Cell Culture-Derived Influenza Vaccines

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Abstract Conventional egg-based vaccine manufacture has provided decades of safe and effective influenza vaccines using the technologies of the 1930–1960s. Concerns over the vulnerability of the egg supply in the case of a pandemic with a high pathogenicity avian influenza strain have spurred the development and licensure of mammalian cell culture-based influenza vaccines, the first major technological innovation in influenza vaccine since the mid-twentieth century. Mammalian cell culture provides a readily expansible, secure substrate for influenza vaccine manufacture, free from the need to suppress the bioburden associated with eggs. Most current cell culture-based vaccines still rely on seed viruses isolated in eggs. Conversion to a fully egg-free process is likely to increase the range of seed viruses available and improve the match between vaccine seed strains and circulating strains. The risk of adventitious agent introduction during manufacture in thoroughly characterized mammalian cell substrates is certainly low and probably significantly lower than the risks in egg-based manufacture. In clinical trials, cell-based influenza vaccines have proven safe and equivalent in immunogenicity to egg-based influenza vaccines. The higher containment that is possible with cell-based production proved valuable during the 2009 pandemic, when large-scale production of vaccine bulks could begin in cell culture manufacturing systems at biosafety level 3, while egg-based production was delayed, waiting for the biosafety level of the pandemic stain to be decreased. For cell-based production to replace egg-based production of influenza vaccine, the new technology will need to demonstrate its robustness over multiple strain changes and its economic competitiveness.

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1 Introduction

Like the Apollo space program, the global system of influenza vaccine production is a testament to what could be accomplished with mid-twentieth century technology, applied with creativity and determination. Even in the early twenty-first century, the goal of making a vaccine that is updated with up to three new strains every year (indeed, twice a year due to the staggered seasonality of the hemispheres), combined with the occasional pandemic vaccine, remains the most impressive feat in vaccinology. We look at the first manned moon landing in 1969 and ask "How could this have been accomplished with a guidance system that had only 38K of memory?" Although biology has undergone a revolution since that time, most of the technology in conventional influenza vaccines dates from the Apollo era or earlier [1]. Indeed, some of the technologies are relics of an agrarian past. Conventional flu vaccine manufacture still relies on production in fertilized chicken eggs, release tests based on bleeding immunized sheep, and immunogenicity tests based on mixing human serum with red blood cells from turkeys, chickens, horses, or guinea pigs. The standard assays required for testing flu vaccine antigen content (single radial immunodiffusion – SRID) and immunogenicity (hemagglutination inhibition – HI) have simple visual read-outs that require no analytic equipment more sophisticated than a ruler. Their simplicity is a strength, but it also reflects their exclusive reliance on components that were readily available around the time of the Second World War. The assays carry with them the variability and idiosyncrasies of tests devised before well-defined, pure biological reagents and modern analytical tools were generally available.

Fertilized, farm-fresh eggs (used 9–12 days after laying) are an essential component of conventional influenza vaccine production. They are used to isolate viruses from clinical specimens, generate reassortant viruses, and produce the prodigious quantities of virions from which vaccine antigens are extracted. With a productivity of approximately one vaccine dose per egg, the hundreds of millions of doses administered each year require hundreds of millions of eggs delivered on schedule within a defined manufacturing season. Planning starts approximately a year in advance. Chickens must beget more chickens to have enough inseminated female chickens to lay all the fertilized eggs needed. Were a severe avian influenza outbreak to wipe out the flocks, influenza vaccine production would collapse. What if the same virus strain also caused severe disease in humans? Because high pathogenicity H5N1 avian influenza strains can infect humans with high lethality (but, fortunately, with low transmissibility between humans thus far), such a scenario is well within the realm of possibility. Concerns about the reliability of the egg supply have been a chief motivator for efforts to develop cell culture-based influenza vaccine production. The reasons for the continued use of eggs include regulatory barriers, decades of experience with egg-based manufacture, technical challenges with cell-based manufacture, and the large investments required for updating the dominant technology used for human influenza vaccine production.

The barriers are surmountable. Veterinary flu vaccines have been produced in cultured mammalian cells for years. One cell culture-based seasonal trivalent influenza vaccine (Optaflu® from Novartis Vaccines) is licensed in the EU, and two cell culture-based monovalent influenza vaccines (Celtura® from Novartis Vaccines and Celvapan® from Baxter) were deployed as part of the 2009 pandemic response. Attenuated seed virus generation by reverse genetics for high pathogenicity H5N1 strains [2] and the introduction of cell culture-based manufacturing are the first fundamental technological advances in influenza vaccines used for protection of human populations since the discovery of efficient growth of influenza viruses in embryonated chicken eggs in 1930s and early 1940s [3], the introduction of "splitting" to separate HA and NA from nucleocapsids in 1964 [4], the development of cold-adapted live-attenuated vaccines in 1965 [5], the purification of influenza viruses by continuous flow ultracentrifugation in 1967 [6], and the generation of high growth vaccine strains by reassortment in 1969 [7].

