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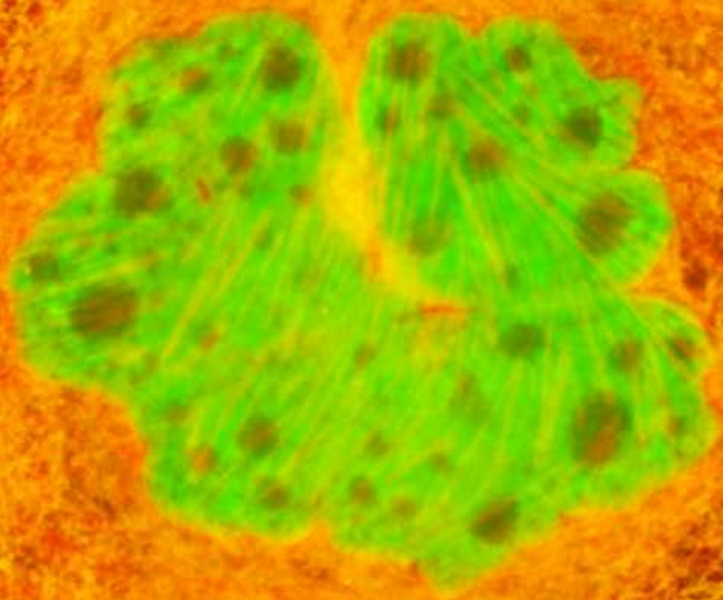

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Characterization and Qualification of Cell Substrates for Manufacturing Viral Vaccines in the United States

By ARIFA S. KHAN*

Cell substrates are used in various stages of viral vaccine manufacturing, as in the isolation, selection, and propagation of the virus seed or virus vector stock, as well as for the amplification of the virus to produce the final vaccine product. The various stages of cell substrate use, including cell banking, are shown in a generic manufacturing scheme in Figure 1.

Traditionally, viral vaccines have been produced in animal tissues, primary cell cultures, and cell lines that either have a finite life span, such as normal diploid cells, or a theoretically infinite life span, as achieved with continuous or immortalized neoplastic cells.[1-3] The cell substrates used in viral vaccines currently licensed in the US,[4] are listed in Table 1. In the past, primary African green monkey cells were used for live oral polio vaccine, and a diploid fetal rhesus monkey lung cell line (FRhL-2) for a live rotavirus vaccine. In addition to animal cell substrates, yeast cells are used for inactivated vaccines for hepatitis B virus and human papillomavirus.[4] For development of some new vaccines, novel cell substrates are emerging such

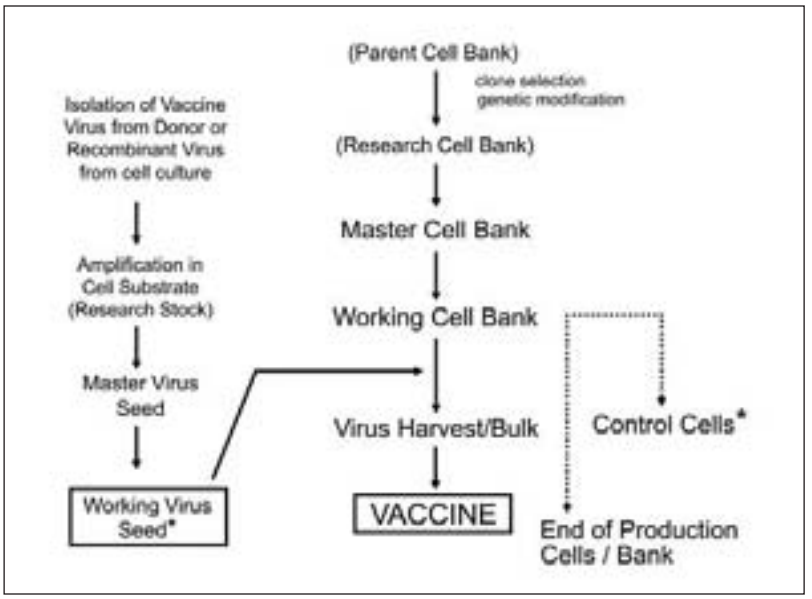

FIGURE 1. Generic vaccine manufacturing scheme with cell banking stages. (*Not in every case.)

