

**Use of Cell Lines for the Production  
of Influenza Virus Vaccines:**

**An Appraisal of Technical, Manufacturing, and  
Regulatory Considerations<sup>1</sup>**

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## 1.0 Background

Manufacture of influenza virus vaccine has traditionally relied on embryonated hens' eggs for virus propagation prior to concentration, inactivation, further purification, formulation, aseptic filling, final testing and release. The basic production scheme has changed very little for the past 60 years, aside from the development and use of high-growth reassortants as seeds for type A strains, additional steps to further process whole virus antigen via disruption (e.g., through the use of detergents) and further purification of the key surface antigens that confer protection, particularly hemagglutinin. Egg-derived vaccines have had an extraordinarily long and successful track record of safety and efficacy in Europe, the USA, and elsewhere, where annual vaccination has been consistently shown to reduce the incidence of influenza-related illness, complications and mortality.

Despite the long-standing and remarkable success of egg-derived vaccines, current supply and demand considerations underscore an urgent need to pursue alternative approaches for influenza vaccine production. These considerations include:

- Increased demand for seasonal influenza vaccination. Over the past 25 years, there has been increasing emphasis on reducing the public health impact of influenza, especially the high rates of mortality observed in both industrialized and developing countries. The annual death toll is especially high in persons with relative or absolute deficits in immune function, including very young children, the elderly, and persons of any age who have chronic underlying medical conditions. As a consequence of these observations and abundant literature demonstrating the value of influenza vaccination, the global demand for influenza vaccine has dramatically increased during the past decade and will likely continue to do so for the foreseeable future. Increasing demand is especially notable in the USA, Canada and Western Europe, although demand in other countries, especially in Australia, Eastern Europe, Asia and South America is also on the rise.
- Reduced and/or variable vaccine supply. Despite the large and increasing demand for influenza vaccine, established manufacturers in the USA and Europe have exhibited increasing difficulty in providing sufficient numbers of doses. For

example, in the USA, two large-scale manufacturers ceased production altogether in the late 1990s, based largely on difficulties in meeting increasingly strict standards for production under current Good Manufacturing Practices (cGMP) in the face of reduced profit. More recently, one USA-licensed manufacturer was prevented from distributing more than 30 million doses during the 2004-2005 influenza season because of concerns about product quality. The sudden withdrawal of this vaccine from the market, combined with the inability for other manufacturers to ramp up production because of long lead-times required to obtain additional eggs, forced USA public health officials to implement a complex prioritization and distribution scheme in which many individuals were denied vaccination. In that same year, a new variant of influenza began circulating in October 2004, which further increased demand. This dramatic convergence of all these events clearly underscores the urgent need to find new and more reliable ways to supply vaccine needs in the longer-term.

- Legitimate Threat of Pandemic Influenza. Increased circulation of avian influenza A/H5N1 viruses in Asia and elsewhere has been well documented during the past 10 years, with sporadic transmission to nearly 300 humans, ~50% of whom have died. H5N1 variants have been increasingly detected in migratory ducks and other birds, and also isolated from pigs. Most experts believe that it will only be a matter of time before the virus undergoes sufficient mutation to become more easily transmissible among humans, or to reassort with another human or animal virus to become immediately adapted to the human host. Because this and many other avian viruses are highly lethal for chickens (and for embryonated eggs, unless the viruses are genetically modified), and because a pandemic strain may emerge at any time, efforts to augment embryonated eggs or other avian-derived substrates as the principal substrates for vaccine production must be pursued as rapidly as possible.

## **2.0 Cell Culture-Derived Influenza Vaccines**

### **2.1 Potential Advantages**

In response to the urgent and growing need for alternative means for influenza vaccine production, a number of vaccine manufacturers have considered new approaches. Of

particularly interest has been the potential use of tissue culture cell lines to be used either in addition to, or in lieu of, egg-based production. The chief advantages of such a system are summarized below:

- Enables utilization of the same basic and clinically proven approach used in egg-based production systems – i.e., production of whole-viruses, multiple disrupted (“split”), more highly purified influenza virus antigens, or live attenuated vaccines – while at the same time eliminating the long lead times and supply-chain vulnerabilities required for egg-based production systems.
- Enables utilization of a modern and controlled seed lot system, with uniform characterization of the production cells and reduced risk of introduction of exogenous or endogenous adventitious agents.
- Enables a more robust, consistent and reproducible means of vaccine production, utilizing a scalable and a closed (or largely closed) bioreactor process, which may be initiated at any time and extended for a prolonged period if needed.
- Enabling the capability of producing influenza vaccines with avian strains, which generally cannot grow in eggs without genetic modification.

