LEADING ARTICLE

Drugs 2008; 68 (11): 1483-1491
0012-6667/08/0011-1483/$53.45/0

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Cell-Based Influenza Vaccines
Progress to Date

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Abstract

Human vaccines against influenza have been available for almost 60 years and, until recently, were prepared almost entirely from viruses grown in the allantoic cavity of 9- to 11-day-old embryonated chicken eggs. Manufacture involving eggs is not sufficiently flexible to allow vaccine supplies to be rapidly expanded, especially in the face of an impending pandemic. Other problems may arise from the infections of progenitor flocks that adversely affect egg supplies, and from the manufacturing process itself, where breakdowns in sterility can occur from the occasional contamination of large batches of viral allantoic fluid. In addition, egg-grown viruses exhibit differences in antigenicity from viruses isolated in mammalian cell lines from clinical specimens. These concerns and the probable need for greatly expanded manufacturing capability in the future have been brought into focus in recent years by the limited spread of H5N1 avian influenza infections to humans in several Asian countries. Alternative approaches involving the use of accredited anchorage-dependent and -independent preparations of the African Green monkey kidney (Vero), Madin-Darby canine kidney (MDCK) and other cell lines have been pursued by several manufacturers in recent years. Yields comparable with those obtained in embryonated eggs have been achieved. These improvements have occurred in parallel with newer technologies that allow the growth of cells in newer synthetic media that do not contain animal serum, in order to allay the concerns of regulators about the potential for spread of transmissible spongiform encephalopathies.

Influenza is a highly infectious, acute upper respiratory tract infection of humans and some animal species. Influenza viruses have been classified into three genera (A, B and C) based on antigenic differences between the internal matrix and nucleoproteins (figure 1). Influenza viruses are members of the family Orthomyxoviridae and possess segmented, negative-sense RNAs. The genomes of influenza A and B viruses have eight segments that code for ten proteins, two of which are surface glycoproteins. Influenza C viruses are structurally distinct from influenza A and B viruses, and only occasionally produce disease in humans. Influenza B virus infections are restricted to human hosts and are capable of producing outbreaks of regional significance. Influenza A viruses have been further classified into subtypes based on differences between their haemaglutinin (HA) and neuraminidase

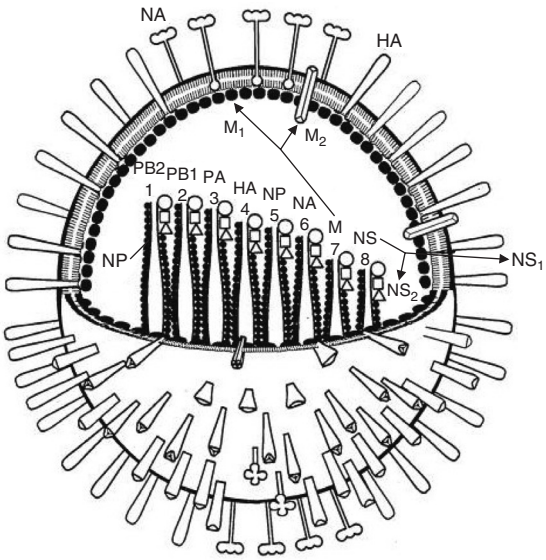

Fig. 1. Diagram of a typical influenza A virion. Nucleoprotein (NP) and three polymerase proteins, PB1, PB2 and PA associates individually with each of the RNA genome segments forming the ribonucleoprotein (RNP) complex. The major structural protein M_1 is found directly underneath the host-derived envelope, forming a shell that encloses the RNP. The NS_2 protein associates with M_1. Haemaglutinin (HA) and neuraminidase (NA) glycoproteins project from the virion surface, as does the M_2 membrane protein (reproduced from Wright and Webster,[5] with permission).

