines.

Although the role of immune response in fighting cancer is unquestionable, accumulating evidence in the literature also implicates the immune system in tumor progression. The tumor microenvironment consists of malignant as well as nonmalignant stromal cells, such as immune cells, fibroblasts, endothelial cells, and extracellular matrix. A complex set of molecular and cellular communications within this unique microenvironment determine the fate of tumor progression versus tumor elimination. A robust effector response is associated with tumor elimination, while a chronic immune response may be beneficial for tumor progression as it generates various soluble factors involved in angiogenesis, tumor growth, metastasis, and resistance to standard-of-care treatments (Allavena et al. 2008). Although various effector mechanisms, including humoral and innate immune responses, depending on the cancer type are associated with the control and elimination of altered-self cancer cells, Th1-mediated cellular immunity, particularly $CD8^+$ T-cell cytotoxic response, plays the most determining role. In response, tumor cells have developed direct and indirect evasion mechanisms to counterattack the immune system. Indeed, tumor-modulated regulatory immune responses may serve as one of the most important hurdles affecting the efficacy of therapeutic cancer vaccines. Although these immune evasion mechanisms are complex and yet to be fully elucidated, T-cell anergy or nonresponsiveness (Nind et al. 1973), T regulatory cells (Nishikawa and Sakaguchi 2014), regulatory NK T cells (Terabe et al. 2000), myeloid-derived suppressor cells (Gabrilovich and Nagaraj 2009), various soluble factors (such as TGF-β and IL-10), indoleamine 2,3-dioxygenase, downregulation of costimulatory ligands (such as CD80, 4-1BBL, and MHC molecules), upregulation of co-inhibitory ligands (such as PDL-1), or death-inducing molecules (such as FasL) represent some (Zou 2005). Given this complex cancer/immune system interplay, cancer vaccine design that incorporates adjuvants or adjuvant systems to shift the overall balance from immune evasion that facilitates tumor progression to immune effectors, such as Th1 immune responses, that combat tumor may achieve therapeutic efficacy.

12.3 Efficacy of Prophylactic Versus Therapeutic Vaccines

Prophylactic vaccines against infections have been extremely effective and are considered the miracle of modern medicine. In marked contrast, the promise of therapeutic vaccines against tumors is yet to be fully realized. There are several factors that may contribute to this discrepancy. Preventive vaccines use foreign strong exogenous antigens for the induction of humoral immune responses in a healthy population with an intact and functional immune system. Therapeutic vaccines, on the other hand, use TAAs for the generation of cellular immune responses required for the eradication of tumors in diseased individuals. The nature of antigens used for immunization and the immune status of the vaccinated individuals may be the key to the observed efficacy differences between prophylactic and therapeutic vaccines. Pathogen-derived proteins serve as strong antigens and as

such generate robust immune responses. In marked contrast, TAAs, by their nature of being self-antigens, lack the ability to generate robust T cell-mediated immune responses required for tumor eradication. The status of the immune system in vaccinated individuals is most likely the pivotal factor dictating the efficacy of prophylactic versus therapeutic vaccines. Unlike prophylactic vaccines administered to healthy individuals, therapeutic vaccines are given to cancer patients whose immune system not only has failed to control the tumor but most likely has also been altered by standard-of-care cancer treatments.

Finally, tumors have evolved to combat the immune system by a series of intrinsic and extrinsic mechanisms. Most importantly, some of the evasion mechanisms involve the tumor's ability to utilize the immune system for its own progression (Zou 2005). In particular, various immunoregulatory mechanisms required for self-tolerance have been exploited by tumors to cheat the immune system (Gabrilovich and Nagaraj 2009; Nishikawa and Sakaguchi 2014; Zou 2005). Therefore, the therapeutic efficacy of the cancer vaccines will depend on their ability to generate robust immune effector responses against tumors as well as overcome various immune evasion mechanisms employed by the progressing tumor. These effects need to be achieved in patients who have undergone standard-of-care cancer treatments, and as a consequence, most likely have compromised immune responses. Therefore, therapeutic vaccines need to be formulated with these considerations in mind and will require novel adjuvants that can drive effective immune responses against tumor. An adjuvant that not only generates a robust Th1 response against tumor but also overcomes the tumor employed regulatory/suppressive mechanisms may have the best chance for achieving efficacy in cancer patients.

12.4 Therapeutic Vaccines

Therapeutic cancer vaccines are designed to generate a productive antitumor immune response that translates into efficacy in cancer patients. Cancer vaccines can be classified into cell-free or cell-based vaccines. Cell-free vaccines include DNA-based vaccines, viral vectored vaccines, oncolytic viral vaccines, and protein-based subunit vaccines. Cell-based vaccines, on the other hand, comprise irradiated or chemically fixed whole tumor cells or dendritic cells (DCs) pulsed with TAAs, such as FDA-approved sipuleucel-T, also known as "Provenge." This book chapter will focus on subunit, protein-based therapeutic cancer vaccines. Therapeutic cancer vaccines against well-defined TAAs emerged as a promising treatment modality. These subunit vaccines are attractive because of their ease of production, cost-effectiveness, off-the-shelf availability, and ease/practical nature of administration into the patients.

The concept of subunit vaccine formulation is rather simple as it involves the addition of one or more whole TAA proteins or synthetic peptides representing T-cell epitopes of such TAAs along with an adjuvant or adjuvant system that not

only drives the desired Th1 antitumor immune responses but is also capable of reversing the unwanted tumor-mediated immunosuppressive mechanisms. Inasmuch as DCs are critical for the generation of adaptive immune responses in general and against cancer in particular, as exemplified by the clinical efficacy of Provenge, vaccine formulations may benefit from incorporating vehicles to deliver TAAs into DC in vivo for optimal antigen presentation and effective T-cell activation, proliferation, acquisition of effector function, and establishment of long-term memory. Several TAAs, such as human epidermal growth factor receptor 2 (HER-2), prostate-specific membrane antigen (PSMA), are expressed on cancer cell surface. An adjuvant system that can optimally prime $CD8^+$ T cell-mediated cytotoxic responses along with B cell-mediated antibody responses may prove to be more effective in these settings. Therefore, therapeutic subunit vaccines need to be formulated based on the cancer type, utilized TAAs, and anticipated effector immune responses necessary for tumor elimination. In this context, careful consideration of adjuvants or adjuvant systems as component of vaccine formulations will be critical to a desired therapeutic outcome.

12.5 Problems and Prospects for the Design of Subunit TAA-Based Cancer Vaccines

Some of the major challenges in vaccine design are the selection of appropriate TAAs, adjuvant or adjuvant systems that are capable of priming/boosting the anticipated antitumor immune responses, and vehicles/systems to ensure the delivery of TAAs into DCs for accomplishing a robust therapeutic efficacy. Antigenic drift, accumulated mutations in T- and B-cell epitopes due to immune pressure, is a major mechanism of tumor escape from immune attack. Therefore, the selection of a TAA that is not only specifically and/or highly expressed by tumor cells but also is essential for tumor survival, progression, and metastasis is important. Discovery of universal TAAs at least for the same tumor type across a patient population will be a key step for designing a generalized vaccine against a specific type of cancer. Analysis of tumors in humans has shown great TAA heterogeneity among the same cancer type and even within the same tumor tissue. Therefore, the choice of a TAA for the development of cancer vaccines should be dictated by a comprehensive understanding of its expression pattern in the selected cancer type and at various stages of the cancer. Although emerging understanding of cancer immunology provides better opportunities to design more specific vaccines, it also brings greater challenges for vaccine customization for a particular type of cancer. Vaccine formulations may need to be tailored to be best suited for the patient's cancer profile with respect to TAA expression as well as effector immune responses required for the eradication of tumor. This issue not only presents a challenge for the design of cancer vaccines but also customization of the current standard-of-care treatments for individual patients for a more effective outcome.

The question is if it is feasible to develop a universal vaccine that may have utility for different cancer types. Realistically, vaccine formulations may need to be customized for the patient, but not tumor, for the desired therapeutic efficacy after tumor biopsy followed by genomic and proteomics analyses to determine the precise status of genetic variations and TAA expression profiles. Accumulating evidences indicate that the immune system can adapt to the antigenic changes within a tumor through the process of inter- or intramolecular antigen or epitope spreading (Hardwick and Chain 2011). In response to the tumor, T-cell repertoire expands and recognizes epitopes that are not part of the initial TAA in the vaccine formulation. As tumor cells are damaged and eliminated by the immune system, new TAAs are released within the tumor milieu or systemically and picked up by DCs for cross-presentation to $CD4^+$ and $CD8^+$ T cells for the generation of a broader cellular immune response than has been primed by the vaccine. These findings indicate the feasibility of developing vaccines that may have utility for different tumor types. However, if the initial recognition of TAA within the vaccine formulation is critical to the antigen/epitope spreading, then all the targeted tumor types need to express this TAA for the vaccine to manifest its efficacy. In this context, it may also be feasible to design vaccine formulations containing tumor-related and/or unrelated antigens that serve as universal T-cell epitopes admixed with adjuvants having robust immune stimulatory activities. These vaccines can then be administered to patients in conjunction with tumor-damaging agents, such as standard-of-care chemo and/or localized radiotherapy, to initiate a self-perpetuating immune response against cancer. In this scenario, adjuvants will initiate and boost T-cell responses against the antigen component of the vaccine, while the tumor damage will provide endogenous TAAs initiating the process of epitope spreading. Inasmuch as immune evasion mechanisms are one of the most important hurdles for achieving the efficacy of vaccines in the clinic, vaccine composition must contain adjuvants or adjuvant systems that are not only capable of inducing the anticancer immune responses but also overcome various immune evasion mechanisms employed by the progressing tumor.