In addition to these documented benefits, vaccine production in mammalian cells also has at least one theoretical advantage: whereas the isolation and replication of influenza viruses in eggs generally leads to selection of certain phenotypes, the majority of which differ from the actual human clinical isolate, the isolation and replication of influenza viruses in cell culture, in contrast, has no appreciable passage-dependent selection. This should theoretically allow for expression of the hemagglutinin antigen in a more native (natural) conformation, thereby potentially improving the specificity and potential avidity of antibody formation and cell-mediated immunity in humans.

## 2.2 Ideal characteristics of cell lines that could be utilized for production

The selection of the specific cell substrate for influenza vaccine production is influenced by the need for a robust and commercially viable manufacturing process and numerous,

specific production requirements unique to influenza. Major desirable characteristics are summarized below:

- Replication of influenza strains to sufficient titer. Aside from embryonated eggs, chicken embryo fibroblasts, and other avian cells (see additional discussion below), replication of influenza viruses has otherwise been documented in hamster cells (BHK21-F and HKCC), MDBK cells, PER.C6 cells, Vero cells, and MDCK cells. A prerequisite for a successful infection is the addition of proteases to the medium, preferably trypsin or similar serine proteases, as these proteases extracellularly cleave the precursor protein of hemagglutinin (HA<sub>0</sub>) into active hemagglutinin (HA<sub>1</sub> and HA<sub>2</sub>). Only cleaved hemagglutinin leads to the adsorption of the influenza viruses on cells with subsequent virus assimilation into the cell, which leads to further replication. Of the various continuous mammalian cell lines that have been adequately tested – including diploid cell lines (MRC-5, WI-38, and FRhI-2) and continuous cell lines (PER.C6, NIH-3T3, BHK, CHO, Vero and MDCK) – only Vero, PER.C6 and MDCK have consistently yielded influenza viruses titers that are sufficiently high enough to be considered commercially viable. This and other aspects of these three cell lines are discussed in greater detail below.
- Established Regulatory Precedent. As noted above, immortalized (continuous) cell lines are the only mammalian cell lines that have been documented to support sufficient replication of influenza viruses. Of the three leading candidates (Vero, PER.C6 and MDCK [see above]), Vero cells have been the only ones to have been used in a widely used, commercially available vaccine.<sup>1</sup> The main regulatory concern related to these cell lines is that each of them has been documented to be tumorigenic in animals at some point during their passage history. Of the three, MDCK cells are the most tumorigenic, the mechanism for which remains unknown. This and other regulatory aspects related to cell line

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<sup>1</sup> In 2001, Influvac® TC, a split-virus influenza vaccine produced in MDCK cells (Solvay Pharmaceuticals) was approved in The Netherlands, but never commercially distributed. In 2002, Influject®, a whole-virion influenza vaccine produced in Vero cells (Baxter Vaccines, Vienna, Austria) was approved 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 trial participants.

characterization (see additional discussion below) have posed significant challenges for the approval of mammalian cell-derived influenza vaccines.

