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Vaccine Cell Substrates 2004

29th June – 1st July, Rockville, MD, USA

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Vaccine Cell Substrates 2004 was held 29th June – 1st July, 2004, in Rockville (MD, USA). This conference was cosponsored by the Division of AIDS (DAIDS), the National Institute of Allergy and Infectious Diseases (NIAID)/National Institutes of Health (NIH) [101] and the International Association for Biologicals (IABs) [102]. The authors cochaired this international conference for the following purposes: viral vaccine development is tied closely with cell substrate choice and the limitations that the currently acceptable cell substrates impose on vaccine development. Many scientific issues have hampered significant progress in widening the available choices of cell substrates for vaccine production, particularly continuous cell lines [1–5]. The purpose of *Vaccine Cell Substrates 2004* was to inform the field of existing data on progress in addressing the specific scientific issues identified below, to discuss the continued validity of existing tests and appropriateness of new ones, and to develop consensus recommendations for the field to address these issues, either by implementing suggested recommendations or identifying research gaps that preclude decision making.

The specific scientific issues covered included the following topic areas:

- Oncogenicity of cellular components, including latent viruses and cellular DNA
- *In vivo* viral adventitious agent test methods
- Level of assurance provided by current tests
- Bovine (and porcine) viruses in bovine-derived raw materials (particularly serum)
- Bovine spongiform encephalopathy (BSE) agents as potential cell substrate contaminants

In addition, a bonus session included presentations concerning testing performed to ensure the safety and quality of specific novel vaccine cell substrates used for investigational vaccine production (or in one case, a European licensed vaccine).

To meet the aims of the conference, a panel discussion was held at the end of each session on each topic area in order to address specific unresolved questions by forming consensus recommendations and identifying research gaps.

Conference highlights

Slides from this meeting may be found on the DAIDS website [101]. The proceedings will be published by the IABs and can be ordered via their website [102] after publication in 2005. However, in order to highlight some of the presentations at the conference, one talk from each session will be described. During the session on the oncogenicity of residual cellular DNA, Jose Lebron of Merck, presented the work they have performed to compare the uptake and biodistribution of DNA delivered parenterally versus that delivered orally. Mice were administered 100 µg of Vero cell DNA, either by intramuscular injection or by oral feeding. Necropsies were performed on days 1, 2, 3 and 7 post inoculation and tissues specimens were taken for polymerase chain reaction (PCR) analysis. A timecourse of DNA clearance was shown. The level of DNA taken up and biodistributed in mice inoculated orally, was 3 logs less than intramuscular inoculation by day 1, and was at least 4 logs less on days 2 and 3 post inoculation. By day 7, no DNA remained in animals inoculated orally, while animals inoculated by intramuscular injection still had 10^5 fg of Vero cell DNA detectable. They concluded that a limit of 100 µg of residual cellular DNA (from a cell line) in an oral dose of vaccine would be of comparable safety with the currently recommended limits of 10 ng for parenteral products.

In the session on oncogenicity of viruses which may be latent in cell lines, Ruth Jarrett from the University of Glasgow presented on new methods of detecting oncogenic viruses or virus families. She discussed the use of redundant PCR, representational difference analysis (RDA) and rolling circle amplification methods. Redundant PCR methods have been developed to detect most, if not all, members of the virus families of herpesviruses, polyomaviruses and retroviruses. These assays can be robust and sensitive (detecting <100–1000 copies per µg of cellular DNA). However, they require knowledge of the conserved sequences of a multitude of members of the virus family to be detected in order to design appropriate primers. RDA methods do not require a prior knowledge of the sequences of the virus but do require two specimens of cellular DNA, one that contains the viral genome and one of the identical cell line that does not. This powerful technique has been used to identify HHV-8, which was then a new member of the herpesvirus family, as an example of the utility of the method for screening for known or unknown viruses. However, other differences that have nothing to do with viruses might also be detected and the method is time consuming and technically demanding and thus is not sufficiently robust to be used as a general screening method at present. Rolling circle amplification may be useful for detecting viruses with double-stranded circular DNA genomes but would otherwise not be generally applicable.