The in vivo half-life and bioavailability of the vaccine is another issue worth considering when designing vaccine formulations. The depot effect of alum is still believed to be largely responsible for its superb adjuvant properties for augmenting B cell-mediated antibody responses (Kool et al. 2012). As such, numerous vaccine delivery systems, including liposomal and nano/microparticles-based adjuvant systems, have been developed to enhance T cell-mediated responses (Gregory et al. 2013). The success of DC-based vaccines in preclinical and clinical studies served as impetuous to target these cells for antigen delivery to ensure the optimal vaccine efficacy. DCs have been manipulated ex vivo by various means to present TAAs and achieve clinical responses. However, DC-based cellular vaccines are time and labor intensive, costly, and, most importantly, patient customized, which severely limit their broad clinical application. Therefore, intense efforts have been devoted to target DCs in vivo for the improvement of therapeutic efficacy of TAA-based conventional vaccines (Tacken et al. 2007). Studies in humans demonstrated that DC maturation is obligatory for the generation of effective immunity

(de Vries et al. 2003). Therefore, various strategies have been attempted to deliver antigens to DCs in vivo by targeting specific receptors, such as DEC205 (Bonifaz et al. 2002), Clec9A (Sancho et al. 2008), the mannose receptor (He et al. 2007), and Dectin-1 (Carter et al. 2006). These strategies also required adjuvants, such as agonists of Toll-like receptor or CD40, to mature the targeted DCs for the generation of endogenous cytotoxic T-cell responses and effective antitumor immunity. Therefore, adjuvants with dual functions, as antigen delivery vehicle and modulator of DC activation, antigen uptake, and cross-presentation, may significantly improve the therapeutic efficacy of the vaccines. In summary, the efficacy of cancer vaccines, irrespective of their formulation, will depend not only on their ability to prime or boost the existing immune responses but also overcome various immune evasion mechanisms that help tumor progression in cancer patients. In this context, the choice of adjuvants is of paramount importance and those that modulate innate, adaptive, and regulatory immunity for the generation of effective Th1 cellular responses without adverse effects or with tolerable toxicity will deliver the promise of cancer vaccines.

12.6 Adjuvants for Therapeutic Cancer Vaccines

Adjuvants are molecules, compounds, or macromolecular complexes that traditionally are admixed with antigens to enhance the magnitude, breadth, quality, and longevity of the immune response to the antigens. As such, adjuvants may substantially reduce the amount of antigen and/or number of immunizations required for the generation of an effective immune response. Despite the fact that adjuvants are crucial vaccine components determining their success or failure, there has been great deal of pessimism regarding their use for the development of therapeutic cancer vaccines. This is mainly due to potential toxicity arising from the lack of full understanding of mechanistic insight and precise knowledge of the constituents of many adjuvants (Marrack et al. 2009; Pashine et al. 2005). Some of the tested vaccine formulations, like viral vectors, are designed to express their own adjuvants, while others, like peptide-based vaccines, do not and hence require coadministration of adjuvants for the induction of potent immune response.

The choice of adjuvants available for cancer vaccines has been very limited, mostly or in part due to the toxicity concerns, which raise significant regulatory hurdles. In fact, aluminum-salt-based adjuvants were the only ones used clinically in the United States until 2010 when monophosphoryl lipid A (MPL) in combination with aluminum hydroxide was approved by the FDA (Vacchelli et al. 2013) as adjuvant component of the preventive vaccine, Cervarix, against human papillomavirus (HPV). Recent advances in molecular technologies, in particular genomics and proteomics, led to a better understanding of the immune system and the nature and magnitude of immune responses required for the clearance of infections and to a certain extent, control of tumors. This collective knowledge in turn has

enormously contributed to the development of vaccines in general and rationalized the design of adjuvants with known mechanisms of action in particular.

Adjuvants achieve their activity by acting as pathogen-associated molecular patterns (PAMPs) that work on evolutionary conserved innate immune receptors to mimic natural infections. Therefore, almost all clinically approved adjuvants and most under development primarily target innate immunity, particularly antigen-presenting cells that serve as a bridge between innate and adaptive immunity. The receptors targeted by PAMPs are called pattern recognition receptors (PRRs). As oppose to adjuvants whose characterizations are poorly understood, a growing focus has been shifting towards the use of natural ligands or synthetic agonists for well-defined PRRs as adjuvants. Therefore, we will focus on agonists of PRRs because of their well-characterized immune actions, advanced development, and one of the agonists, MPL, being approved for clinical use (Vacchelli et al. 2013).

12.6.1 PRR Agonists as Vaccine Adjuvants

The innate immune system provides first line of defense to the host against invading organisms, such as viral, microbial, and fungal pathogens. Cells of innate immune system express PRRs to identify PAMPs associated with a wide variety of infectious agents (Table 12.1). PRRs initiate defense mechanisms via several conserved signaling pathways that lead to the production of inflammatory cytokines and type I interferons (IFNs). These inflammatory responses recruit and activate circulating immune cells and are essential for priming adaptive immune responses. There are two main classes of PRRs that have been identified in mammalian cells: membrane-bound receptors, such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), and cytoplasmic receptors, such as NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs).

Among all PRRs, TLRs are the largest and most well-characterized family of a diverse set of germ line-encoded receptors that recognize broad classes of conserved molecular structures common to groups of microorganisms (Akira et al. 2006; Janeway and Medzhitov 2002; Sansonetti 2006). Due to the critical role TLR signaling plays for the regulation of innate, adaptive, and regulatory immune responses, TLR agonists have emerged as ideal adjuvants for cancer immunotherapy. These agonists include TLR-3 (poly I:C), TLR-4 (MPL), TLR-5 (flagellin), TLR-7 (Aldara), TLR-7/8 (Resiquimod), and TLR-9 (CpG). Alone or in combination with various other immunomodulators, the TLR agonists have been demonstrated to enhance vaccine efficacy. In preclinical studies, TLR agonists were shown to generate antitumor immunity by enhancing innate immunity through the activation of DCs, NK cells, monocytes, and macrophages and induction of cytokines with both direct and indirect antitumor activities (Kim et al. 2004; Ishii and Akira 2007; Davis et al. 1998; Akira and Takeda 2004). Engagement of TLRs on APCs, such as DCs, results in their maturation and migration to lymph nodes where they initiate adaptive immune responses and generates long-lasting memory against

Table 12.1 Pattern recognition receptors (PRRs), ligands, and effectors

PRR	Ligand	Source of ligands	Immune action (s)
Toll-like receptors (TLRs)			
TLR1	Triacyl lipopeptides	Bacteria	Inflammatory cytokines
TLR2	Lipoproteins, peptidoglycan, LTA, zymosan, mannan	Bacteria	Inflammatory cytokines
TLR3	dsRNA	Viruses	Inflammatory cytokines, type I interferons
TLR4	LPS, RSV and MMTV fusion protein, mannans, glycoinositol phosphate from *Trypanosoma spp.*	Gram-negative bacteria, viruses	Inflammatory cytokines, type I interferons
TLR5	Flagellin	Bacteria	Inflammatory cytokines
TLR6	Diacyl lipopeptides, LTA, zymosan	Bacteria	Inflammatory cytokines
TLR7/ TLR8	ssRNA	Bacteria, viruses	Inflammatory cytokines, type I interferons
TLR9	CpG DNA, hemozoin from *Plasmodium spp.*	Bacteria, viruses, protozoan parasites	Inflammatory cytokines, type I interferons
TLR10	Unknown	Unknown	Unknown
TLR11	Profilin, flagellin	Apicomplexan parasites, bacteria	Inflammatory cytokines
TLR12	Profilin	Apicomplexan parasites	Inflammatory cytokines
TLR13	Bacterial 23S rRNA with CGGAAAGACC motif	Gram-negative, Gram-positive bacteria	Inflammatory cytokines
NOD-like receptors (NLRs)			
NOD1	iE-DAP (PGN)	Bacteria	Inflammatory cytokines
NOD2	MDP (PGN), ssRNA	Bacteria, RNA viruses	Inflammatory cytokines
NLRP3	ssRNA, dsRNA, bacterial mRNA, oxidized mitochondrial DNA	RNA viruses, bacteria, cellular damage	Inflammatory cytokines
RIG-I-like receptors (RLRs)			
MDA5	Long dsRNA	Picornavirus, vaccinia virus, Flaviviridae, reovirus, bacteria	Inflammatory cytokines, type I interferons
RIG-I	PPP-ssRNA, RNA with base pairing, polyI:C	ssRNA viruses, DNA viruses, Flaviviridae, reovirus, bacteria	Inflammatory cytokines, type I interferons
LGP2	dsRNA	RNA viruses	Inflammatory cytokines, type I interferons

dsRNA double-stranded RNA, *LTA* lipoteichoic acid, *LPS* lipopolysaccharide, *MMTV* mouse mammary tumor virus, *ssRNA* single-stranded RNA, *iE-DAP*, gamma-D-glutamyl-meso-diaminopimelic acid, *MDP* muramyldipeptide, *PGN* peptidoglycan

tumors. In case of clinical studies, MPL has already been licensed in the United States as the adjuvant component of a preventive vaccine against HPV (Vacchelli et al. 2013). MPL was also tested as a component of allogeneic tumor cell lysate or defined TAA-based vaccines against melanoma in clinical trials (Marchand et al. 2003; Vantomme et al. 2004). A non-small-cell lung carcinoma (NSCLC) vaccine using MPL as adjuvant is in late-stage clinical trials (Atanackovic et al. 2004; Vansteenkiste et al. 2013). RC-529 (GSK, Dynavax), another synthetic TLR-4 agonist, has been licensed for an HBV vaccine in Europe (Baldwin et al. 2009). A combination of MPL and basic fibroblast growth factor (bFGF) has been shown to enhance IgG titers and IFNγ levels in the serum and antitumor activity in mice (Zhong et al. 2010). A polymeric form of TLR4 agonist, lipopolysaccharide (LPS), known as SP-LPS in combination with paclitaxel showed promising antitumor effects through induction of apoptosis (Roy et al. 2012). CpG oligodeoxynucleotides (ODN) as agonist of TLR9 have also shown great promise as an adjuvant for TAA-based cancer vaccines (Kim et al. 2004). Immunization of mice with hepatitis B virus surface antigen (HBsAg) along with type B CpG-ODN (1826) enhanced HBsAg-specific IgG2a Abs (Davis et al. 1998).