- Ability to Propagate Cells in a Chemically Defined Medium Under Serum-free Conditions. In order to develop a process suitable for large-scale commercial production, cells can be propagated and maintained as either anchorage-dependent (adherent) or non-anchorage dependent. In the case of adherent cells, after the initial proliferation phase, the nutrient medium is removed and fresh medium is added to the cells, with infection of the cells with influenza viruses taking place simultaneously or shortly thereafter. After a specified time post infection, a protease (most often trypsin) is added in order to obtain an optimum virus replication. The initial and subsequent additions of trypsin later on in the typical process are generally labor-intensive, with an increased potential for contamination of the cell culture by adventitious agents. A more cost-effective alternative is cell proliferation in fermenter systems utilizing cells growing adherently on microcarriers under serum-free conditions, but such a process also requires opening of the culture vessels several times and thus brings with it an increased risk of contamination. The most desirable system would be the propagation of cells in suspension. This not only eliminates the need for trypsin, but also reduces cost, labor and the risk of contamination and also allows for the formation of microvilli on the entire cell surface, thus improving process yield and efficiency.

Although the primary focus of most developers has been on mammalian cell lines, avian-based cell lines, including chicken embryo fibroblasts (CEF), chicken embryo kidney (CEK) and blastoderm-derived embryonic stem cells (e.g., EB14, Vivalis) are additional possibilities, particularly for vaccine producers that are already working with these cell lines (chiefly CEF) for other vaccines (e.g., measles, mumps). The only companies that appear to have developed a large-scale process at the present time that could be adapted to influenza are Vivalis and Bavaria Nordic. The latter company reportedly has utilized CEF cells grown in suspension in Wave® bags to propagate the company's proprietary strain of Modified Vaccinia Ankara (MVA), a process that might be modified to grow influenza virus. However, some investigators have reported relatively low or

variable yield of certain influenza virus strains from CEF, CEK and EB14 cells, which, if replicated by others, could impede use in commercial production.<sup>2</sup>

## 2.3 Considerations Related to Specific Cell Lines

### 2.3.1 *Madin Darby Canine Kidney (MDCK)*

Of the three principal candidate mammalian cell lines, MDCKs are by far the most studied and advanced of the three. At least four companies, including Novartis Vaccines (formerly Chiron), Solvay, GlaxoSmithKline (which acquired, via the 2005 acquisition of ID Biomedical, the technology developed originally by IAF BioVac) and MedImmune have developed and characterized proprietary Master and Working Cell Banks derived from the original MDCK cell line that was initially derived in 1958. All four companies have reportedly adapted the cells to grow under serum-free conditions, including at least one (Novartis) in suspension. At least two of the companies, Novartis and Solvay, have extensively tested and characterized their cell banks according to USA, EU and ICH requirements. Candidate vaccines produced by Novartis, Solvay and IAF Biovac have been tested clinically and found to be non-inferior to conventional, egg-derived comparator vaccines, and one of them (Novartis) is now under consideration for marketing in the EU under the centralized procedure. The most detailed, publicly available information about Novartis' and Solvay's vaccines can be found at the US Food and Drug Administration Website as part of the proceedings of a meeting of the Vaccines and Related Biological Products Advisory Committee (VRBPAC) held on 16 November 2005.<sup>3</sup> Both companies have also been awarded contracts by the U.S. Department of Health and Human Services to develop MDCK cell-derived influenza vaccines (for both seasonal and pandemic use) for consideration for licensure in the USA, as has MedImmune. The EMEA has also recently issued guidance on cell culture-based, inactivated influenza vaccines.<sup>4</sup>

### 2.3.2 *Vero Cells*

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<sup>2</sup> See Coelingh K. FluMist case study. Harnessing novel technologies. In: Proceedings of the World Vaccine Congress, Washington, DC, USA, 20 March 2007.

<sup>3</sup> See <http://www.fda.gov/ohrms/dockets/ac/cber05.html#VaccinesandRelatedBiological>

<sup>4</sup> See <http://www.emea.europa.eu/pdfs/human/bwp/249000en.pdf>

As noted previously, Baxter International, Inc., has developed a whole-virus inactivated influenza vaccine utilizing Vero cells that have been further adapted to grow under serum-free conditions. The process reportedly involves the use of wild-type influenza viruses as seeds, rather than the typical high-growth reassortant strains for type A used by most other manufacturers. The company is reportedly investigating the cause for the observed increased risk of adverse reactions (primarily fever) in its Phase II/III trials in Europe, the cause of which has not been publicly disclosed. In May 2006, the company announced a collaboration with DVC LLC, a Computer Sciences Corporation, to develop cell-culture based influenza vaccines as part of a USA Government contract award to DVC. Baxter will manufacture the candidate vaccines at its production facility in the Czech Republic. More recently, the company announced that the Vero cell-derived, candidate H5N1 vaccine developed under this agreement was safe and immunogenic, and reportedly plans to proceed with Phase IIb trial in 2007.