TABLE 1. Animal cell substrates for licensed viral vaccines currently used in the US.[4]

Substrate	Species of Tissue or Cell Origin	Vaccine	
		Inactivated	Live
Animal Tissue			
Brain	Mouse	Japanese encephalitis virus	
Skin (lymph)	Calf		Smallpox
Embryonated eggs	Chicken	Influenza	Influenza, yellow fever
Primary Cell Culture			
Embryo fibroblasts	Chicken	Rabies	Measles, mumps
Diploid Cell Line			
MRC-5 (fetal, lung fibroblast)	Human	Hepatitis A, rabies	
WI-38 (fetal, lung fibroblast)	Human		Varicella-zoster, rubella
Continuous Cell Line			
Vero (adult, kidney)	African green monkey	Poliovirus	Rotavirus, smallpox

Arifa S. Khan, PhD *(Arifa.Khan@fda.hhs.gov), is a Senior Investigator in the Division of Viral Products, Office of Vaccines Research and Review, Center for Biologics Evaluation and Research, US Food and Drug Administration, Bethesda, Maryland.*

****Dr. Khan is the corresponding author.***

as those that have been transformed by known agents like viral genes or chemicals, by unknown mechanisms, and by genetic engineering, which provides the complementation needed for some vectored viral vaccines.

The initial step to assure the safety of a viral vaccine is extensive characterization and testing of the cell substrates used in manufacturing the product. Historically, primary cell cultures were used for vaccine production because of the tumorigenicity safety concerns that could be associated with continuous cell lines. But since primary cells cannot be banked for continued use, extensive testing was needed on each new lot. In addition, donor history, donor testing, and the use of specific pathogen-free (SPF) donors were needed wherever possible. In 1967, during discussions on the safe use of human diploid cells, a cell banking system was proposed in which extensive characterization would be required for the master cell bank, and limited testing would be done on the working cell bank.[5-10] While the initial objective of this testing was to assure that the bank would be free of adventitious agents, a number of additional assays are needed for the cell banks and virus seeds to assure product safety.[14]

Then in 1986, the need for continuous cell lines in vaccine manufacturing was formally discussed due to their ability to propagate indefinitely, and the belief that they could therefore better support product scale-up and manufacturing.[11-13] But their use for virus seed preparation and vaccine production has progressed cautiously due to safety concerns related to their potential tumorigenicity. Thus far, the only continuous cell line that has been used for a US licensed vaccine is a low-passage, non-tumorigenic Vero[15] (Table 1). Primarily, Vero was chosen due to its broad susceptibility to infection by diverse viruses, and its ability to support high replication levels for viruses needed in many licensed and investigational vaccines.

Recently, due to the emergence of new diseases and potential threats from naturally-occurring or genetically-modified biological agents, there has been increased activity to develop novel vaccines and new cell substrates (Table 2). The urgent need to develop pandemic influenza vaccines in novel cell substrates, including those with tumorigenic potential, provided impetus for new testing strategies that could address the safety issues related to potential oncogenic agents and novel adventitious viruses. To meet the challenges anticipated with the use of "non-traditional" cell substrates, CBER has been working with national and international experts to use a defined risk approach for developing testing strategies that can assure vaccine safety. Some of the critical public meetings are listed in Table 3.

TABLE 2. Introduction of novel cell substrates in viral vaccine manufacture.

Traditional Cell Substrate	Novel Cell Substrate	Advantages	Vaccine Examples
Egg-based	Cell lines	• Higher virus yield • Easy scalability • Availability of cells to meet production demand • Well characterized cell banks • Less redundant testing due to reduced risk of unknown agents from the animal species of origin	• Influenza virus
Non-tumorigenic	Tumorigenic	• Higher virus yield • Susceptibility of cells to vaccine virus	• Influenza virus • Adeno-associated virus-vectored HIV vaccines
Unmodified	Genetically-engineered	• Requirement for complementation for some vaccine vectors	• Defective adenovirus-vectored vaccines
	Development of new cell lines	• To replace depleting stocks of limited-passage diploid cell lines • For novel vector development • For high virus particle or protein yields • To remove adventitious agent concerns related to mammalian cell substrates or raw materials of mammalian origin	

TABLE 3. Public discussions on novel cell substrates.