Despite promising results, safety profile of TLR agonists has been a major hurdle for clinical development and needs to be addressed for the use of these agonists as a component of vaccine formulations. TLR agonists as vaccine adjuvants caused severe toxicity in selected settings due to nonspecific activation of lymphocytes as well as signaling into nonimmune cells (Akira and Takeda 2004; den Haan et al. 2007; Krieg 2007). The limited efficacy of TLR-signaling in the induction of adaptive immune responses, required for the establishment of long-term immunological memory and prevention of tumor recurrences, has also been one of the major challenges of TLR agonists as adjuvant component of therapeutic cancer vaccines (Gavin et al. 2006; Ishii and Akira 2007; Meyer-Bahlburg et al. 2007). Most importantly, TLR signaling in selected settings is involved in the generation of regulatory immunity, which plays a critical role in immune evasion and allows tumors to counterbalance the antitumor immunity. For example, TLR-4 signaling allows the expansion of $CD4^+CD25^+FoxP3^+$ T regulatory cells (Treg cells) ex vivo and induces IL-10-producing $CD4^+$ Treg cells in vivo (den Haan et al. 2007). Similarly, CpG, a TLR-9 agonist, was shown to convert $CD4^+$ T effector cells into Treg cells via plasmacytoid DCs (Moseman et al. 2004). This agonist also was found to induce $CD19^+$ DCs to acquire potent T-cell suppressive functions through the production of indoleamine 2,3-dioxygenase (Mellor et al. 2005). Due to undesired outcome of TLR agonists as vaccine adjuvants, there is a dire need for the discovery and development of alternative adjuvants that not only have potent immunomodulatory activities on cells of innate, adaptive, and regulatory immunity with a final outcome measured in the generation of Th1 immune responses critical for cancer eradication and control of recurrences but also demonstrate safety at therapeutic doses.

12.6.2 Costimulatory Ligands as Alternative Adjuvants

An effective therapeutic cancer vaccine should aim to enhance the activity of DC, T cells, and NK cells for the generation of antitumor immune responses effective in eradicating the existing tumor and promoting immunological memory for control of recurrences. Most importantly, therapeutic cancer vaccines should also ideally prevent the generation and/or function of Treg cells and other immune evasion pathways, which serve as major hurdles for the efficacy of cancer vaccines (Schabowsky et al. 2007). In this context, costimulation plays a critical role in modulating innate, adaptive, and regulatory immune responses. Unlike TLRs, costimulation directly targets adaptive immunity and is critical for the generation of primary as well as memory T- and B-cell responses (Croft 2009). As such, agonistic ligands to costimulatory receptors have the potential to serve as effective immunomodulatory components of therapeutic cancer vaccines. Tumor cells have propensity to downregulate costimulatory signals as a means of immune evasion mechanism. Lack of costimulatory signals limits the magnitude of primary T-cell activation against tumors, leading to T-cell anergy (Cuenca et al. 2003). Therefore, ectopic expression of costimulatory molecules in tumor cells via various means has been a successful strategy for the generation of effective antitumor immune responses with preventive and therapeutic efficacy in various preclinical tumor models (Guckel et al. 2005; Singh et al. 2003).

Costimulatory molecules can be divided into two superfamilies: CD28 and TNFR (Croft 2003). The CD28 family includes molecules with costimulatory, CD28 and ICOS, and co-inhibitory functions, CTLA-4 and PD-1, and those that have both inhibitory and stimulatory functions, such as B7-H3 receptor. The TNFR superfamily includes costimulatory CD30, 4-1BB, OX-40, CD40, CD70, and glucocorticoid-induced TNFR-related protein (GITR) (Table 12.2). In contrast to the members of CD28 superfamily, except CD28 receptor, that are involved in the generation of Th2 responses (ICOS), regulatory immunity (ICOS, PD1), or inhibition of immune responses (PD1, CTLA-4, B7-H3R), the majority of TNFR family members are involved in the generation of Th1 and $CD8^+$ T-cell immune responses critical to the elimination of cancer (Croft 2003). As such, the agonists of TNFR family have drawn considerable attention as potential adjuvants for the development of therapeutic cancer vaccines.

Activation of DCs by PAMPs leads to their activation, enhanced antigen uptake and presentation, expression/upregulation of costimulatory ligands and MHC molecules, and cytokine production critical to the initiation of adaptive immune responses. Naïve T cells that have recognized antigens as peptides in the context of MHC molecules respond to DC-generated cytokines and costimulatory cues by proliferating and acquiring effector functions (Janeway and Medzhitov 2002; Jenkins et al. 2001; Banchereau and Steinman 1998). In principal, the initial costimulatory signals are provided by B7 ligands interaction with the constitutively expressed CD28 receptor on naïve T cells. Once activated, T cells upregulate various members of the TNFR superfamily, such as 4-1BB and OX-40, which in

Table 12.2 Expression and key function of receptors/ligands of TNF superfamily

Receptor	Ligand	Receptor distribution	Ligand distribution	Physiological functions
4-1BB (CD137)	4-1BBL (CD137L)	Activated T cells, NK cells, NKT cells, neutrophils, mast cells, eosinophils, and endothelial cells Resting monocytes, DCs, and Treg cells	Activated APCs (DCs, B cells, and macrophages), T cells, mast cells, NK cells, and smooth muscle cells Resting hematopoietic progenitors	T cell activation, survival, effector, and memory function DC-T cell communication, renders T effectors resistant to Tregs
OX40 (CD134)	OX40L (CD252)	Activated T cells Resting Treg cells, NK cells, NKT cells, and neutrophils	APCs (DCs, B cells, and macrophages) Activated T cells, NK cells, endothelial cells, smooth muscle cells, and mast cells	T-cell activation, expansion, and survival Important for $CD4^+$ T-cell memory Inhibits the development and suppressive function of Tregs
CD40	CD40L (CD154)	APCs (DCs, B cells, macrophages), smooth muscle cells, fibroblast, epithelial cells, and basophils	Activated T cells, APCs (DCs, B cells, and macrophages), and endothelial, epithelial, and muscle cells	T-cell activation and survival, B-cell proliferation, maturation, class switching, and DC maturation
CD27	CD70	Naive T cells, Tregs, thymocytes, memory B cells, NK cells, and NKT cells	APCs (DCs and B cells) Activated T cells	T-cell activation and survival, regulation of B-cell activation, and immunoglobulin synthesis
CD30	CD30L	Activated T cells B cells, monocytes, NK cells, and eosinophils	Activated T cells B cells	T cell regulation, proliferation, apoptosis, and cytotoxicity of lymphoid cells
HVEM	LIGHT, LT-α	Resting T cells DCs, NK cells, monocytes, and Tregs	Monocytes, immature DCs Activated T cells, and NK cells	T-cell costimulation, B-cell costimulation in cooperation with CD40/CD40L, plasma cell differentiation and Ig secretion, and DC maturation
GITR (CD357)	GITRL	Resting and activated T cells Constitutive expression on Tregs, NK cells, NKT cells, B cells, macrophages, and DCs	APCs (DCs, B cells, and macrophages), and endothelial cells	Proliferation and survival of activated T cells Inhibits TCR-induced apoptosis Renders T effectors resistant to inhibitory effects of Tregs

turn interact with their upregulated ligands on DCs to further drive T-cell proliferation, survival, differentiation into effectors, and establishment of long-term memory (Harding et al. 1992; Jenkins et al. 1991; Norton et al. 1992; Watts 2005; Croft 2003). Several studies have demonstrated the utility of agonistic Abs against TNFRs in inducing effective Th1 immune responses with therapeutic efficacy in settings of infection and cancer preclinical models (Melero et al. 1997; Weinberg et al. 2000). Among all the TNFR family members, 4-1BB appears to have the desired attributes for the development of therapeutic cancer vaccines as it is a potent inducer of Th1 responses, critical to long-term $CD8^+$ T-cell memory, and overcomes $CD4^+CD25^+FoxP3^+$ Treg inhibitory responses by various means (Myers et al. 2006; Sharma et al. 2009). These attributes led us to recently propose 4-1BB costimulatory ligands as adjuvants of choice for the development of therapeutic cancer vaccines (Sharma et al. 2009).