In the session on adventitious agent test methods, Ray Nims of BioReliance presented on the relative rarity of detecting adventitious agents in biologics. He discussed the use of the routine cell culture *in vitro* adventitious agent test methods. Generally, human diploid cells and monkey kidney cells, as well as cells of the same species and tissue type as the production substrate, are used in these tests. Alternatively, the tests for bovine or porcine viruses require use of specific indicator cells promulgated in the 9 Code of Federal Regulations (CFR) Part 113 [6]. The sample size of the test article used includes 15% serum for testing serum lots, 5 g of trypsin centrifuged and reconstituted in a trypsin inhibitor for trypsin lots, three-times the concentration of other raw materials used in cell culture, 3 ml of viral harvest from bulk lots or viral seeds, or 10^7 cells/ml in conditioned medium from a cell bank. Readouts for these assays include observation for cytopathic effects and tests for hemagglutination/hemadsorption. If evidence suggests the presence of an adventitious agent, then further methods may be utilized to identify the contaminant, including electron microscopy, PCR or immunofluorescence. Finally, he shared their experience in testing biologic samples over the years. They have never identified a contaminant in a cell bank or in a vaccine or monoclonal antibody product. In gene therapy products, they have identified replication-competent adenovirus as a contaminant of a replication-incompetent adenoviral vectors. In addition, in recombinant protein products, they have identified Cache Valley Fever, reovirus, or minute virus of mice in Chinese hamster ovary (CHO) cell-derived products but no contaminants in non-CHO cell-derived products. When using well-characterized cell banks (as opposed to primary cell substrates) for the production of biologics, adventitious agent contamination appears to be quite rare.

In the session on level of assurance gained by current methods, Alan Moore, of Althea Technologies, presented on how technology has improved our level of assurance. In addition to the older adventitious agent test methods, newer methods, such as PCR, are also routinely used to screen for specific agents of concern in particular products or cell lines. In addition, newer manufacturing technologies continuously help to reduce or eliminate the risk of adventitious contamination. Use of banked and well-characterized cell substrates as starting materials, validation of processes for viral clearance and redundancy of testing strategies at various stages of production, synergize to reduce risk. Technologic improvements in manufacturing, such as clean-in-place/steam-in-place, automated closed systems, use of disposable (thus, single-use materials) and tightly controlled environmental monitoring programs also reduce risk. He presented a case study example of a cell-based biologic for which the shelf-life is less than 72 h. In such a case, the routine test for sterility, which is a 14-day test, is supplemented by the BacT/Alert System, a colorimetric technology for detecting carbon dioxide produced by the growth of a variety of bacterial or fungal contaminants, usually within 24 h.

During the bonus session, four manufacturers shared their experience in characterizing novel cell substrates for investigational vaccine production and in one case, a licensed European vaccine. The cell lines for which characterization was described included the following: Madin–Darby Canine Kidney, Adenovirus Type 5 transformed human embryonic kidney (293-ORF6), Adenovirus Type 5 transformed human embryonic retinoblasts (PerC.6) and insect cells (Hi-5). In the presentation by Brian Ledwith of Merck, testing beyond the usual characterization recommended in the International Conference on Harmonization Guidance Documents [7] or the Center for Biologics Evaluation and Research (CBER) Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals [8] was described [9]. In particular, in addition to the routine tumorigenicity test performed by inoculating 10^7 cells per animal (in immunosuppressed rodents such as nude mice), they performed a titration inoculating PerC.6 or HeLa cells at multiple doses and compared the relative tumorigenicity. While HeLa cells formed tumors at a dose of 10^5 cells per animal, PerC.6 cells only formed tumors in animals at the highest dose level of 10^7 cells. In addition, oncogenicity assessments were described. PerC.6 cellular DNA, lysed PerC.6 cells or adenoviral vector products were inoculated by the subcutaneous route into various animal species (TABLE 1) and animals were observed for several months for the formation of tumors. No animal developed tumors at the site of injection, however, one or more animals (depending on the total sample size) did develop spontaneous tumors at other distal sites. None of these tumors could be attributed to the test article. These data demonstrate that the PerC.6 cell bank used by Merck to produce an investigational vaccine is not grossly contaminated with an oncogenic virus released during cell lysis, nor is the residual cellular DNA from this weakly tumorigenic cell line overtly oncogenic. Furthermore, the product manufactured in this cell line is not grossly oncogenic in newborn rodents. Merck performed these additional characterization experiments to address regulatory concerns regarding the use of a weakly tumorigenic cell line to produce a preventive vaccine candidate. However, these data also demonstrate the limitations of reliance on such experiments, which are not validated tests and are without a positive control, since relevant controls would contaminate the animal facility in which the experiments were carried out. It is likely that the tumors observed in the conduct of these large and lengthy experiments all occurred by random chance, but without demonstration that these are the relevant animal models and without a positive control, it is difficult to draw conclusions regarding the utility of these data for the purpose. The authors commend Merck for sharing these data with the field, thereby highlighting the difficulty of addressing these theoretical regulatory safety concerns and why this remains a research gap.