(NA) surface antigens;[1-3] there are currently 16 HA (H1–H16) and 9 NA (N1–N9) subtypes.[4]

The constantly changing nature of the influenza genome ensures that influenza infections will always be a major public health problem. Antigenic 'shift' and 'drift' are brought about by changes to the HA and NA glycoprotein surface antigens. Antigenic shift involves the complete substitution of the HA and sometimes the NA gene. Changes due to antigenic drift are caused by the accumulation of point mutations in specific regions of the HA and NA genes, and occur more frequently with influenza A than influenza B viruses.[6]

Despite the protection that has been afforded by vaccines over 50 years and the likely future use of antiviral chemotherapy, the influenza disease burden remains significant, an issue that is particularly cogent with the possibility of a new influenza pandemic. In 2005, the annual global attack rate from non-pandemic influenza infections has been estimated to be 5–10% in adults and 20–30% in children; total annual costs are estimated in the US and Europe to be $US1–6 million per 100 000 inhabitants.[7]

Electronic searches using MEDLINE were used to review the literature. The 'snow-balling' method was used on articles identified in the MEDLINE search to further identify relevant articles. Key words used in the MEDLINE search included influenza vaccines, influenza cell culture, Vero, MDCK and serum-free media.

1. Current Egg-Based Influenza Vaccine Production

The most effective way to provide protection against influenza infections is through vaccination. Current vaccines are usually trivalent and contain representative influenza A H1N1, H3N2 and influenza B surface antigens, which are re-evaluated each year to ensure that there is antigenic match with current epidemic strains. The majority of viruses used in the preparation of inactivated vaccines are still prepared by growth in the allantoic cavity of embryonated chicken eggs. Allantoic virus is purified, concentrated and inactivated. Reactogenicity associated with purified influenza virus is greatly reduced by treatment with a detergent or splitting agent ('split' vaccines). An additional step, involving the isolation and purification of surface glycoprotein antigens, is used by some vaccine manufacturers ('subunit' vaccines). Purified whole virus vaccines are more immunogenic than split/subunit vaccines but are not widely used in interpandemic immunization programmes because of their reactogenicity. Influenza vaccines may still contain trace amounts of potentially reactogenic endotoxins, egg proteins, formaldehyde and preservative.[5] Yields of egg-adapted viruses are usually high, but there are a number of problems associated with the use of eggs, which include (i) the limited flexibility they afford for expanded vaccine manufacture; (ii) interruption of the embryonated-egg supply chain due to the presence of diseases in layer flocks; (iii) the possibility of sterility problems arising during the processing of infected allantoic fluids; and (iv) poor

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growth of some reassortant vaccine strains in eggs. Additionally, the growth of epidemic viruses in eggs often selects variants that differ in their glycosylation patterns from the original clinical isolates that are antigenically distinct. These problems do not appear to arise for viruses isolated in the Madin-Darby canine kidney (MDCK) and African Green monkey kidney (Vero) continuous cell lines. The use of stable cell lines for vaccine virus growth could largely overcome these problems if yields were satisfactory and safety issues could be successfully addressed.[8]

2. Host-Selected Virus Variants

Influenza B clinical isolates propagated in eggs or the MDCK continuous cell line exhibited clear differences in antigenicity based on double immunodiffusion and haemagglutination-inhibition tests using anti-HA monoclonal antibodies.[9] The molecular basis of variation is a single amino acid substitution at amino acids 196–198 of the HA molecule, which results in a change in glycosylation at the HA distal tip.[10] Host cell selection of antigenic variants have also been reported for both H1N1 and H3N2 influenza A viruses.[11-13]

Viruses isolated in MDCK cells from clinical samples collected from a single patient over 48 hours following infection by an H3N2 virus were shown to possess antigenic and structurally identical HA glycoproteins.[14] However, viruses from the same clinical samples, after isolation in eggs, showed three distinct sub-populations based on antigenic and/or structural differences. This pattern of heterogeneity for egg-grown isolates was confirmed in studies on isolates from larger numbers of patients. Three variants with substitutions in HA1 at residues 156, 145 or 138 were described.[15] All clinical isolates that were egg-passaged contained at least two of these variants and similar findings have been reported for influenza A H1N1 and influenza B isolates.[16] Isolation was determined by the polymerase chain reaction, using primers specific for the HA1 region on clinical samples, prior to and following passage in MDCK cells or eggs. Results indicated that the original clinical isolates and MDCK-passaged viruses possessed HA glycoproteins that were similar and relatively homogeneous, whereas the HA glycoproteins of egg-passaged viruses were heterogenous.[17-20] H3N2 viruses passaged once or twice in MDCK, LLC-MK_2 (monkey kidney), MRC-5 (human fetal lung), WI-38 (human embryonic lung), primary guinea pig kidney and chicken kidney cells were all antigenically similar and had identical HA1 amino acid sequences.[21]