12.7 4-1BB and 4-1BBL Expression and Signaling in Immune Regulation

4-1BB (also known as CD137) is a member of the TNF receptor superfamily that was first discovered to be overexpressed at mRNA levels in activated T cells (Kwon and Weissman 1989). Subsequent studies confirmed the inducible expression of 4-1BB receptor not only on activated T cells but also various cells of innate immunity, such as NK, NKT cells, monocytes, macrophages, mast cells, and eosinophils (Futagawa et al. 2002; Lee et al. 2005a; Melero et al. 2008; Kim et al. 2008a). Constitutive expression of 4-1BB has been shown for Treg cells, neutrophils, a sub-subpopulation of DCs, and also under selected conditions NK and NKT cells (Futagawa et al. 2002; Lee et al. 2005a, 2009a; Melero et al. 1998, 2008). These cells with constitutive 4-1BB expression further upregulate the expression of the receptor following activation. The duration of 4-1BB receptor expression on activated T cells is variable, lasting hours to days depending on the experimental setting. It has recently been reported that around 10 % of $CD8^+$ T memory cells maintain sustained expression of 4-1BB (Lin et al. 2012, 2013). However, such sustained expression was contextual and limited to $CD8^+$ T memory cells in the liver and bone marrow. Importantly, the sustained expression of 4-1BB on memory $CD8^+$ T cells was regulated by GITR in T cell-intrinsic manner (Lin et al. 2013). The expression of 4-1BB is not restricted to hematopoietic lineage only. Hypoxic endothelial cells in tumor beds, fibroblasts, inflamed blood vessels, and lymphatic epithelial cells in response to cytokines or TLR agonists express 4-1BB, suggesting a role for global homeostatic control of this receptor within and beyond the immune regulation (Teijeira et al. 2012).

4-1BB signaling leads to recruitment of TNFR-associated factor (TRAF) adopter proteins, TRAF-1 and TRAF-2, initiating proinflammatory signaling pathways involving phosphatidylinositol-3-kinase (PI3K) and mitogen-activated

protein kinase (MAPK) that eventually converge on the activation of NF-κB (Arch and Thompson 1998; Sabbagh et al. 2008; Saoulli et al. 1998). This signaling also promotes the upregulation of antiapoptotic molecules, such as Bcl-2 and bcl-X_L, and protects antigen-specific T cells from activation-induced cell death (Sabbagh et al. 2008; Kroon et al. 2007). Although signaling into T cells from 4-1BB receptor is predominantly associated with a positive immune response, $CD4^+$ T cells from 4-$1BB^{-/-}$ mice displayed enhanced proliferation when stimulated with mitogens in vitro and showed improved antigen-specific responses following adoptive transfer into wild-type mice (Kwon et al. 2002; Lee et al. 2005b). This observation indicates that 4-1BB signaling in $CD4^+$ T cells may also have a regulatory role, fine-tuning the pursuing immune responses. Recently, it was shown that agonistic 4-1BB Abs induce a unique $CD4^+$ T-cell subpopulation with robust cytotoxicity against melanomas (Curran et al. 2013). This cell population expresses KLRG1 and the T-box transcription factor eomesodermin and requires 4-1BB signaling in both T cell and APCs and IL-27, IL-15, and IL-10 cytokines for development. Besides the potent proliferative, survival, and functional advantages for effector T cells, 4-1BB ligation on NK and NKT cells is important for their expansion, survival, and secretion of IFNγ, which collectively contribute to the critical role of these cells in immune responses against cancer (Melero et al. 1998).

The ligand of 4-1BB, 4-1BBL (also known as CD137L or TNFSF9), is a member of the TNF superfamily and was discovered to be present mostly on APCs, such as macrophages, B cells, and DCs. The reverse signaling of 4-1BBL in APCs induces the production of cytokines such as IL-6 and IL-12 (Ju et al. 2009; Kim et al. 2009a). Reverse signaling has been shown to involve the direct interaction of 4-1BBL via its extracellular domain with TNFR1 on the plasma membrane of human monocytes (Moh et al. 2013). In as much as DCs and monocytes express 4-1BB upon activation, the engagement of 4-1BB with 4-1BBL on the same cell or two different cells may play a positive feed-forward mechanism for the generation, activation, and survival of DCs for improved immune responses. In addition to a plethora of positive effects of 4-1BB signaling on the effector arms of the immune system, this feature of 4-1BB/4-1BBL system related to APC regulation further provides a strong rationale for the use of agonists as a potential immune adjuvant platform for the development of therapeutic cancer vaccines.

12.8 Targeting 4-1BB Signaling for Immunomodulation

$CD8^+$ T cytotoxic response is important for the elimination of various intracellular infections caused by bacteria and viruses (Lee et al. 2005a, 2009a; Tan et al. 1999; Lin et al. 2009). $CD8^+$ T cells are a critical component of effector immune responses against tumors, and in selected settings, they are required for the elimination of tumors (Lesterhuis et al. 2011; Sharma et al. 2009; Smyth et al. 2000; Uno et al. 2006). Importantly, these cells are often ignorant or tolerant towards cancer cells (Lesterhuis et al. 2011; Zou 2005). Therefore, agents that promote

$CD8^+$ T-cell activation, expansion, and survival and impart strong cytolytic and inflammatory properties are ideal candidates as adjuvants for the development of therapeutic vaccines. The agonists of TNFR family are well suited as strong $CD8^+$ T-cell adjuvants due to their demonstrated roles in the activation, expansion, and survival of these cells and establishment of long-term memory (Croft 2003; Watts 2005; Aggarwal 2003). We primarily focused on 4-1BB/4-1BBL pathway because 4-1BB signaling is (a) the most effective of all the other members of costimulatory pair of the TNFR family in activating T cells (Rabu et al. 2005), (b) critical to the generation and maintenance of $CD8^+$ T-cell responses (Lee et al. 2003; Myers et al. 2006) that play an essential role in the eradication of viral infections and tumors (Feltkamp et al. 1993; Lin et al. 1996), and (c) important in overcoming various immune evasion mechanisms by tumors (Madireddi et al. 2012; Sharma et al. 2009; Wilcox et al. 2004).

The 4-1BB receptor is expressed early after $CD8^+$ T-cell activation and is important to various functions of T cells. Signaling via 4-1BB receptor induces robust amplification of T cell-mediated immune responses, inhibits apoptotic cell death (Laderach et al. 2002; Rogers et al. 2001), and establishes long-term T-cell memory (Bansal-Pakala et al. 2001; Watts 2005). Ligation of 4-1BB on $CD8^+$ T cells can reverse the tumor-induced nonresponsiveness of these cells, leading to regression of established tumors primarily through the activities of $CD8^+$ T and NK cell axis (Sharma et al. 2009, 2010b; Wilcox et al. 2004). Furthermore, the 4-1BB costimulation has recently been demonstrated to induce a distinct $KLRG1^+Emos^+CD4^+$ T cells with robust cytotoxic function against melanomas (Curran et al. 2013; Qui et al. 2011). Most importantly, 4-1BB ligation renders T effector cells resistant to suppression by Treg cells (Sharma et al. 2009) as well as prevents antigen, TGF-β, and tumor-mediated conversion of T effector cells into Treg cells (Madireddi et al. 2012).

12.8.1 4-1BB Signaling in Immunity Against Infections

The importance of 4-1BB signaling in immune responses against infections came from initial observations that 4-$1BBL^{-/-}$ mice have reduced $CD8^+$ T-cell recall response against viruses (DeBenedette et al. 1999; Bertram et al. 2002). Ensuing studies have shown that 4-1BB signaling also contributes to the priming phase of $CD8^+$ T-cell response against various viruses, including influenza, herpes simplex virus-1 (HSV-1), and lymphocytic choriomeningitis virus (LCV) (Bertram et al. 2002; Kim et al. 2005; Tan et al. 1999). Unlike $CD28^{-/-}$ mice that exhibited a severe defect in the expansion of influenza virus-specific primary $CD8^+$ T cells, 4-$1BBL^{-/-}$ mice showed a normal response (Bertram et al. 2002). The number of virus-specific $CD8^+$ T cells, however, was significantly reduced late in primary response. Importantly, 4-$1BBL^{-/-}$ mice did not generate a significant recall response against influenza, and as such implicating 4-1BB signaling in the survival and virus-specific responsiveness of $CD8^+$ T cells late in primary and recall

responses. Treatment of $CD28^{-/-}$ mice with an agonistic Ab to 4-1BB during priming effectively rescued the secondary $CD8^+$ T-cell responses against influenza (Bertram et al. 2004), while in marked contrast, the same treatment regimen in 4-$1BBL^{-/-}$ mice was ineffective in rescuing secondary recall responses against influenza. The secondary response in 4-$1BBL^{-/-}$ mice, however, was restored by treatment with the agonistic 4-1BB Ab during the virus challenge. Importantly, treatment of mice during challenge with influenza virus was effective in increasing the number of $CD8^+$ T cells responding to a dominant epitope, expanded the $CD8^+$ T-cell repertoire to subdominant epitopes, and rescued a defect in the primary $CD8^+$ T-cell response in $CD28^{-/-}$ mice (Halstead et al. 2002). Taken together, these studies demonstrate a critical role for 4-1BB signaling in the generation of primary late and recall responses against influenza.