1998	VRBPAC discussion[16] on neoplastic and tumorigenic cells for vaccine manufacture (November 20)
1999	International meeting: Evolving Scientific and Regulatory Perspectives on Cell Substrates for Vaccine Development[17]
2000	VRBPAC discussion[16] on Vero cells (non-tumorigenic) for live-attenuated vaccines (May 12)
2001	VRBPAC discussion[16] on *in vitro* transformed (genetically-engineered) human cells (HEK293, PER.C6) for defective adenovirus-vectored vaccines (May 16)
2004	IABS/NIAID meeting on vaccine cell substrates[18, 19]
2005	VRBPAC discussion[16] on tumorigenic MDCK cells for inactivated influenza vaccine (November 16)
2008	VRBPAC discussion[16] on MDCK cells for live influenza virus vaccine (September 25)

A major safety issue concerning the use of novel cell substrates is the potential for infectious, latent and occult adventitious agents[20] that may not be detected by the conventional assays described in the 1993 Points to Consider (PTC) document for the characterization of cell lines used to produce biologicals[21] (summarized in Table 4). Tumorigenic cell substrates have additional issues related to whole-cell tumorigenicity and DNA oncogenicity. Therefore, strategies to remove whole cells, and assays to detect latent viruses (endogenous retroviruses, RNA viruses and DNA viruses) and oncogenic agents (viruses and cell-derived DNA), need to be used to demonstrate cell substrate safety. While not discussed in this paper, transmissible spongiform encephalopathies (TSEs) must also be considered.

To address these safety concerns, FDA has provided "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,"[14] which updates the 1993 PTC document.[21] The new guidance details the previous assays for cell line testing, plus further discusses the approaches to characterize novel cell substrates for the development of viral vaccines. Moreover, additional testing recommendations for novel cell substrates are included such as: 1) modification of the existing tumorigenicity assay to characterize the neoplastic cell phenotype by measuring the capacity of cells to form tumors in immune compromised animals; 2) assays to evaluate the oncogenic activity of an adventitious virus, or a cellular component such as DNA, to induce a tumor in an animal; and 3) assays for detection of latent and occult viruses, including endogenous retroviruses, DNA viruses, and RNA viruses that may be present at low levels, or are quiescent in the cell with the potential to reactivate.

While the existing tumorigenicity assay evaluates the capacity of cells to form tumors in immunodeficient athymic nude mice, it may need to be redesigned to use the most sensitive model for tumor formation. For example, the assay may require newborn nude mice, and in some cases, the study observation period may have to be extended up to 4, or even 7 months.[22]

The proposed *in vivo* oncogenicity assays are designed to evaluate DNA oncogenicity[23] and the presence of oncogenic viruses in < 4 day-old newborn hamsters, newborn nude mice and newborn rats. Cellular DNA (>100 µg) or the lysate of 10^7 cell equivalents is injected and the animals monitored for a period of 4–7 months.

Detection of latent and occult viruses involves the treatment of cells with chemical inducers. Examples of these agents include 5′-iodo-2′-deoxyuridine (IUdR) and 5-azacytidine (AzaC), which are known activate endogenous retroviruses from cells of different species including avian and mammalian; and 12-O-tetradecanoly phorbol-13 -acetate (TPA) and sodium butyrate (NaB), which can activate various DNA viruses such as herpesviruses, and some retroviruses such as HIV-1. Recently,

TABLE 4. Testing recommendations in 1993 PTC for characterization of cell lines.

Characterization		
Karyology	For diploid cells only	
Identity	Isoenzyme	
Tumorigenicity	Not necessary for rodent cells	Nude mice; ATG-treated newborn hamsters, mice or rats; irradiated newborn mice (up to 12 week observation period)
		Colony formation in soft agarose and growth in organ culture
Oncogenes	Not necessary	
Non-Viral Agents		
Mycoplasma and spiroplasma	21CFR 610.30 (agar and broth media culture method); indicator cell culture procedure	
Bacterial and fungal sterility	21CFR 610.12	
Mycobacteria	21CFR 650.13 (b)(2) (culture method); guinea pig test	
Viral Agents: General		
In vitro cell culture tests for cytopathic and hemadsorbing/ hemagglutinating viruses	Same species and tissue type as used in production	
	Human diploid cells	
	Monkey kidney cells	
In vivo assays	Adult mice; suckling mice; embryonated hens' eggs; (guinea pigs, rabbits, monkeys if suitable) [21CFR 630.35]	
Retrovirus detection	Reverse transcriptase assay	
	Infectivity assays	
	Transmission electron microscopy	
Viral Agents: Species-specific		
Animal viruses due to raw materials (e.g., bovine or horse serum, porcine trypsin)	9CFR113.47 and 113.53	
Rodent cell lines	Antibody production assays: MAP, RAP, HAP	
	Challenge assay for LCM virus	
Human cell lines	EBV, CMV, HBV, HCV	
For known viruses based upon donor species and passage history	PCR assays, DNA hybridization, infectivity assays, antibody-based assays	