2 Isolation of Viruses for Vaccine Seeds

Avian influenza viruses are the primary repository of influenza genetic diversity, although pigs and horses are also potential sources of zoonotic strains [8]. All 16 hemagglutinin (HA) and 9 neuraminidase (NA) types of influenza A viruses circulate among waterfowl. In birds, influenza is primarily an enteric infection. Avian influenza viruses enter bird intestinal epithelial cells after attaching to α-2,3-linked sialoside moieties on cell surface glycoproteins or glycolipids [9]. The adaptation of avian viruses to infect humans efficiently requires mutation of the receptor binding region of HA so that it can mediate virus attachment to α-2,6-linked sialosides, the predominant sialoside linkage found in epithelium of the human upper respiratory tract [10]. A difference in overall contour facilitates the discrimination between these glycans by HA [11]. Sialosides with an α-2,3 linkage have a gently angled structure (resulting in a "cone-like" topology); those with an α-2,6 linkage have a more sharply angled structure (resulting in an "umbrella-like" topology) [12]. Nevertheless, a single amino acid substitution can be sufficient to alter the preferred sialoside linkage bound by HA [13, 14]. Because the dominant antibodies that neutralize influenza virus bind near the sialoside recognition site [15, 16], preventing virus attachment to cells [17], adaptation of influenza virus to replication in humans alters the antigenic region that is the main target of protective antibodies.

When human respiratory secretions are injected into eggs to isolate influenza viruses for vaccine seeds, the virus must re-adapt from its mammalian sialoside specificity to replicate efficiently in avian cells by attaching to α-2,3-sialosides. The allantoic cavity is the principal compartment of embryonated chicken eggs in which influenza viruses replicate [3]. The cells of the allantoic membrane primarily bear α-2,3-sialosides on their surface [14]. To facilitate adaptation to growth in the allantoic cavity, virus from clinical samples is selectively inoculated into the

relatively small amniotic cavity [3]. The amniotic membrane bears both α-2,6- and α-2,3-linked sialosides [14]. Presumably, replication in the amniotic cavity expands the number and sequence diversity of viruses, increasing the chances that a variant will arise with α-2,3-sialoside specificity and become competent for replication in the allantoic cavity [18]. This switch in receptor specificity again selects for a mutation in the dominant antigenic region around the receptor binding site. Thus, egg-isolated virus strains and the HA in vaccines derived from them have an obligate sequence difference and, in many cases, a detectable antigenic difference from the strains that cause human disease [19, 20]. During the adaptation of B strains to growth in eggs, the loss of a glycosylation site can cause substantial antigenic changes [21].

MDCK cells bear both α-2,3- and α-2,6-sialosides on their surface [14]. Therefore, MDCK cells support the isolation of the influenza viruses shed from the mammalian cells that line the human respiratory tract with no obligate change in receptor specificity [22]. Accordingly, isolation rates are substantially higher on MDCK cells than in eggs and are higher still on an MDCK cell line (MDCK-SIAT1) engineered by the introduction of the cDNA of human 2,6-sialyltransferase to increase the proportion of α-2,6-linked sialosides on the cell surface [23, 24]. The difference in isolation rates is greatest for H3N2 strains. Since the introduction of this subtype into humans in the pandemic of 1968, H3N2 influenza viruses have adapted to human hosts and correspondingly become more difficult to isolate in eggs. In 2006 and 2007, of 264 H3N2 clinical specimens inoculated into both eggs and MDCK cells at the US Centers for Disease Control, only 4% produced viral isolates in eggs, but 65% produced viral isolates in MDCK cells [25]. The greater permissiveness of mammalian cells for the isolation of human influenza strains has led to their widespread use to isolate viruses for the purpose of epidemiologic surveillance. However, as of the writing of this chapter, these cell-isolated viruses may not be used for vaccine seeds. If a promising strain is identified by culture on mammalian cells, an attempt is made to re-isolate the strain from the original clinical specimen in eggs. This re-isolation is not always successful, restricting the range of seeds available for manufacture, including mammalian cell-based manufacture, to only those viruses that can be isolated in eggs [25]. Once the virus, shed from humans and isolated in mammalian cells during surveillance, is adapted to eggs and sent to cell-based vaccine manufacturers, it must then be re-adapted back to growth in mammalian cells. The re-adaptation typically requires two to three passages. This alternation between substrates wastes weeks during the tight annual vaccine production cycle, and there is no evidence that egg-adaptive mutations in the receptor binding region revert upon subsequent passage in mammalian cells.

Has the limited choice of influenza vaccine seeds imposed by continued reliance on egg isolation had a public health impact? During the 2003/2004 influenza season, no strain that was antigenically “like” the A/Fujian/411/2002 H3N2 strain could be isolated in eggs in time for vaccine preparation [26]. Therefore, the vaccine contained the antigenically mismatched A/Panama/2007/99 H3N2 strain. Retrospective analyses indicated that the vaccine had decreased effectiveness compared to vaccines produced during years in which the vaccine H3N2 strain was well matched to circulating H3N2 strains [27]. That influenza season was

unusually severe and marked by an increase in pediatric mortality [28]. The degree to which a better matched vaccine would have ameliorated the severity of the influenza season is unknown.