The 4-1BB signaling was also shown to be important for the generation of primary and secondary $CD8^+$ T-cell responses to herpes simplex virus 1 (HSV-1). Treatment of mice with an agonistic 4-1BB Ab during HSV-1 challenge resulted in increased numbers of virus-specific primary and memory $CD8^+$ T cells that also expressed CD11c as a distinct marker (Kim et al. 2005). Unlike influenza, the 4-1BB signaling appears to play a dual role in $CD8^+$ T-cell responses to mouse cytomegalovirus (MCMV) infection (Humphreys et al. 2010). Although the 4-$1BB^{-/-}$ mice exhibited exaggerated primary $CD8^+$ T-cell responses to MCMV, the recall responses to the virus were significantly reduced as compared with wild-type mice. The 4-1BB signaling was shown to rescue HIV-specific $CD8^+$ T-cell cytotoxic function from functionally impaired $CD8^+$ T cells that was correlated with the TRAF1-dependent downregulation of the proapoptotic molecule Bim (Wang et al. 2007). Most significantly, it was shown that HIV-specific $CD4^+$ T cells expressing 4-1BB produced more IL-2 than cells lacking 4-1BB (Kassu et al. 2009). The expression of 4-1BB was found to be lower on virus-specific $CD4^+$ T cells producing both IL-2 and IFNγ. Treatment with antiretroviral drugs resulted in increased 4-1BB expression on virus-specific, IL-2 producing $CD4^+$ T cells, and the percentage of HIV-specific $CD4^+$ T cells expressing 4-1BB was inversely correlated with viremia. Similarly, DCs transfected to express 4-1BBL were shown to enhance the proliferation, function, and survival of HIV-specific $CD8^+$ T cells (De et al. 2011). Signaling via 4-1BB resulted in the downregulation of the inhibitory function of Treg cells on $CD8^+$ T-cell proliferation. In a macaque model, an agonistic Ab against 4-1BB was shown to enhance the efficacy of a DNA subunit vaccine against simian immunodeficiency virus (SIV) by increasing IFNγ production (Hirao et al. 2011).

The 4-1BB signaling also plays an important role in immune responses against bacterial infections. 4-1BB stimulation of neutrophils in the early phase of *Listeria monocytogenes* infection causes rapid production of inflammatory cytokines/chemokines, which leads to subsequent infiltration of neutrophils and monocytes crucial for the elimination of infection (Lee et al. 2005a). Moreover, a recent study demonstrated that 4-1BB/4-1BBL interaction regulates the innate and adaptive immune responses of the host against *Mycobacterium tuberculosis* (Fernandez Do Porto et al. 2012). Collectively, these studies demonstrate that agonists of 4-1BB

signaling can serve as potential adjuvants for the development of vaccines against intracellular infections.

12.8.2 4-1BB Signaling in Immunity Against Cancer

The impact of 4-1BB signaling on immune responses is extensively studied in settings of cancer vaccines or other cancer immunotherapy modalities. Stimulation via this receptor was shown to have multiple effects on tumor-specific effector immune responses that include (a) DC activation, survival, and enhanced antigen uptake and processing (Sharma et al. 2009; Futagawa et al. 2002; Choi et al. 2009); (b) T-cell activation, expansion, survival, acquisition of Th1 effector function, and establishment of long-term memory (Sharma et al. 2009; Melero et al. 1997; Lee et al. 2003; Futagawa et al. 2002); and (c) activation and improved function of various innate immune cells, including NK cells, NKT cells, γδT cells (Lee et al. 2013), macrophages, neutrophils, eosinophils, and mast cells (Futagawa et al. 2002; Lee et al. 2005a; Melero et al. 2008). Most significant in the context of tumor vaccines is the role of 4-1BB signaling in overcoming various immune evasion mechanisms employed by the progressing tumor. Stimulation with agonistic Abs to 4-1BB was shown to result in reversal of tumor-induced $CD8^+$ T-cell anergy (Sharma et al. 2009; Wilcox et al. 2004). $CD4^+CD25^+FoxP3^+$ Treg cells constitutively express 4-1BB receptor, and stimulation through this receptor was shown to block their inhibitory function (Choi et al. 2004). Collectively, these immune attributes of 4-1BB qualify this signaling pathway as an important target to be exploited for cancer immunotherapy.

A series of studies using agonistic Abs to 4-1BB have demonstrated the robust efficacy of 4-1BB signaling in eradication of established tumors in various preclinical models (Melero et al. 1997; Shuford et al. 1997). Immunizations with agonistic Abs to 4-1BB as monotherapy or in combination with other immunomodulators generated $CD8^+$ T cell- and NK cell-driven potent antitumor immune responses that translated into tumor eradication in various animal tumor models, including colon carcinoma, P815 mastocytoma, Ag104A sarcoma, and lymphomas (Melero et al. 1997; Shuford et al. 1997). The impressive therapeutic efficacy of agonistic 4-1BB Abs in preclinical cancer models led to the development of these reagents for clinical trials. Indeed, a humanized agonistic Ab has been tested in several Phase I and a Phase II clinical trials for cancer (Li and Liu 2013). Although impressive, one potential drawback to the use of Abs is their reported toxicity both in experimental systems (Mittler et al. 1999; Niu et al. 2007; Schabowsky et al. 2009) and in clinical trials (Pardoll and Drake 2012). The effective exploitation of the 4-1BB signaling for cancer immunotherapy will, therefore, depend on the development of novel agonists that generate robust immune responses in the absence of or tolerable toxicity at therapeutic doses.

12.9 SA-4-1BBL as an Adjuvant for the Development of Therapeutic Cancer Vaccines

We have recently hypothesized that natural ligand to 4-1BB may serve as a more effective and safe alternative to agonistic Abs for the development of immune therapies (Schabowsky et al. 2009; Sharma et al. 2009, 2010a, b; Srivastava et al. 2012). The 4-1BBL exerts its costimulatory function as trimers expressed on the surface of APCs. The soluble trimers have no costimulatory function (Rabu et al. 2005). Our laboratory has pioneered a novel technology designated as Protex™ that involves the generation of chimeric ligands with a modified form of core streptavidin (SA), modification of the cell membrane with biotin, and engineering of chimeric proteins as an alternative to gene therapy for immunomodulation (Fig. 12.1) (Sharma et al. 2009, 2010c; Singh et al. 2003; Yolcu et al. 2002). In addition, these chimeric proteins have two distinct advantages over native ligands. First, chimeric ligands exist as tetramers and higher-order structures and effectively cross-link their receptors on immune cells in soluble form for effective signal transduction (Sharma et al. 2009, 2010b; Elpek et al. 2007). Second, SA portion of chimeric molecules allows for conjugation to

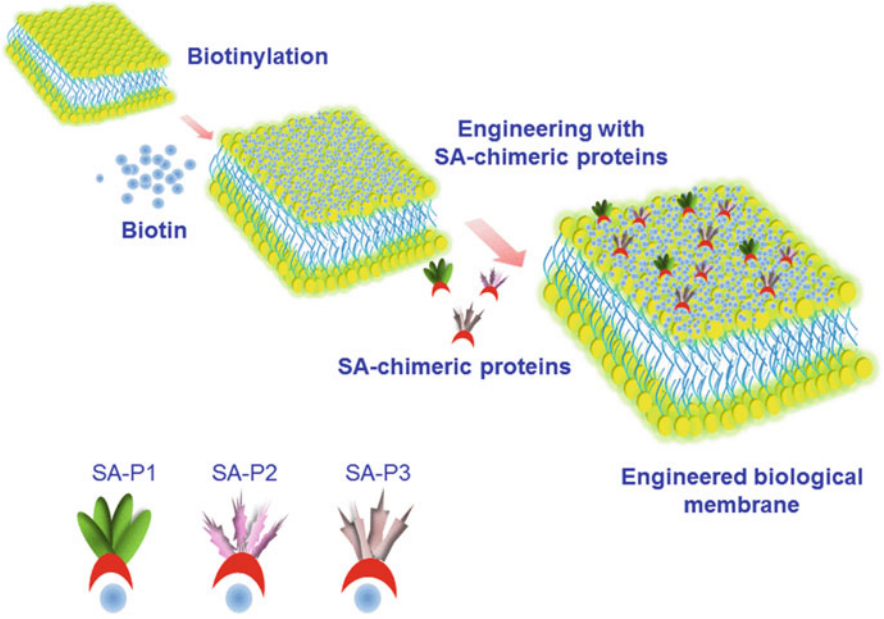

Fig. 12.1 Protex™ technology for cell surface engineering of exogenous proteins of interest for immunomodulation. Cells, tissues, or organs can be modified with biotin followed by engineering with SA-chimeric proteins in a rapid, efficient, and cost-effective manner as an alternative to gene therapy for immunomodulation. SA-P1, SA-chimeric protein 1. Modified from Shirwan et al., Cancer Vaccines Methods and Protocols, series Methods in Molecular Biology, Vol. 1139, Lawman, Michael J.P., Lawman, Patricia D. (Eds.), Springer 2013

any biotinylated antigen of interest for the development of conjugate vaccines. Inasmuch as a subpopulation of DCs constitutively express 4-1BB receptor, 4-1BBL in the conjugate vaccine may serve as a vehicle to deliver Ags to DCs in vivo for better antigen uptake and cross-presentation to T cells (Fig. 12.2).