an algorithm for chemical induction of latent viruses has been developed that may be used to evaluate the safety of a vaccine cell substrate.[24]

When developing a comprehensive testing scheme for a cell substrate, as well as the vaccine product, the most important factors to consider are indicated in Figure 2. But the manufacturing process can also influence the testing requirements. For instance, in the case of subunit or inactivated whole virus vaccines, safety can be assured by incorporating purification steps into the manufacturing process that inactivate or remove adventitious agents or reduce the amount of contaminating host cell materials (Table 5). However, more extensive testing is necessary to demonstrate the safety of cell substrates for live attenuated or recombinant vector vaccines, which are minimally processed and purified, and may therefore contain residual host cell DNA and proteins.

A summary of the general approaches to designing safety into viral vaccine manufacturing is outlined in Figure 3. It should be noted that redundant testing and complementary assays are needed to evaluate product safety with confidence. These principals of selecting appropriate tests and introducing them at relevant steps in product development provide a platform to develop customized testing strategies in accordance with the recommendations of relevant international or national regulatory agencies.[14, 25-30]

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TABLE 5. Different vaccine types and characteristics with regard to adventitious agent safety concerns.

Vaccine Type	Protection	Potential for Extraneous Agents	Testing to Address Adventitious Agent Concerns
Live	Long-term protection with initial injection	High: minimally processed and purified	Extensive testing of: - virus seed - biological raw materials - cell substrate(s) - in-process
			Removal of whole cells
			Reduction of host cell DNA (size and amount)
			Reduction of host cell protein
Inactivated	Need multiple doses for a protective immune response	Reduced: moderately processed with some reduced levels of cellular materials	Virus inactivation
			Process validation
Subunit	Need multiple doses for a protective immune response	Minimal: highly processed with minimal levels of cellular material	Virus inactivation
			Virus clearance
			Reduction in cellular DNA
			Reduction in cellular proteins
			Process validation

Risk assessment of cell substrate contamination

- ❑ Host species of origin
  - Health of donor
  - Naturally-occurring exogenous and endogenous viruses
  - Specific-exposure to other potential infectious agents (e.g., animal vaccines)
- ❑ Cell characteristics
  - Genotype: diploid versus neoplastic/continuous cell line
  - Phenotype: non-tumorigenic versus tumorigenic
- ❑ Cell passage history
  - Cross-contamination in previous facilities (*if applicable*)
    - Propagation in different labs
    - Other viruses or cell lines used
    - Raw materials (serum, trypsin, etc.)
  - Potential introduction of adventitious agents during manufacture
    - Handling
    - Raw materials

Evaluation for risk reduction

- ❑ Susceptibility to known viruses
- ❑ Inactivation or clearance due to manufacturing process
- ❑ Use of qualified reagents

FIGURE 2. Considerations for development of a testing scheme for vaccine and cell substrate safety.

- ❑ **Characterization of cell substrate**
  - Evaluation of cell phenotype: tumorigenicity may be associated with oncogenic viruses or DNA oncogenicity
- ❑ **Qualification of cell banks, virus seed and biological raw materials**
  - Extensive testing of vaccine virus seed and cell substrate
  - Use of certified raw materials or test to demonstrate absence of detectable virus
- ❑ **In-process testing**
  - Developing a comprehensive testing plan to evaluate bulk/production lots for known and novel viruses
- ❑ **Process validation**
  - Designing an efficient process
    - to avoid risk of contamination
    - eliminate or reduce potential virus load
    - inactivate potential, contaminating virus
- ❑ **Reduction of residual host cell material in final product**
  - Whole-cell removal
  - Cellular DNA and protein reduction

FIGURE 3. Designing safety in viral vaccines.

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