As of the writing of this chapter, all seasonal influenza vaccines in commercial use are produced from influenza strains that have been passaged through chicken eggs, regardless of whether their final manufacture is in cultured mammalian cells or chicken eggs. Therefore, the strains have one or more amino acid sequence differences and possibly antigenic differences from the circulating strains that cause human disease. How significant are such differences for the efficacy of influenza virus vaccines? There are no human data to definitively answer this question, because no influenza virus that has been propagated exclusively in mammalian cells has been used in a clinical trial that could answer this question. The results of animal studies are not definitive. Infection of small numbers of ferrets with egg-isolated and MDCK-isolated viruses cross-protects the animals against influenza viruses grown on either substrate equivalently, but such experiments would only detect gross differences in efficacy [22]. Immunization of ferrets with formalin-inactivated MDCK-grown virus provided better protection against either MDCK-grown or egg-grown challenge virus than did formalin-inactivated, antigenically distinguishable egg-grown virus with a single amino acid difference in HA from the MDCK-grown virus [29]. Although this finding suggests that an all-cell-produced vaccine might have enhanced efficacy, this interpretation is tempered by the obscure mechanism of the difference in efficacy, which did not correspond to the degree of antigenic relatedness of the immunizing and challenge viruses.

Manufacturers and World Health Organization (WHO) Collaborating Centers have launched a multilateral effort to introduce mammalian cells to vaccine seed isolation [30]. Manufacturers of non-live influenza vaccines obtain strains from a common set of Collaborating Center laboratories, and it is impractical for these laboratories to isolate viruses for each manufacturer on a different cell line. Therefore, comparative studies will identify a common cell line that permits efficient isolation followed by ready adaptation to the various manufacturing processes. Isolation of viruses in mammalian cells could even facilitate the adaptation of additional strains to growth in eggs. The expanded pools following efficient isolation and expansion in mammalian cells will have greater sequence diversity than the small number of viable viruses present in the original clinical specimens. This higher viral titer and expanded diversity could increase the likelihood of successful adaptation of a strain to eggs.

3 Reassortment and Backbone Selection

To make vaccine seeds for inactivated vaccines from influenza type A strains, the HA and NA genome segments of egg-adapted clinical isolates are reassorted onto the "backbone" of A/Puerto Rico/34, an attenuated, egg-adapted strain [7]. To make the cold-adapted live-attenuated vaccine manufactured by MedImmune, the HA

and NA of A strains are reassorted onto A/Ann Arbor/6/60 and the HA and NA of B strains are reassorted onto B/Ann Arbor/1/66 [31]. During reassortment, the individual genome segments of two or more viruses that coinfect a single cell are swapped in the progeny viruses – a mating process analogous to the assortment of chromosomes during sexual reproduction of eukaryotes. The backbone consists of the genome segments encoding the matrix protein (M), the nonstructural proteins (NS1 and NS2), the nucleoprotein (NP), and the polymerase complex (PA, PB1, and PB2). Selective pressure against viruses bearing the HA and NA of the backbone strain is provided by antisera specific for these determinants [7]. An ideal 6:2 reassortant would contain only the HA and NA genome segments of the clinical strain on a full set of other genome segments from the intended backbone donor. In practice, one or more backbone genome segments of the clinical isolate may also be incorporated into reassortants that are selected for manufacture.

The egg-adapted current backbones may not be optimal for the productivity of mammalian cell-based manufacture. Therefore, new cell-adapted backbones may increase the efficiency of manufacture in mammalian cells. Wild type strains that grow efficiently in mammalian cells can provide donors for cell-adapted backbones. Because HA and NA are major determinants of viral growth, selecting a backbone donor requires comparing consistent sets of HA and NA pairs on alternative backbones. With a donor selected, reverse genetics can allow the rational modification of backbone genome segments to increase productivity from cultured cells and consistency of downstream processing. Efficient polymerase complexes resulting in rapid replication in mammalian culture are found in some highly virulent strains, such as the 1918 pandemic H1N1 virus and a variant of PR8 with high virulence for mice [32, 33]. Therefore, safe vaccine manufacture with engineered highly productive strains may be facilitated by additional mutations, such as NS-1 deletions or truncations, that attenuate the viruses in mammals, including humans, while preserving replication efficiency in some mammalian cells [34–36]. In principle, reverse genetic engineering of backbones could produce strains that are produced more efficiently in eggs, too. The ability to manipulate both the cell and the virus in cell culture production systems allows greater application of modern viral and cellular genetics to cell optimize influenza virus vaccine manufacture.