We thus generated a novel form of 4-1BBL by fusing the extracellular domains of mouse or human 4-1BBL molecules C-terminus to a modified core streptavidin. This chimeric 4-1BBL (SA-4-1BBL) molecule has various desired features relevant for the development of cancer vaccines. First, SA-4-1BBL exists as stable tetramers and higher-order structures owing to the structural features of SA (Sharma et al. 2010a), which endows this molecule with robust costimulatory activity in soluble form for both $CD4^+$ and $CD8^+$ T cells (Schabowsky et al. 2009). This is plausibly due to the ability of SA-4-1BBL to effectively cross-link 4-1BB receptors on immune cells for potent signal transduction. Second, SA-4-1BBL effectively activates DCs in vivo for antigen uptake and cross-presentation to $CD8^+$ T cells (Sharma et al. 2009, 2010a). Third, SA-4-1BBL endowed T effector cells resistant to suppression by $CD4^+CD25^+FoxP3^+$ Treg cells and blocked antigen, TGF-β, and

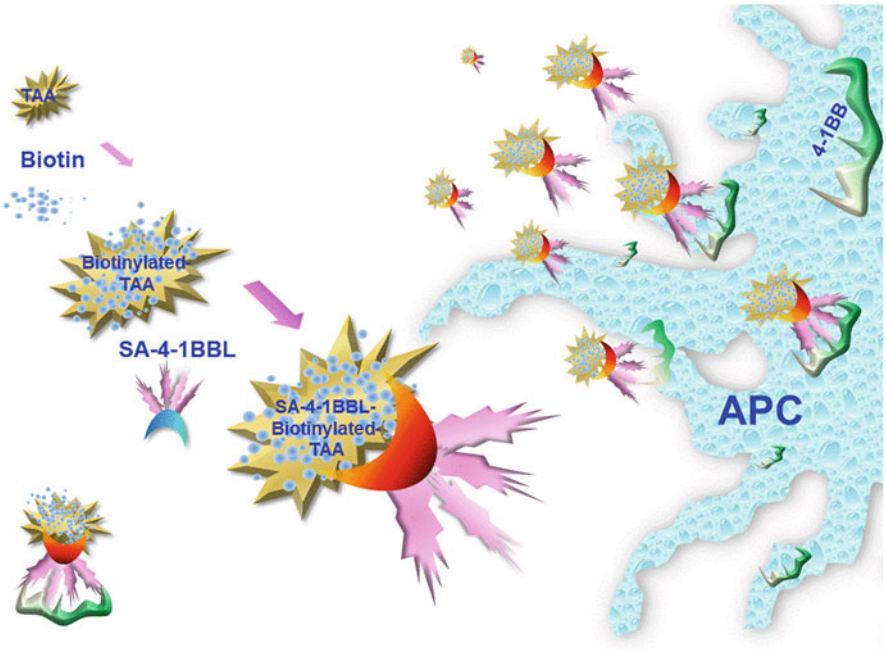

Fig. 12.2 SA-4-1BBL as a vehicle for targeted delivery of conjugated TAAs into 4-1BB expressing DCs. TAAs can be biotinylated and mixed with SA-4-1BBL for the generation of a conjugate vaccine. In vivo, 4-1BBL portion of the vaccine facilitates the delivery of TAAs to 4-1BB expressing DCs for activation and enhanced antigen uptake and presentation to T cells. SA-4-1BBL at a subsequent stage interacts with the upregulated 4-1BB receptor on newly activated T cells and drives their expansion, survival, acquisition of effector function, and establishment of long-term memory

tumor-induced conversion of T effector into Treg cells (Madireddi et al. 2012; Sharma et al. 2009). Fourth and most importantly, treatment of mice with SA-4-1BBL alone or in combination with various antigens did not result in measurable acute toxicity, immune abnormalities, or autoimmunity (Schabowsky et al. 2009; Srivastava et al. 2012) as had been reported for agonistic Abs (Niu et al. 2007).

As the adjuvant component of various TAA-based vaccine formulations, SA-4-1BBL demonstrated robust therapeutic activity in various tumor models. A single vaccination with SA-4-1BBL and a peptide representing the dominant $CD8^+$ T-cell epitope for human papillomavirus (HPV) E7 oncogene ($E7_{49\text{-}57}$) resulted in the eradication of E7 TAA-expressing TC-1 tumor cells (Sharma et al. 2009). SA-4-1BBL in this setting performed better than three other TLR agonists, LPS, MPL, and CPG (Sharma et al. 2009). SA-4-1BBL as the adjuvant component of complete E7 protein vaccine formulation also proved effective in eradicating the TC-1 tumor (Sharma et al. 2010b). This therapeutic efficacy was not restricted to E7 as a xenoantigens as a vaccine formulation containing SA-4-1BBL and survivin as a bona fide self-TAA also demonstrated robust therapeutic efficacy in survivin overexpressing 3LL lung carcinoma model (Sharma et al. 2009; Srivastava et al. 2012). Importantly, therapeutic efficacy of SA-4-1BBL-based vaccines in both models was enhanced to 100 % by multiple vaccinations (Srivastava et al. 2012; Sharma et al. 2013). The vaccine showed no efficacy against tumors grown in 4-1BB mutant mice, demonstrating the requisite role of signaling through 4-1BB receptor (Sharma et al. 2010b).

The therapeutic efficacy of the vaccines correlated with augmented $CD8^+$ T-cell effector/memory responses, IFNγ production, reversal of $CD8^+$ T cell anergy, and increased intratumoral ratio of $CD8^+$ T/Treg cells (Sharma et al. 2009, 2010a). $CD8^+$ T cells played a requisite role for the vaccine therapeutic efficacy, while NK cells played a non-requisite, secondary role (Fig. 12.3) (Sharma et al. 2010b; Srivastava et al. 2012). Importantly, $CD4^+$ T cells did not appear to play a critical role in vaccine therapeutic efficacy at the effector phase, but were required at the priming phase (Sharma et al. 2013). The depletion of $CD4^+$ T cells 1 day before vaccination had no effect on the therapeutic efficacy of SA-4-1BBL/E7 and SA-4-1BBL/survivin-based vaccines in both TC-1 and 3LL established tumor models. However, the depletion of $CD4^+$ T cells was associated with lack of long-term immune memory in the 3LL, but not TC-1 model, suggesting that priming with a weak self-antigen requires $CD4^+$ T-cell help for the establishment of long-term memory as demonstrated for various infection models (Sharma et al. 2013). Importantly, there was a significant decrease in the efficacy of these vaccines when $CD4^+$ T cell was depleted 1 day before tumor challenge. This finding suggests that challenge with the tumor is sufficient to prime $CD4^+$ T cells, which plausibly set in motion a tumor-specific $CD8^+$ T cell response targeted for augmentation by vaccination with SA-4-1BBL/Ags. These observations are not only important specifically in the context of SA-4-1BBL-based vaccines but also in general for all cancer vaccines as they demonstrate the requisite role of $CD4^+$ T cells for effective immune responses against cancer. The therapeutic efficacy of the vaccines was achieved in the absence of detectable acute toxicity or autoimmunity. Potent

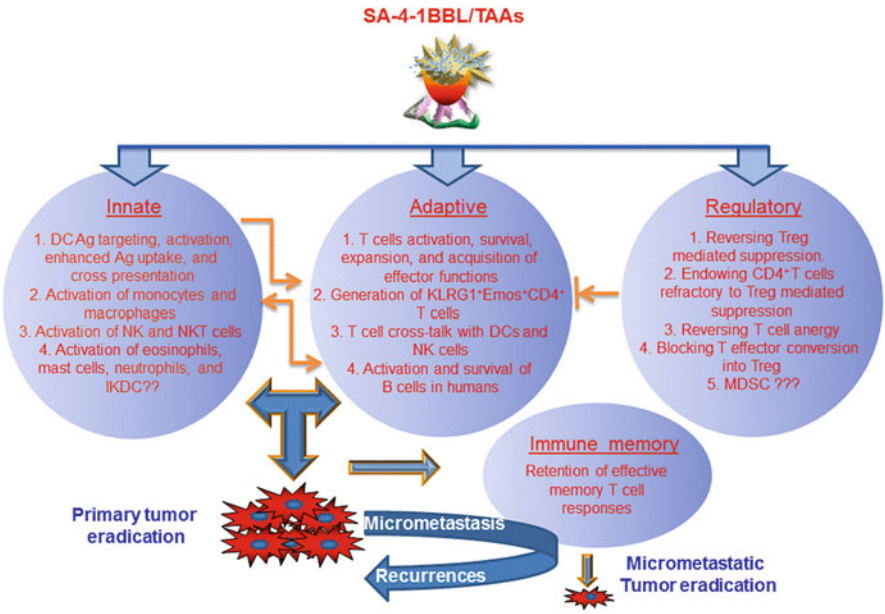

Fig. 12.3 Pleiotropic effects of SA-4-1BBL as the adjuvant component of subunit protein vaccines on tumor-specific immune responses. SA-4-1BBL works on various cells of innate, adaptive, and regulatory immunity to generate antitumor effector immune response that eradicate primary tumors. SA-4-1BBL also exploits 4-1BB signaling to establish and retain long-term T-cell responses for the eradication of micrometastasis and control of tumor recurrences locally and systemically

pleiotropic immune activities (Fig. 12.3) combined with the lack of toxicity highlight the potential SA-4-1BBL holds as an adjuvant platform for the development of therapeutic cancer vaccines (Schabowsky et al. 2009; Sharma et al. 2009, 2010b).