Influenza A viruses passaged by the amniotic route, as distinct from the allantoic route, usually retain their sequence identity.[22] Amniotic cells possess both SAα2,3Gal linkage receptors (specific for avian influenza viruses) and SAα2,6Gal receptors (specific for human influenza A viruses), whereas allantoic cells possess only SAα2,3Gal linkage receptors.[23] The absence of SAα2,6Gal receptors in the allantois provides pressure for the selection of variants with altered specificities.[23]

Nucleotide changes associated with the egg adaptation of human influenza A and B viruses are predominantly located around the HA receptor binding site of the HA1 region. Changes at the receptor binding site can influence the pattern of host-dependent glycosylation and, conversely, the position of carbohydrate side chains can affect the capacity of the virus to bind to receptors with specific carbohydrate linkages.[24]

3. Immune Responses to Egg-Adapted Influenza Viruses

Accredited seed viruses used to prepare the most current vaccines are grown in eggs. Virus variants have been found in vaccine virus seed stocks[25,26] and a number of studies have been undertaken to investigate responses to vaccines prepared from egg- and cell culture-derived viruses in animals and humans.

In several studies, experimental MDCK-grown vaccine viruses were prepared from MDCK isolates and passaged seed viruses. Live H3N2 vaccine viruses grown in eggs or MDCK cells provided equivalent cross-protective immunity in ferrets challenged with homologous virus grown in either substrate.[12] Sequencing of the HA glycoprotein of the

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egg- and MDCK-derived viruses did not reveal any amino acid differences, but antigenic differences were observed using anti-HA monoclonal antibodies. Similar findings in cross-protection were noted with an influenza B recombinant vaccinia virus vaccine, containing either an egg-derived or an MDCK-derived HA glycoprotein.[12,27,28] H1N1 inactivated vaccines prepared from viruses grown in eggs or MDCK cells were similarly immunogenic in ferrets, guinea pigs and hamsters, although the MDCK vaccine induced antibodies that were more broadly cross-reactive when measured by single-radial haemolysis.[29] In hamsters, higher rates of protection against homologous challenge were obtained with the MDCK vaccine. Antigenically distinct egg-grown H3N2 variants have been shown to be equally immunogenic and protective after homologous or heterologous challenge in mice.[30] A comparison of inactivated, whole H3N2 vaccines prepared from viruses grown in eggs or MDCK cultures revealed that both vaccines induced cross-reactive neutralizing antibody in ferrets, although the MDCK vaccine induced higher antibody titres and greater protection against challenge by virus grown in either substrate.[31] The same clinical sample isolated and passaged in eggs or MDCK cells resulted in two egg-grown variants, while the cell culture-grown viruses retained an identical HA1 sequence to that present in the original clinical isolate. One egg-adapted variant with a single amino acid substitution resulted in a lowered response to an H3N2 inactivated reassortant vaccine in mice, as measured by B-cell responsiveness.[32] The other egg-adapted variant with a single substitution at a different HA1 location induced an immune response similar to that observed with MDCK-grown viruses. Two subunit egg-grown H1N1 vaccines prepared from variant viruses derived from the same clinical sample, one 'cell culture-like' and the other 'egg-like', induced high levels of cross-reactive protective antibodies. However, the cell culture-like virus induced higher levels of strain-specific antibody response.[33]

Analysis of H3N2 reference strains from the US and UK over the period 1987–9 and field strains from 1988–90 revealed that three of seven reference strains and all the egg-grown field strains contained virus variants.[34] Only the MDCK-grown field strains had HA1 sequences identical to those of the original clinical isolates. When MDCK cells were co-infected with an excess of MDCK-derived virus over egg-adapted virus, the progeny virus was largely egg-adapted.[35]

Immune responses to vaccines prepared from egg-adapted viruses appear to vary according to the location of amino acid substitution(s) in the HA glycoprotein following egg passage. Although selection of variants does not usually occur following passage in mammalian cell culture, it would be prudent to monitor for changes, especially in viruses being considered as candidate vaccine seeds.