4 Eliminating Adventitious Agents from Vaccine Seeds

The possibility that passage of influenza viruses from human secretions through eggs provides a “filter” that prevents the propagation of potential adventitious agents has been cited as a potential advantage of egg-based seed generation and manufacture [25]. Concerns have been also been raised that mammalian cell substrates themselves could be a source of adventitious agents [25]. From 1955 to 1963, simian virus 40 (SV40) from primary monkey kidney cells contaminated batches of polio virus vaccine [37], although there is no evidence of increased cancer risk in those who received polio virus vaccine during that period [38].

Cell lines, like intact organisms, can harbor endogenous retroviruses with the potential for re-activation [39]. For this reason, cell lines used for manufacture of vaccines undergo rigorous testing for absence of adventitious agents. Tests include PCR for known agents, enzymatic assays to detect viral polymerases, electron microscopy to detect viral particles, inoculation of cultured cells, and inoculation of animals [40]. In addition, influenza vaccine processing includes steps to eliminate or inactivate adventitious agents [41]. Chickens also carry viruses, such as Rous sarcoma virus, avian leukosis virus, and reticuloendotheliosis virus, which could potentially be introduced into egg-based manufacturing. Therefore, eggs used for manufacture of vaccines for the USA must be derived from flocks certified to be free of a list of specific pathogens, the production process must be shown to eliminate listed agents, or the absence of the agents from the vaccine must be demonstrated [40]. There is a key difference in the adventitious agent testing possible for cultured cell-based and egg-based production. Representative frozen aliquots of a banked continuous cell line are scrutinized for microbiological safety before a vaccine produced from equivalent frozen aliquots of that cell bank is licensed. It is not possible to apply the same level of rigor in biological control to the hundreds of millions of freshly laid eggs used in vaccine production each year.

A model for systematically assessing the risk of adventitious agent introduction by different influenza vaccine manufacturing schemes has been developed [41]. This model weighs the relative ability of viruses found in human nasopharyngeal secretions or in chickens to propagate in eggs or several mammalian cell types, to survive inactivation and other steps in the downstream manufacturing process, and to cause disease in a vaccine recipient. Analyzing available data using this model indicates that the use of eggs to isolate the virus seeds used for mammalian cell manufacture does not "filter" viruses found in human nasopharyngeal secretions more effectively than isolation on MDCK cells, but it does risk introducing avian viruses [41]. The physical hardiness of some prevalent avian viruses, particularly reoviruses, renders chicken eggs a chief potential source of agents that are relatively difficult to inactivate during the downstream processing of vaccines [41]. The risk that an adventitious agent will survive manufacturing is much lower for split and subunit influenza vaccines than it is for live-attenuated vaccines. The manufacture of non-live vaccines includes a virus inactivation step, typically using chemical agents such as β-propiolactone or formalin. These agents would destroy the infectivity of a live-attenuated vaccine. In addition, the "splitting" of most inactive influenza virus vaccines (except for whole virus vaccines) – that is, the use of detergents and sometimes solvents to separate HA and NA from the nucleoprotein core – adds an additional inactivation step that is particularly effective against enveloped viruses [41].

The use of eggs rather than cultured cells for isolation of seed viruses and for reassortment precludes the use of plaque purification, the chief technique employed in modern research settings to ensure that a viral isolate is clonal – a pure, genetically homogeneous (to the degree that an RNA viral quasi-species can be homogeneous) population derived from a single infectious virus. The direct visualization and harvesting of well-separated plaques on a cultured cell monolayer under

a semi-solid overlay allow assurance that a single virus isolate is being obtained and allow the selective isolation of viruses with a large-plaque phenotype, which may correlate with efficient growth in culture [42]. Although decades of experience indicate that the blind technique of terminal dilution cloning in eggs is an adequate procedure to provide seeds for safe influenza vaccine manufacture, plaque purification in cultured cells can provide an additional margin of safety and selectivity.

The risk that adventitious agents from clinical samples could be introduced into the vaccine manufacturing process will be eliminated when the generation of influenza vaccine seeds by gene synthesis followed by reverse genetic rescue becomes the industry standard [2, 43, 44]. Current reverse genetics rescue starts by reverse transcribing DNA clones from viral RNA purified directly from an original clinical specimen or from a cultured virus isolated from such a specimen. The purification of RNA under biochemically harsh conditions can greatly reduce the risk of adventitious agent carry-over. Generating influenza-encoding DNA by chemical synthesis [44] could completely eliminate this source of adventitious agent risk. In a synthetic scheme, wet bench experiments with patient-derived specimens would generate sequence information. That information would be transmitted electronically to a DNA synthesizer to make the nucleic acids used for virus rescue, breaking any conceivable chain of adventitious agent transmission from an influenza patient's specimen to a vaccine lot. This advance in the hygiene of influenza vaccine manufacture will only be possible by using cultured cells to generate vaccine seeds.