12.10 Qualitative and Quantitative Differences Between SA-4-1BBL and Agonistic 4-1BB Antibody

SA-4-1BBL in its signaling outcome shows quantitative and qualitative differences with an agonistic 4-1BB Ab (3H3) widely used in various preclinical settings (Melero et al. 1997; Uno et al. 2006). These differences are summarized as follows: (a) SA-4-1BBL generates better primary $CD4^+$ and $CD8^+$ T-cell responses compared with the agonistic 4-1BB Ab (Schabowsky et al. 2009); (b) the agonistic 4-1BB Ab preferentially activates the proliferation of $CD8^+$ T cells, while at equimolar levels, SA-4-1BBL activates proliferation of both $CD4^+$ and $CD8^+$ T cells (Mittler et al. 1999; Niu et al. 2007; Schabowsky et al. 2009); (3) SA-4-1BBL

lacks toxicity and immune perturbation reported for the agonistic 4-1BB Ab (Schabowsky et al. 2009); (4) the agonistic 4-1BB Ab acts as superagonist and activates naive T cells for proliferation and type I cytokine release, whereas SA-4-1BBL lacks such effects and it appears to expand antigen-activated T cells (Schabowsky et al. 2009); (5) a single vaccination with SA-4-1BBL with $E7_{49\text{-}57}$ peptide has better efficacy than the agonistic Ab for the eradication of established E7-expressing TC-1 tumor (Sharma et al. 2009); (6) SA-4-1BBL induces greater infiltration of $CD8^+$ T cells into tumors than the agonistic 4-1BB Ab (Sharma unpublished observation), which may suggest a differential chemokine effect; (7) agonistic Ab induces in vivo expansion of $CD4^+CD25^+$ Treg cells and increased infiltration into tumor, while SA-4-1BBL has minimal favorable effect on Treg cells (Sharma unpublished observation); (8) vaccination with the agonistic Ab leads to reduction in NK cell numbers as well as diminished B cell-mediated antibody responses (Lee et al. 2009b; Mittler et al. 1999), while SA-4-1BBL vaccination enhances the NK cell-mediated killing responses (Sharma et al. 2010b; Srivastava et al. 2012); (9) the agonistic Ab appears to directly affect the suppressive function of Treg cells (Choi et al. 2004), while SA-4-1BBL makes $CD4^+$ T effector cells resistant to suppression by Treg cells (Sharma et al. 2009); and (10) SA-4-1BBL blocks in vitro and in vivo conversion of $CD4^+$ T effector cells into Treg cells (Madireddi et al. 2012), while the effect of agonistic 4-1BB Abs is yet to be assessed. The mechanistic basis of these key differences between the agonistic 4-1BB Abs and SA-4-1BBL is yet to be fully elucidated. These divergent efficacy/safety features of these agents will require further studies to delineate their mechanistic basis of efficacy/toxicity, which will further guide their development for safe and effective use in cancer immunotherapy.

12.11 Prospect of 4-1BB Signaling in Combination with Standard-of-Care and Other Cancer Immunotherapies

Given that cancer has evolved to overcome immune surveillance by various mechanisms and TAAs are weak antigens, therapeutic subunit vaccines may benefit from formulations that incorporate more than one adjuvant or immune potentiator to recruit multiple immune effector arms for efficacy. Agonists of 4-1BB receptor may also improve the efficacy of standard-of-care chemo and/or localized radiotherapy by capitalizing on tumor cell death induced by such treatments and the ensuing TAA cross-priming and immune responses.

12.11.1 Combinatorial Use of 4-1BB Agonists with Other Positive and Negative Costimulatory Molecules

The presence of numerous costimulatory pathways involved in T-cell responses may reflect the fact that individual pathways program unique facets of T-cell functions. For example, although OX40 and 4-1BB are both expressed on activated $CD4^+$ and $CD8^+$ T cells, OX40 costimulation preferentially impacts the $CD4^+$ T-cell effector function (Dawicki et al. 2004; Gramaglia et al. 1998, 2000; Kopf et al. 1999), while 4-1BB more significantly drives $CD8^+$ T-cell responses (Dawicki et al. 2004; Sharma et al. 2009; Pollok et al. 1993). Moreover, while costimulation through OX-40 directly affects the suppressive function of $CD4^+CD25^+FoxP3^+$ Treg cells (Voo et al. 2013), 4-1BB costimulation makes T effector cells resistant to suppression by Treg cells (Sharma et al. 2009). The combination of agonists of 4-1BB and OX40 may program $CD4^+$ T cells to differentiate into cytotoxic Th1 effector cells. These cytotoxic $CD4^+$ T cells may not only produce IFNγ but also possess the ability to kill target cells presenting cognate MHC class II-restricted peptides (Qui et al. 2011), which might be useful in targeting tumors that have propensity to downregulate their MHC class I (Ferrone and Marincola 1995). Consistent with this notion, we have shown that combination of SA-4-1BBL and SA-OX40L was effective in inducing potent proliferative responses in both $CD4^+$ OT-II and $CD8^+$ OT-I cells in vivo and improved therapeutic efficacy, as compared with single agents, in the established TC-1 tumor model (Srivastava et al. unpublished data).

The combinatorial use of an agonistic 4-1BB Ab and a blocking CTLA-4 Ab resulted in significant improvement in the therapeutic efficacy of a whole tumor cell-based vaccine, whereas individual agents had no effect (Curran et al. 2011). Therapeutic efficacy was associated with 4-1BB-driven intratumoral increase in both $KLRG1^+CD4^+$ and $CD8^+$ T effector cells, increase in inflammatory cytokines, and decrease in Treg cells. A triple therapy with Abs to 4-1BB, CD40, and DR5 (apoptosis-inducing receptor for TNF-related apoptosis-inducing ligand, TRAIL) resulted in robust therapeutic efficacy in primary fibrosarcomas, initiated with the carcinogen 3-methylcholanthrene, multiorgan metastases, and primary tumor resistant to DR5 Ab treatment (Uno et al. 2006). Importantly, therapeutic efficacy of this triple Ab treatment was neither associated with detectable toxicity nor autoimmunity and required $CD8^+$ T cells and IFNγ production.

Significant in the context of this book chapter are the results of two recent studies comparing the efficacy of Abs to various costimulatory, CD137 and CD40, and co-inhibitory, CTLA-4, PD-1, TIM-3, and LAG-3, molecules as single agents or in various combinations in the stringent ID8 mouse ovarian cancer model (Wei et al. 2013; Guo et al. 2013). In a 3-day established tumor model, treatment with Ab to 4-1BB resulted in a better therapeutic efficacy as compared with treatments with Abs to PD-1 or CTLA-4 as monotherapy. However, treatment with single agents in a 10-day established ID8 model had no therapeutic benefit, whereas combinations of Abs to 4-1BB and PD-1 or CTLA-4 or all three Abs significantly

prolonged survival as compared with any other double or multiple combinations without 4-1BB Ab. Importantly, the combination of Abs to 4-1BB and PD-1 had the most antitumor effect as compared with any other combinations. Therapeutic efficacy of anti-4-1BB and PD-1 Abs was associated with increased number of splenic $CD8^+$ T effector cells, IFNγ production, and decreased numbers of myeloid-derived suppressor cells (MDSCs) and Treg cells (Wei et al. 2013; Dai et al. 2013). Therefore, the combination of positive costimulatory agonists with co-inhibitory agents, i.e., immune checkpoint blockers, may work in synergy to influence the overall functional outcome of T effector cell responses and manifest better therapeutic efficacy against cancer.

12.11.2 Combinatorial Use of 4-1BB Agonists with Other Immune Potentiators

Several studies have demonstrated enhanced antitumor responses culminating into therapeutic efficacy when agonistic 4-1BB Ab is used in combination with other immunostimulatory agents. Treatment with an agonistic 4-1BB Ab in combination with IL-12 gene transfer resulted in robust therapeutic efficacy in the poorly immunogenic pulmonary metastatic B16.F10 melanoma model, where the individual agents alone had no measurable efficacy (Huang et al. 2010; Xu et al. 2004). Both NK and $CD8^+$ T cells were critical to the observed therapeutic efficacy with $CD4^+$ T cells having no measurable contribution. Similarly, intratumoral injection with an oncolytic adenovirus vector expressing either 4-1BBL or IL-12 resulted in significant immune responses and therapeutic efficacy in the B16-F10 subcutaneous tumor model (Huang et al. 2010; Xu et al. 2004). However, the combination of both agents significantly improved immune and therapeutic efficacy. An agonistic 4-1BB Ab when used in combination with irradiated tumors engineered to secrete granulocyte-macrophage colony-stimulating factor (GM-CSF) as vaccine resulted in increased infiltration of $CD8^+$ T cells into the tumor, their expansion, and long-term memory, which collectively translated into therapeutic efficacy in the established B16 melanoma model (Li et al. 2007). Taken together, these observations justify the combinatorial use of 4-1BB agonists with other immune potentiators for the treatment of cancer.

12.11.3 Combinatorial Use of 4-1BB Agonists with Standard-of-Care Cancer Treatments

The 4-1BB costimulation was exploited in combination with standard-of-care treatment agents in various preclinical tumor models. Treatment with an agonistic 4-1BB Ab (BMS-469492) along with single-dose irradiation therapy at 5, 10, or

15 Gy resulted in measurable therapeutic efficacy at all radiation doses for a breast and only at higher radiation dose for a lung carcinoma model (Shi and Siemann 2006). Treatment with the 4-1BB and PD-1 Abs worked in synergy with cisplatin to achieve an impressive therapeutic efficacy in the aforementioned 10-day-established ID8 tumor model (Wei et al. 2013). Similarly, agonistic 4-1BB Abs worked in synergy with cisplatin resulting in robust therapeutic efficacy in the CT-26 colon carcinoma model (Kim et al. 2008b). The therapeutic efficacy of agonistic Ab was associated with a rapid recovery of T and B cells from cisplatin-induced lymphopenia and generation/expansion of $CD11c^+CD8^+$ T cells expressing IFNγ. Importantly, cisplatin treatment induced the expression of 4-1BB on kidney tubular epithelium and enhanced 4-1BB expression on antigen-primed T cells. Agonistic Ab treatment blocked cisplatin-induced apoptosis of both T cells and kidney epithelium by increasing the expression of antiapoptotic molecules.