4. The Growth of Influenza Viruses in Cell Culture

The growth of influenza viruses in cell cultures was initially investigated as tool for research into their genetic, biochemical and biological properties. Studies with primary cultures prepared from fetal pig lungs, pig kidneys, calf kidneys, monkey kidneys, chicken embryo fibroblasts, chicken and chicken embryo kidneys produced variable results and many were difficult to prepare.[36] The addition of crystalline trypsin improves the yield in cell lines that lack specific host proteases necessary for the proteolytic cleavage of the HA glycoprotein to produce infectious virus.[37] The use of primary cell lines presents a number of drawbacks including the availability and cost of source organs, batch variation and their potential for contamination with adventitious agents.[38]

Some continuous lines of epithelial origin support the growth of influenza viruses in the presence of added trypsin to levels approximating those achieved by primary epithelial cells.[39,40] These cells can be maintained frozen and expanded for large-scale use as required. The MDCK cell line was derived from the kidney of a healthy female cocker spaniel in 1958.[41] This cell line has been extensively studied as it provides an excellent model for the study of kidney function and epithelial development.[42] MDCK cells exhibit differentiated kidney-

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specific transport properties and retain their ability to regenerate kidney tubular structures under appropriate conditions.[43] MDCK cells have been used for the plaque assay of influenza A and B viruses since the mid-1960s and are commonly used for the clinical isolation of influenza viruses.[40,44-46]

The suitability of a number of other cell lines for the growth of influenza viruses has been investigated. Yields of the cold-adapted donor strain A/Ann/Arbor/6/60 from the diploid cell line MRC-5 (human fetal lung fibroblast) were about 100-fold less than those obtained in primary chicken kidney and chicken embryo kidney cells.[47] A porcine lung epithelial cell line established from a normal 4-week old female Yorkshire pig has been shown to support the growth of influenza A and B viruses.[48] The PER.C6, BHK-21/BRS and Novartis MDCK lines are anchorage-independent and have been developed for the growth of vaccine viruses in the absence of serum. They can be rapidly expanded for growth in bioreactors and are potentially more suited to rapid scale-up than anchorage-dependent lines, such as MDCK and Vero. The PER.C6 line has been prepared from a human fetal retinoblasts, which have been immortalized by transfection with an E1 minigene of adenovirus type.[49] The PER.C6 line can be propagated to high concentrations and supports the growth of influenza viruses to titres comparable with those attainable in eggs.[50] The BHK-21/BRS line is used for the preparation of veterinary vaccines but yields of influenza viruses are relatively low.[51] Acceptability of transformed cell lines as a substrate is dependent on the particular cell line and its passage history. Acceptability criteria also vary according to regulations imposed by different jurisdictions.

A proprietary cell line, EBx™ developed by Vivalis, is currently licensed by GlaxoSmithKline, CSL and Nobilon for the production of influenza vaccines. EBx™ is an anchorage-independent non-transformed avian embryonic stem cell line that can be propagated in serum-free medium and appears to have potential as a substrate for the growth of vaccine viruses.[52]

HA and NA glycoproteins have been successfully expressed in both insect cells and larvae inoculated with recombinant baculoviruses since the late 1980s.[53-55] Baculovirus vaccines in humans induce serum antibody responses and are well tolerated, although effective rates of protective neutralizing antibody responses were only observed in those receiving the highest doses.[56,57] These responses may be improved with the judicious use of newer adjuvants.

5. Serum and Protein-Free Media

The growth of cells in culture requires the use of media that provide all the components necessary for cell attachment, proliferation and normal function. Serum (usually fetal calf) has been regarded as a critical supplement to medium to achieve growth in culture. Serum provides a complex mixture of hormones, growth factors, carrier proteins, attachment and spreading factors, and nutrients.[58] However, its use presents problems that arise from lot variability, presence of undefined components, cost and the possible introduction of contaminating agents.[59] Contamination by fungi, bacteria, viruses or the agents of transmissible spongiform encephalopathies (TSE) has raised regulatory concerns for the safety of human vaccines. The first step towards avoiding these problems was the development of serum-free media, although many such formulations still contain animal- or human-derived proteins, including peptones, hydrolysates and albumin fractions.[60] Animal- and human-derived proteins can also be replaced by synthetic, recombinant or plant proteins. The use of chemically defined protein-free media minimizes the risks from TSE and viruses, but development is complicated and usually depends on specific requirements for particular cell lines.[59]

6. The Development of Cell-Culture Based Influenza Vaccines

A number of experimental MDCK-grown vaccines have been developed and have been reported to be safe, well tolerated and immunogenic in healthy young adults, the elderly and children.[61-65]