5 The Cell Lines Used in Influenza Vaccine Manufacture

Several influenza vaccine manufactures are developing cell-based production processes. Details of these processes and their productivities are, in general, proprietary. Thus, a survey of the published literature would necessarily give an incomplete and dated impression of the state of the field. Therefore, this section does not attempt to provide a comprehensive review or a comparison of the relative merits of different manufacturers' processes, but rather focuses on key issues in the development of cell-based influenza vaccine manufacturing. Detailed reviews of information that can be gleaned from published literature on different manufacturers' cell-based processes are available [45, 46]. This chapter also does not review advances in making recombinant influenza virus vaccines containing antigens from sources other than cultured influenza viruses. Such candidates as purified protein, virus-like particle, vectored, and peptide vaccines are reviewed elsewhere [47].

As of the writing of this chapter, five cell culture-based influenza vaccines have been licensed for human use. In 2001, Influvac TC® from Solvay (seasonal trivalent, split, produced in MDCK cells) was licensed in the Netherlands but was never commercially distributed due to manufacturing delays [45, 48]. In 2002, Influject® from Baxter (seasonal trivalent, whole virion, produced in Vero cells) was licensed in the Netherlands, but subsequent phase II/III trials of this vaccine were suspended

due to a higher than expected rate of fever and associated symptoms among vaccinees [45]. In 2007, Optaflu® from Novartis Vaccines (seasonal trivalent, subunit, produced in MDCK cells) was approved in the EU [48]. In 2009, Celvapan® from Baxter (H1N1 pandemic, monovalent, whole virion, produced in Vero cells) was authorized in the EU and sold commercially. In 2009, Celtura® from Novartis Vaccines (H1N1 pandemic, monovalent, MF59-adjuvanted subunit, produced in MDCK cells) was authorized in the EU and sold commercially. Sanofi Pasteur, MedImmune, GlaxoSmithKline, and Crucell all have or have had programs to develop cell culture-based influenza vaccines.

There are distinctions between the approaches taken by different manufacturers. For example, Celvapan® is produced by growing wild type virus in Vero cells that adhere to microcarrier beads. Of the cell lines used to produce influenza vaccines, Vero cell lines have the longest history in vaccine manufacture. They were first used to produce inactivated poliovirus and rabies virus vaccines in the 1980s [49]. Because Vero cells at limited passage number do not form tumors when injected into infant nude mice [50], the defined passage cells used in vaccine manufacture are considered nontumorigenic. Optaflu® and Celtura® are produced from conventional egg-isolated and (for type A strains) reassorted seeds obtained from WHO Collaborating Centers and adapted to replicate efficiently in a proprietary MDCK 33016 cell line that grows in suspension culture [48]. Infection of suspended, rather than adherent, cells simplifies the upstream manufacturing process. The Crucell vaccine candidate is also produced in a suspension cell line, PER.C6, a human fetal retinoblast cell line that was immortalized through a defined genetic manipulation – the introduction of the E1 minigene of adenovirus type 5 [51]. Vero and MDCK cells were isolated as continuous cell lines by empiric techniques [46, 48, 49].

Influenza viruses, with the exception of highly pathogenic H5N1 strains with furin-cleavable HA, require the addition of exogenous trypsin to cleave HA into the HA1 and HA2 fragments, activating the viruses for infection [16]. Trypsin can also loosen the attachment of adherent cells, a constraint during manufacture with adherent cell lines. The cell culture media used in influenza vaccine manufacture are always serum-free and generally animal-product-free, although trypsin and insulin of animal origin may be added. Animal-product-free preparations of insulin and trypsin-like serine proteases are available [52]. There is a movement toward chemically defined media, in which the chemical constituents are explicitly determined, for cell-based vaccine manufacture.

6 Egg-Based and Cell-Based Vaccine Production Processes

Production in cell culture allows greater control of infection parameters than egg-based production. Media can be changed or supplemented during the infection. Oxygen content, nutrient levels, agitation, and pH can be monitored and adjusted. Known multiplicities of infection can be optimized for each virus strain. A production process based on the infection of cultured cells is more complicated than the

"fermentations" for most biopharmaceutical products [53]. In a standard fermentation, all cells in a culture produce a product continuously, possibly after induction, with steady accumulation of a secreted or retained product. Standard parameters of cell number, cell viability, pH, lactate production, oxygen tension, and product accumulation are monitored. All of these parameters are also relevant for an influenza virus infection. However, the proportion of cells infected, the production and release of virus, level of tryptic activity, and virus-mediated cell lysis must also be monitored for a well-controlled process. To limit the size of the viral seed pool, starting multiplicities of infection may be very low. Therefore, as the infection progresses, the initially infected cells undergo apoptosis and lysis, while other cells are just entering productive infection. Rates of virus production, HA production, and virus release from cells may change during the infection as may the morphology of released virus and even the viral genotype. The infection continues during the separation of virus and cells at harvest, which may take hours for thousands of liters of infected culture. The product, for a subunit influenza vaccine, is primarily HA. Yet, HA on budded spherical viruses, on budded filamentous viruses, or retained on cell membranes may behave quite differently in the downstream purification. Therefore, a number of virological parameters, in addition to the usual fermentation parameters, must be monitored to optimize infection. Successful optimization requires close collaboration between virologists and process engineers.