### *2.3.3 PER.C6 cells*

PER.C6 cells were originally derived from embryonic human retinal cells that were immortalized by a known means of transformation, i.e., the E1 sequences from Adenovirus type 5. As with MDCK cells, PER.C6 cells have been extensively characterized by the developer, Crucell, and have now been utilized in the production of a number of investigational vaccines against numerous diseases such as HIV, West Nile, SARS, and influenza. Most of the influenza-related work has been conducted by Sanofi, which obtained an exclusive license from Crucell for this purpose. Limited information available from the public domain indicates that PER.C6 cells are broadly permissive to wide variety of influenza strains, with titers ranging from  $10^{7.6-8.2}$  pfu/mL. The production process reportedly propagates cells in suspension using animal protein-free and serum-free media. The results of clinical trials, if any, have not been disclosed.

## 2.4 Other Considerations Related to All Three Cell Lines

### *2.4.1 Intellectual Property and Know How*

The actual production cell lines currently being used by the companies mentioned in this report were all developed internally after many years of work, and will likely not be made available to other public or private organizations. Therefore, additional companies

interested in establishing cell culture processes will need to establish and characterize banks on their own, an extremely intensive, lengthy and costly process, or to license in the cell lines from the developer.

#### 2.4.2 Other regulatory considerations

The primary regulatory and other general requirements pertinent to the establishment and characterization of cell lines used for the development and manufacture of influenza vaccines can be found in the following documents:

- FDA Draft Guidance for Industry: Characterization and Qualification of Cell Substrates and Other Biological Starting Materials Used in the Production of Viral Vaccines for the Prevention and Treatment of Infectious Diseases. Issued September 2006.
- EMEA/CPMP/BWP/214/96: Note for Guidance on Harmonisation of Requirements for Influenza Vaccines. Issued March 1997.
- CPMP/BWP/2490/00: Cell Culture Inactivated Influenza Vaccines. Annex to Note for Guidance on Harmonisation of Requirements for Influenza Vaccines (CPMP/BWP/214/96). Issued January 2002.
- EMEA/CHMP/VWP/263499/2006: Guideline On Dossier Structure And Content Of Marketing Authorisation Applications For Influenza Vaccines Derived From Strains With A Pandemic Potential For Use Outside Of The Core Dossier Context.
- The International Conference on Harmonisation (ICH) Guidance, *Q5A: Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin*, (63 FR 51074; September 24, 1998).
- ICH Guideline, *Q5D: Derivation and Characterisation of Cell Substrates Used for Production of Biotechnological/Biological Products*, (63 FR 50244; September 21, 1998).

- ICH Guideline, Q2A: *Text on Validation of Analytical Procedures*, (60 FR 11260, March 1, 1995).
- ICH Guideline, Q2B: *Validation of Analytical Procedures: Methodology*, (62 FR 27463, May 19, 1997).
- US and EU Pharmacopoeias.
- World Health Organization. Recommendations for the production and control of influenza vaccine (inactivated). Annex 3. WHO Technical Report Series. 2005;927:99-134.
- World Health Organization. WHO Guidelines on Nonclinical Evaluation of Vaccines. Annex 1. WHO Technical Report Series. 2005;927:31-63.