In a separate study, combinatorial treatment with a 4-1BB agonistic Ab and 5-fluorouracil (5-FU) resulted in the eradication of radiotherapy- and chemotherapy-resistant renal cell carcinomas in more than 70 % of the mice, where individual agents had no significant effect (Ju et al. 2008). Combination therapy was associated with enhanced proportion of apoptotic cells and higher number of lymphocytes in the spleen and tumor-draining lymph nodes of treated mice. Importantly, mice that had eradicated primary tumors were completely resistant to rechallenge with the original tumor, demonstrating establishment of long-term immune memory. In the B16 melanoma study, combinatorial treatment with 4-1BB Ab and cyclophosphamide resulted in significant antitumor immune responses and tumor eradication, which was associated with increased numbers of IFNγ-producing effector $CD11c^+CD8^+$ T cells (Kim et al. 2009b). Agonistic 4-1BB Ab treatment facilitated rapid recovery of T and B cells from drug-induced lymphopenia and protected naïve T cells from drug-induced toxicity. Importantly, treatment with cyclophosphamide increased the expression of 4-1BB on both $CD4^+$ and $CD8^+$ T cells and suppressed peripheral Treg cells. Taken together, these studies provide compelling rationale for exploiting 4-1BB signaling using agonistic Abs or 4-1BBL in combination with standard-of-care agents for cancer treatment.

12.11.4 Combinatorial Use of 4-1BB and TLR Agonists

Critical to the activation and maintenance of an immune response are the signals transduced by TLR and costimulatory receptor pathways (Croft 2009; Kawai and Akira 2010). As such, agonistic ligands to receptors of these two pathways have significant potential as adjuvants for therapeutic vaccines. Consistent with this notion is the approval of TLR-4 agonist MPL by FDA to be used as the adjuvant component of the preventive vaccine against HPV infection (Romanowski et al. 2009). However, the efficacy of MPL as the adjuvant component of therapeutic vaccines against cancer remains to be fully assessed. MPL primarily targets innate immunity, leading to the recruitment, activation, and maturation of APCs,

such as DCs, that facilitate the generation of adaptive immune responses (Didierlaurent et al. 2009). MPL primarily targets APCs for the initiation of adaptive immunity (Didierlaurent et al. 2009) and 4-1BBL targets $CD8^+$ T cells for activation, acquisition of effector function, survival, and long-term memory (Watts 2005; Bukczynski et al. 2004; Cannons et al. 2001). Given the critical role of $CD8^+$ T cells for tumor eradication, we recently hypothesized that an adjuvant system composed of both of these molecules may have potent therapeutic efficacy as the component of TAA-based vaccine formulations against cancer. In preliminary studies, we demonstrated that a single vaccination with a formulation containing both adjuvants and E7 as TAA resulted in the effective eradication of E7-expressing TC-1 tumor in all mice (Srivastava et al. unpublished data). This effect was extendable to the 3LL pulmonary lung carcinoma model where survivin was used as a bona fide self-TAA. Importantly, the therapeutic efficacy of the vaccines required $CD8^+$ T cells and associated with high intratumoral $CD8^+$ T effector/Treg cell ratios (Srivastava et al. unpublished data).

A series of recent studies demonstrated synergy between 4-1BB and TLR4 signaling. Stimulation of macrophages with a TLR agonist was recently shown to upregulate 4-1BBL expression on macrophages (Kang et al. 2007). Importantly, 4-1BBL expression on macrophages was critical to sustained expression of proinflammatory cytokines, particularly TNFα, and achieved this effect by physically interacting with TLR4. The effect of 4-1BBL was 4-1BB receptor independent and required TLR4-induced activation of transcription factors CREB and C/EBP to sustain the late TNFα response. This new and direct interplay between 4-1BB and TLRs is more likely not unique to TLR4 and provides another means of regulation between innate immunity and adaptive immunity. Furthermore, reverse signaling through 4-1BBL in human monocytes converts them into mature DCs with potent antigen-presenting function and production of cytokines such as IL-6 and IL-12 (Ju et al. 2009). Such DCs inhibit the development of Treg cells and induce the expression of perforin, IFNγ, IL-13, and IL-17 in T effector cells (Kwajah and Schwarz 2010). The 4-1BB signaling is also critical for the activation and survival of DCs and trafficking to the T-cell zone in lymph nodes (Choi et al. 2009). Given the demonstrated role and importance of TLRs in activation and effector function of APCs, the synergy between 4-1BB and TLRs provides an important opportunity for the combinatorial use of their agonists as adjuvant systems for the development of therapeutic vaccines against chronic infection and cancer.

12.12 Conclusions

Cancer immunotherapy field without question is at the brink of exciting developments. The FDA approval of DC-based Provenge therapeutic cancer vaccine against hormone refractory prostate cancer in 2010 followed by anti-CTLA-4 Ab for melanoma (Hodi et al. 2010) marked the beginning of many immunotherapies to

follow. The impressive clinical results with anti-PD-1 and PDL-1 Abs are good indication of their approval in a near future as another class of therapeutics (Brahmer et al. 2012; Topalian et al. 2012). These clinical developments combined with our better understanding of the immune system in general and immune response against cancer in particular will further accelerate the development of cancer immunotherapies. In particular, immunomodulation to boost effector immune responses and control immune escape mechanism by progressing tumors combined with the positive impact of some standard-of-care agents on tumor immune responses provides a plethora of possibilities for rationale development of combinatorial therapies. The efficacy of such therapies will require careful consideration of various parameters, which include the nature of tumor, stage/burden of cancer, characteristic of required antitumor immune responses for efficacy, prior standard-of-care treatment history of the patient, and potential treatment to be applied during or post vaccination.

Cancer vaccines stand a good chance of changing the course of cancer treatment. However, their efficacy will require careful consideration of not only TAAs but most importantly also adjuvants for vaccine formulations. Adjuvants having pleiotropic effects on various cells of the immune system with a net outcome of generating tumor destructive immune responses without significant toxicity harbor great potential. In this context, SA-4-1BBL stands a good chance of serving an adjuvant of choice because of several desired features of 4-1BB expression and signaling. In the context of T cells, 4-1BB is expressed in activated T effector cells and plays a critical role for their proliferation, survival, and acquisition of Th1 function, which is critical for the eradication of many tumors. Most importantly, 4-1BB expression marks tumor-specific T cells, and signaling via this molecule has been exploited for effective expansion of these cells ex vivo for cancer adoptive immunotherapy (Lin et al. 2010). The 4-1BB signaling is also involved in DC activation, survival, and enhanced antigen uptake and presentation (Choi et al. 2009; Sharma et al. 2009, 2010a). Most importantly, 4-1BB regulates the suppressive function of Treg cells directly or indirectly for the benefit of generating potent effector immune responses (Sharma et al. 2009; Choi et al. 2004). SA-4-1BBL or agnostic 4-1BB Abs as a single agent has shown impressive therapeutic efficacy in various tumor models (Sharma et al. 2009, 2010b; Srivastava et al. 2012). In combination with other immune potentiators or standard-of-care treatments, agonists of 4-1BB have shown even improved therapeutic efficacies (Ju et al. 2008; Kim et al. 2008b, 2009b; Li et al. 2007; Wei et al. 2013). Importantly, 4-1BB is expressed by nonmalignant cells, such as endothelial or epithelial, within tumor microenvironment in response to tumor-induced hypoxia, and signaling via 4-1BB was shown to generate various cytokines and chemokines that feed on 4-1BB signaling in T effector cells for a more pronounced therapeutic efficacy against tumors (Melero et al. 2008). Signaling via 4-1BB on tubular epithelium cells also protects against standard chemotherapy-induced toxicities (Kim et al. 2008b), further making this pathway an attractive target for cancer combinatorial immunotherapy/chemotherapy/radiotherapy.

SA-4-1BBL may also serve as a platform to develop adjuvant systems using agents with different mechanisms of action and as such expected potential therapeutic synergy. Moreover, the next-generation vaccines may benefit from the targeted delivery of TAA/tumor-specific antigens (TSAs) into DCs given the importance of these cells in the initiation of potent adaptive immune responses against tumors. In this context, our published studies demonstrating that SA-4-1BBL conjugated with TAAs effectively delivers the antigens to DCs constitutively expressing 4-1BB for activation, enhanced antigen uptake, and cross-presentation to T cells are significant (Sharma et al. 2010a). The ability of SA-4-1BBL to endow T effector cells refractory to suppression by $CD4^+CD25^+FoxP3^+$ Treg cells (Sharma et al. 2009), block the conversion of T effector cells in the Treg cells (Madireddi et al. 2012), and reverse tumor-induced $CD8^+$ T cell anergy (Sharma et al. 2009) are important added advantages of this molecule as an immune adjuvant (Fig. 12.3). Although SA-4-1BBL did not show detectable toxicity and autoimmunity in rodent tumor models at therapeutic doses, it remains to be demonstrated if this safety profile translates to humans. Inasmuch as mouse SA-4-1BBL does not cross-react with human 4-1BB, it remains to be seen if the human version of SA-4-1BBL will have the same immune and therapeutic attributes of the mouse version of the molecule. Most importantly, it is critical to evaluate if the therapeutic doses of the mouse version will be applicable to the clinic, or much higher dose will be required, and if the pharmacokinetics and pharmacodynamics of these two molecules are different. Testing this novel form of SA-4-1BBL in clinical trials will be important for its development, and if proven efficacious in therapeutic clinical settings, this vaccine concept may have broad application to all types of cancers with well-defined TAAs as well as chronic infections since the antigenic component of the vaccine can easily be tailored to formulate into conjugate or non-conjugate vaccines for the targeted indications.

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Conflict of Interest The Protex™ technology and SA-4-1BBL described herein are patented by the University of Louisville Research Foundation. Haval Shirwan and Esma S. Yolcu are inventor on these patents. All the other authors declare no conflicts of interest.

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