The complexity of infection optimization is particularly challenging due to the short interval between the announcement of a new vaccine strain and the start of the validation runs needed for a seasonal vaccine update or pandemic vaccine release. The constraints of a product license limit the permissible adjustments of the process. Therefore, a well-designed and efficient optimization strategy is an essential component of cell-based influenza vaccine manufacture. For egg-based processes, many years of experience provide a database of historically optimal parameters for each viral subtype. This experience provides a starting point for annual optimization of egg-based production. That experience is now being obtained, in a compressed time frame, for mammalian cell-based manufacturing processes.

The material harvested after infection in egg-based and cell-based manufacture is different. Egg allantoic fluid has a high content of non-influenza proteins. Harvested cell culture medium is relatively low in protein but contains the trypsin or trypsin-like proteases added to promote virus spread. The harvest of cell culture production is sterile (except for influenza virus); the harvest of egg-based production is not. Flocks of chickens used to produce the eggs used in vaccine manufacture may be specific pathogen-free or may meet the lower standards of a "clean" flock [82]. Neither specific pathogen-free nor clean chickens are germ-free. They have an abundant bacterial flora. Eggs pass through a hen's cloaca (Latin for "sewer"), the common orifice of the chicken digestive, urinary, and reproductive tracts. Eggs for vaccine manufacture are sanitized to reduce their bacterial load.

The infection in cell-based manufacture takes place in one or a few large (up to thousands of liters) tanks or bags. The cell culture medium is sterile, and sterility is maintained throughout the closed production process. In contrast, egg-based manufacture is an inherently open process. Each egg must be opened for virus

inoculation, incubated after the shell is breached, and then accessed again to harvest the virus-containing allantoic fluid. In this process, there is opportunity for the introduction of agents from workers or the environment and for the leakage of influenza virus-containing fluid from the eggs. Although a variety of precautions are taken to minimize the microbiological risks of egg-based manufacture, problems with bioburden do occur. *Serratia marcescens* contamination of vaccine bulks at an egg-based manufacturing plant in Liverpool, UK, led to a plant shutdown and a severe influenza vaccine shortage in the USA in 2004–2005 [54]. If one of the tanks used in cell-based manufacture were contaminated, the loss of product would be large, but readily detected and isolated. Monitoring the sterility of the vast number of eggs used in traditional manufacture is a much more daunting challenge. To limit bioburden, traditional manufacture requires the addition of antibiotics to eggs at the time of virus inoculation and in some cases the addition of antimicrobial compounds to downstream process streams, until the final sterile filtration of the product. Cell-based manufacture can be antibiotic-free from start to finish.

After the virus is separated from other components of the harvests, most commonly by continuous flow ultracentrifugation [6], the downstream processing of egg-based and cell-based vaccines is similar, except for the bioburden control requirements for egg-based vaccines and one additional requirement for cell-based vaccines – control of host cell DNA size and content. Continuous cell lines, because they have been immortalized, have undergone one of the phenotypic changes associated with malignant transformation. Suspension cell lines are anchorage independent, another phenotype associated with malignancy. Some suspension cell lines, including MDCK 33016 cells, can establish tumors in highly immunodeficient infant nude mice [51, 55]. The tumors are not mouse tumors; they are foci of MDCK cells. Such dog kidney cell colonies would be rejected in a mouse (or human) with a functioning immune system. Downstream processing of vaccines produced in MDCK 33016 cells includes multiple filtration steps, detergent treatment steps, and an inactivation step. The redundancy of the cell removal is such that the risk of any individual ever receiving an MDCK cell from immunization, even if every individual who has ever lived or will live until the sun burns out were to receive a flu vaccine every year for 100 years, is estimated at less than 1 in 10^{12} [55].

There is a theoretical concern that the genes responsible for the immortalization and, in some cases, anchorage independence of production cell lines could be present in cell culture-based vaccines, be taken up by host cells, and bring about malignant transformation of a host cell [56]. To alleviate this concern, the host cell DNA content of cell culture-produced, injectable vaccines is limited to less than 10 ng per dose, and the size of DNA fragments is limited to less than 200 base pairs, precluding the presence of intact oncogenes [40, 49, 56]. DNA can be eliminated at multiple process steps, including cell separation, virus purification, splitting, and chromatographic polishing. To further limit DNA content, in-process material may be digested with benzonase. β-Propiolactone, commonly used to inactivate viruses in vaccines, also fragments DNA [57]. The use of a known immortalizing gene to generate "designer" cell lines, such as PER.C6 [58], creates the ability to assay the presence or absence of the specific transforming gene in a vaccine product.