One of the main concerns that have been expressed by both USA and EU regulatory authorities for cell culture-based influenza vaccines is that developers must provide substantial assurance that the final product will be free from adventitious agents. Accordingly, there must be vigorous testing of all materials used in the process, including raw materials, virus seeds, cells, and intermediates, along with careful assessment of the ability of the process to remove any residual infectious (or potentially infectious) agents. Because the mechanism for immortalization of the cells is unknown for Vero and MDCK cells, the developer will have to make every effort to detect any unknown agent that could potentially be oncogenic, particularly residual DNA exceeding more than 200bp. Unlike for egg-derived vaccines, the EU and the USA also expect more rigorous testing of the viral seed for extraneous agents, which, according to the Ph.Eur. monograph 2.6.16 and 21 CFR 630.35 (although revoked), must include neutralization of influenza virus to prevent interference with the different test systems (detector cells, animals). Such a requirement will likely pose challenges to timely production of any cell-culture derived influenza vaccine

### **3.0 Conclusions**

Influenza virus infection has, for many centuries, ranked among the most frequent causes of severe respiratory illness globally. The worldwide increase in the incidence of

avian influenza caused by A/H5N1 viruses highlights increasing concern that another pandemic may occur in the near-term. Should this happen, there will be significant threats to human health, national and global economies, and the normal functioning of communities worldwide. Although the global capacity for producing influenza vaccine has increased significantly during the past 10 years, much more dramatic surges are needed to ensure the timely availability of vaccine for as many individuals as possible. Although embryonated eggs have proven to be a reliable substrate for influenza vaccine production for more than 50 years, new technologies, particularly the use of continuous cell lines, offer a number of practical and theoretical advantages to enhance available vaccine supplies. Despite considerable technical and regulatory challenges, several companies have achieved the ability to produce cell culture-derived vaccine at commercial scale, and at least one of these products is expected to be approved for marketing within the next two years. It is hoped that this success, combined with the continued cooperation among many organizations in the public and private sectors, will further stimulate the development and licensure of many more vaccines in the coming decade.

## OTHER SELECTED REFERENCES

Brands R, Visser J, Medema J, Palache AM, van Scharrenburg GJM: Influvac: A safe Madin Darby Canine Kidney (MDCK) cell culture-based influenza vaccine. In: Brown F, Robertson JS, Schild GC, Wood JM (eds): Inactivated Influenza Vaccines Prepared in Cell Culture. Dev Biol Stand. Basel, Karger, 1999, vol 98: 93-100.

Brown F, Lewis AM, Peden K, Krause P (eds): Evolving Scientific and Regulatory Perspectives on Cell Substrates for Vaccine Development. Dev Biol. Basel, Karger, 2001, vol 106.

Govorkova EA, Murti G, Meignier B, deTaisne C, Webster RG. African green monkey (Vero) cells provide an alternative host cell system for influenza A and B viruses. J Virol 1996;70:5519-5524.

Kistner O, Barrett PN, Mundt W, Reiter M, Schober-Bendixen S, Eder G, Dorner F. Development of a Vero cell-derived influenza whole virus vaccine. Dev Biol Stand 1999;98:101-110.

Merten OW, Hannoun C, Manuguerra JC, Ventre F, Petres S. Production of influenza virus in cell cultures for vaccine preparation. In: Cohen S, Schafferman A (eds.): Novel strategies in design and production of vaccines. Plenum Press, New York, 1996; 141-151.

Merten OW, Manuguerra JC, Hannoun C, Van der Werf S. Production of influenza virus in serum-free mammalian cell cultures. Dev Biol Stand 1999; 98:23-37.

Lewis AM Jr, Krause P, Peden K: A defined-risk approach to the regulatory assessment of the use of neoplastic cells as substrates for viral vaccine manufacture. In: Brown F, Lewis AM, Peden K, Krause P (eds): Evolving Scientific and Regulatory Perspectives on Cell Substrates for Vaccine Development. Dev Biol. Basel, Karger, 2001, 106: 513-535.

Palache AM, Brands R, van Scharrenburg GJ. Immunogenicity and reactogenicity of influenza subunit vaccines produced in MDCK cells or fertilized chicken eggs. J Infect Dis 1997; 176: S20-S23.

Robertson JS, Cook P, Attwell AM, Williams SP. Replicative advantage in tissue culture of egg-adapted influenza virus over tissue-culture derived virus: implications for vaccine manufacture. Vaccine 1995; 13 (16): 1583-1588.

World Health Organization. Cell culture as a substrate for the production of influenza vaccines: memorandum from a WHO meeting. Bull World Health Organ 1995; 73: 431-435.