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A meta-analysis of vaccines prepared from viruses grown in MDCK cultures and trialled in 1995–6 indicated that levels of reactogenicity were comparable with others prepared from egg-grown viruses that both fulfilled European criteria based on serum responses.[65] Solvay Pharmaceuticals have approval from Dutch regulatory authorities for the production of an MDCK-grown influenza vaccine (Influvac® TC)[1] in the Netherlands and are attempting to obtain registration throughout Europe and in the US. The Chiron Corporation (now Novartis) have developed an influenza vaccine (Optaflu®) using MDCK cells grown in suspension and is currently seeking European registration. An MDCK-derived live cold-adapted attenuated trivalent influenza vaccine, prepared on microcarriers in a 10 L culture vessel, was shown to be genetically stable and clinical trials are planned.[66]

Accredited preparations of the Vero cell line have been used in the preparation of poliomyelitis and rabies vaccines for over 20 years as more satisfactory alternatives to primary monkey kidney cells.[67] Initial studies indicated that influenza viruses grew poorly in Vero cells.[68,69] Improvements in yields were later obtained by the addition of trypsin to the cultures, and Vero cells are now considered suitable for use in clinical isolation and for the growth of vaccine viruses.[39,70,71]

Vero-grown vaccines have been studied in animal models. Inactivated whole vaccines were shown to be as immunogenic as egg-grown vaccines in mice and chimpanzees. Analysis of the chimpanzee serological responses to Vero-derived vaccines showed that rates of seroconversion, geometric mean titre increase and percentage of subjects achieving a protective titre all met European guidelines for humans.[72,73] When delivered as a live intranasal vaccine, Vero-grown whole virus vaccines induced levels of influenza-specific secretory IgA levels that were comparable with commercially available egg-grown whole vaccines. The cell-mediated immunity induced was significantly higher in mice receiving Vero-derived compared with egg-derived vaccines, as determined by proliferation and cytokine release assays.[74] Vero-grown inactivated whole virus H5N1 vaccine has been shown to induce cross-protection in mice.[75] Baxter International Inc. has new facilities in Austria and the Czech Republic for the production of their inactivated Vero-grown influenza vaccine, InfluJect®. InfluJect® was approved for use in the Netherlands in 2002 and the company is seeking registration in other European countries and the US.

Live cold-adapted influenza A vaccines have been produced in the Vero cell line, using a donor strain adapted to growth at 25°C in Vero cells.[76] The donor strain retained its temperature-sensitive phenotype and attenuation properties in mice, and was protective in ferrets against a wild type challenge. H1N1 and H2N2 cold-adapted reassortants using the cold-adapted Vero-grown donor strain and prepared in the Vero line were shown to be safe, well tolerated and suitably immunogenic in a small group of healthy adults. Production processes suitable for the preparation of vaccines using viruses grown in the PER.C6-derived influenza vaccine have been developed.[77] A phase I clinical trial of an influenza vaccine PER.C6 grown, licensed from Crucell N.V. in the Netherlands, was conducted in 2006 by Sanofi-Pasteur. A phase II clinical trial commenced in November 2007.

7. Conclusions

The growth of influenza viruses in eggs selects egg-adapted variants that may be antigenically distinct from the original parent strain. In addition, the supply of fertile eggs is limited and long-term planning is necessary to significantly increase egg supply to rapidly increase the supply of vaccines. Such increases can be adversely affected by the sudden and unpredictable occurrence of avian diseases in flocks. Another consideration is the unsuitability of egg-derived vaccines in individuals with egg allergies. The pressures associated with the production flexibility required to supply large volumes of avian pandemic vaccines has been a motivating factor for recent, rapid advances in technologies for the devel-

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opment of cell culture-based influenza vaccines.[8] The use of stable cell lines for influenza vaccine virus growth provides an alternative substrate that can largely overcome these problems, if yield and safety issues can be satisfactorily addressed.

Acknowledgements

No sources of funding were used to assist in the preparation of this review. Professor Tannock acts as a consultant for CSL Ltd and has received grants for clinical vaccine trials from CSL Ltd in the past. Dr Audsley has received part funding for a past PhD project from CSL Ltd. This article was prepared and submitted without consultation with CSL Ltd.

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