Although the transmissibility of some tumors by cell-free extracts was first demonstrated by studying chicken sarcomas in 1911 [59] (the phenomenon is now known to be caused by an avian virus, Rous sarcoma virus, that transmits the src oncogene [60]), the tumorigenic potential of chicken cells and the oncogenic potential of residual egg DNA in vaccines have not been subjected to the same level of scrutiny as that applied to cultured cells and residual cultured cell DNA. Egg-based influenza vaccines are not subject to restrictions on host cell DNA content or size. This disparity reflects, in part, the more relaxed safety standards that prevailed when egg-based vaccines were first introduced. In fact, clinical experience has given no indication that human immunization with any vaccine, whether produced in eggs or cultured cells, predisposes to tumors.

HA content is a key release criterion for inactivated influenza vaccines. For subunit vaccines, this determination is based on SRID, a technique in which the vaccine antigen, often treated with Zwittergent, is placed in a well cut into in a layer of agarose that has been impregnated with a polyclonal, strain-specific sheep antiserum against a crude preparation of HA [61]. As the vaccine antigen diffuses into the gel, a zone of immunoprecipitation between the vaccine antigen and the sheep antiserum forms, visible by scattered light or protein staining. The diameter of the zone of precipitation is considered proportional to the antigen content of the vaccine. The sheep antisera and fixed virus antigen standards are provided by regulatory authorities. The antigens used as standards and as sheep immunogens are produced in eggs. Therefore, a question has been raised whether such egg-produced reagents are suitable for the assay of mammalian cell-produced influenza vaccine antigens [25]. The use of cell-produced reagents to assay cell-produced vaccines would normalize for any relevant differences in posttranslational modification between mammalian cell-produced and egg-produced HA. On the other hand, if the sheep are immunized with HA that is not completely pure, the elicited antiserum will contain a mixture of antibodies against HA and antibody against the impurities from the egg or mammalian cell substrate. Use of reagents derived from the same platform as the vaccine antigen may therefore result in an overestimation of HA content, because both HA and substrate-derived contaminants could form immunoprecipitates. Cross-platform assays (using antisera against egg-produced HA to assay cell-produced vaccines and vice versa) could prevent this potential error. Limited data on cross-platform potency assays have been published [62], and further studies comparing same platform and cross-platform immunoassays are needed to guide this regulatory decision.

7 Clinical Testing of Cell-Based Influenza Vaccines

Clinical trials have assessed the safety, immunogenicity, and protective efficacy of cell-based influenza vaccines [49, 63–74]. In published trials, the reactogenicity, safety, and immunogenicity of cell culture-based vaccines appear to be generally

equivalent to those of comparable egg-based vaccines – whether MDCK-produced, Vero-produced, seasonal trivalent, H5N1 monovalent prepandemic, H1N1 monovalent pandemic, subunit, split, whole virus, or adjuvanted. There are modest exceptions. In trials of a Vero-produced H5N1 whole virion vaccine, there appeared to be less reactogenicity compared with historical egg-produced comparators [65, 74]. In one trial of a MDCK-produced seasonal subunit vaccine, there was a modest increase in mild-to-moderate local pain on injection relative to an egg-produced comparator [63], although this has not been a consistent finding with such vaccines. The cell culture-produced vaccines could differ from each other and from egg-produced vaccines in their posttranslational processing, particularly glycosylation. Although the more authentic glycosylation of mammalian cell-based vaccine antigens is a potential advantage, the clinical data to date do not provide evidence that these differences significantly affect immunogenicity, as measured by HI or single radial hemolysis (SRH). This is consistent with absence of functional anti-influenza antibodies known to bind glycans. Glycan masking of influenza epitopes has been well documented [16, 75], and HA produced in different substrates does vary in the bulkiness of its oligosaccharides [76], raising the possibility of antigenically relevant differences. It remains to be determined whether differences in immunogenicity between egg-produced and mammalian cell-produced vaccines will emerge when mammalian cell isolation is substituted for egg isolation of seed viruses for seasonal influenza vaccines produced in cell culture.

A chief contra-indication to immunization with egg-based vaccines is egg allergy, which has a prevalence estimated between 0.5% and 2.5% [77]. Egg allergens include ovalbumin and ovomucoid, contaminants found in egg-based vaccines [77]. This risk is eliminated by the use of mammalian cell-based vaccines. Questions have been raised whether immunization with influenza vaccines produced in MDCK cells, which were derived from a dog kidney, might elicit hypersensitivity responses in those with dog allergies. The chief dog allergens are found in dander and saliva [78] and have not been detected in MDCK cells [79]. Cultured mast cells, sensitized with IgE from dog-allergic human subjects, do not degranulate upon exposure to an MDCK-produced influenza vaccine [79]. The prevalence of dog allergy is high. Although data are incomplete for many groups, dog allergy is reported in greater than 4% of some pediatric populations in developed countries [78, 80]. Yet, no excess of hypersensitivity reactions has been observed in clinical comparisons of immunization with MDCK-produced and egg-produced influenza vaccines. Thus, available data do not indicate that the dog origin of MDCK cells increases the risk of hypersensitivity reactions to MDCK-produced vaccines. Does immunization with vaccines produced in mammalian cells increase the likelihood of autoimmunity? The clinical trial experience with cell-based influenza vaccines gives no indication of such an association. The more than 25 years of benign experience with simian Vero cell-produced vaccines, including inactivated rabies vaccines and inactivated and live polio vaccines, is particularly encouraging [49].

8 Role of Cell-Based Vaccine Manufacture in the Response to the Swine-Origin H1N1 Influenza Pandemic

The 2009–2010 swine-origin influenza pandemic provided a live test of cell-based manufacture for pandemic response. Much of the initial motivation for the development of cell-based manufacture was the need to ensure a rapid expansion of the influenza vaccine supply in the event of a pandemic. In fact, in 2009, egg supplies were not the main limiting factor for vaccine supply. This was, in part, the result of pandemic preparedness activities to increase the egg supply. However, the early days of the pandemic were marked by a scramble among influenza vaccine manufacturers to secure sufficient supplies of suitable eggs. The slower pace of identifying a suitable vaccine seed, producing sheep antisera for vaccine release, and staffing vaccine production facilities was more rate limiting, giving time to obtain the eggs needed. As these slower components of the pandemic response are accelerated in the future, egg supply could again become limiting, and the egg supply remains vulnerable to catastrophic depletion in an avian influenza pandemic.

Cell culture-based influenza vaccine manufacturing demonstrated a little anticipated benefit during the 2009 H1N1 pandemic response. In the opening days of the pandemic, the pathogenicity of the pandemic strain was not known, and reassortants on a PR8 backbone were not available. Consequently, in the EU, the pandemic strain was handled at BSL3, precluding large-scale testing or production in open egg-based manufacturing. Vaccine bulks could, however, be produced at scale in BSL3 cell culture-based manufacturing facilities in Marburg, Germany (Novartis Vaccines), and Bohumil, Czech Republic (Baxter). The first GMP batch of a H1N1 vaccine candidate produced by a manufacturer that adheres to western quality standards was produced on June 12, 2009, at the Novartis flu cell culture facility. The time advantage provided by cell culture production enabled the start of clinical trials [66] with a reassortant-derived cell culture vaccine even before calibrated SRID reagents had been supplied by regulatory authorities. In contrast, large-scale production at Novartis egg-based manufacturing facilities in Siena and Rosia, Italy, could not start until the beginning of July, once reassortant strains were available and biosafety levels had been lowered. Had the Novartis MDCK-based manufacturing facility also been licensed for growth of genetically modified organisms at BSL3, a potential influenza vaccine seed rescued by reverse genetics on a PR8 backbone in Novartis research laboratories in mid-May could have been tested at scale weeks before reassortant seeds were received from a WHO collaborating center.

9 The Future of Cell-Based Influenza Vaccine Manufacture

The replacement of egg-based influenza vaccine manufacture by cell culture-based manufacture seems inevitable, but the pace remains uncertain. The current system of egg-based manufacture involves the isolation of viruses from the nasal and pharyngeal washings of people with respiratory illnesses, the propagation of these

viruses in fertilized eggs tainted with the other cloacal output of chickens, and an open manufacturing process in which bacterial contamination must be suppressed with antimicrobial agents. Thus, egg-based conventional vaccines, although demonstrated to be acceptably safe through decades of experience, are the product of a process that can reasonably be described as "earthy." When reverse genetic seeds remove human secretions from the process (except as a source of sequence information); cell culture removes the possibility of contamination by chicken flora; and closed manufacturing processes greatly reduce the risk of contamination during manufacture, regulatory authorities and even the public may demand that their vaccine supply be produced by processes that conform to modern standards.

Economics is likely to drive the pace of the transition. Much of the development of flu cell culture has been funded through public–private partnerships to increase pandemic preparedness. The US Flu Cell Culture Facility, which recently opened in Holly Springs, North Carolina, was built through a partnership between Novartis and the United States Biological Advanced Development and Planning Authority [81]. To expand to exclusively privately funded manufacturing sites, flu cell culture vaccines will need to be profitable. The productivity of flu cell culture is a moving target, as the technology matures. The reliability of strain changes in the egg-based processes is built on decades of experience. Egg-based processes will only be abandoned when the new mammalian cell-based processes prove that they can deliver sufficient vaccine supplies as reliably as the egg-based processes.

Finally, the current hybrid egg-based seed generation followed by cell-based production process is proving to be as safe, immunogenic, and effective as the all egg-based processes. Collaborative efforts between manufacturers and public health agencies are underway to enable the production of entirely cell-based reassortant vaccines [25, 30]. In some years, in which no well-matched egg isolate is available, the cell-based vaccines could be dramatically more effective than egg-based vaccines. In non-mismatch years, the HA antigens of cell-based vaccines are expected to be more similar to the HA antigens of circulating strains by at least one amino acid in the receptor binding site. The clinical impact of this improved strain match remains to be determined. Finally, in the event of an avian influenza pandemic that causes high mortality among both humans and chickens, the advantages of cell-based manufacture could be of historic importance.

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