In the session on bovine (and porcine) viruses, Steve Wessman, from the US Department of Agriculture (USDA), described the US regulations for testing of bovine serum for biologics production. He highlighted the viruses (and BSE agent) found in serum, including

Table 1. Oncogenicity experiments by Merck to characterize PerC.6 cells.

Sample	Species	Time (months)	Number	Specimen	Tumors (site of injection)	Tumors (other sites)
DNA	Newborn hamsters	5	40	VC	0	0
			45	VC	0	1[a]
			20	100 µg DNA	0	0
DNA	Nude mice	5	20	VC	0	0
			20	VC	0	0
			20	225 µg DNA	0	1[b]
DNA	Nude mice	9	100	VC	0	3[c]
			100	VC	0	7[d]
			100	250 µg DNA	0	3[e]
Lysate	Newborn hamsters	6	100	VC	0	0
			100	VC	0	0
			100	10^7 cells lysed	0	1[f]
Lysate	Newborn rats	6	102	VC	0	2[g]
			102	VC	0	1[b]
			102	10^7 cells lysed	0	2[g]
Product	Newborn rats	6	100	VC	0	1[b]
			100	VC	0	0
			100	10^{11} virus particles	0	0
Product	Newborn hamsters	6	100	VC	0	0
			100	VC	0	0
			100	10^{10} virus particles	0	1[h]

[a]Malignant ovarian teratoma; [b]Malignant lymphoma; [c]One malignant lymphoma, one benign lung adenoma, one benign skin papilloma; [d]Four malignant lymphomas, one benign lung adenoma, one benign skin papilloma, one malignant skin fibrosarcoma; [e]Two malignant lymphomas, one benign skin papilloma; [f]Mesenteric lymph node histiocytic sarcoma; [g]Malignant mammary adenocarcinomas; [h]Myeloid leukemia. VC: Vehicle control.

vesiculovirus, bovine herpesvirus 4 and the agent which causes BSE, as well as encephalitis viruses. He also described viruses known to be zoonotic in humans. Testing performed on lots of serum over the past two decades have resulted in the rejection of anywhere from 38 to 75% of serum lots because they were positive for viruses or viral antibodies, rendering the lots unacceptable for use to produce biologics. He recommended continued testing, improved sourcing, test method improvement, serum-free media research and utilization of serum viral inactivation methods as a means to remedy the risks that bovine serum may pose by introducing contaminants into the production of biologics.

In the session on BSE, Phil Minor from National Institute for Biological Standards and Control (NIBSC), highlighted the difficulties in assessing the risk of BSE or variant Creutzfeldt–Jakob disease (vCJD) agents in biologic products and raw materials. He presented data on the sequence of the human prion protein (PrP) gene in several human cell strains or lines, including commonly used vaccine cell substrates, MRC-5 (heterozygous at codon 129), WI-38 (homozygous for methionine) and a cell line proposed for investigational vaccine production (HEK-293, homozygous for methionine). Western blots of the PrP protein in these cell strains or lines demonstrated that they all expressed PrP. When exposed to extracts of brain homogenate from a vCJD patient, human fibroblasts took up the vCJD PrP, but upon cell passage, lost this protein – this provided a preliminary demonstration that human vaccine cell substrates may not be capable of propagating vCJD PrP even if exposed at some point in the passage history of the cell substrate. Further experiments of this nature on a broader array of vaccine cell substrates and transmissible spongiform encephalopathy (TSE) agents would be useful.

Conference outcomes

DNA oncogenicity

Information disseminated in this session was that the existing data concerning oncogenicity of residual cellular DNA is

negative – these data come from animal studies and human experience with products safely manufactured and used. In addition, at least five groups have performed risk estimates [10–15] and although the actual estimates vary by logs, all resulted in estimates that were exceedingly low. Consensus recommendations from the panel discussion from this session were that the theoretical risk from residual cellular DNA can be lowered by reducing the size and amount present in the final product. In addition, viral inactivation methods may also serve to inactivate the biologic activity of residual cellular DNA and that this inactivation could be validated, although there are no currently standardized methods to do so. For some vaccines, however, inactivation methods may not be introduced during production – particularly for live viral vaccines. Finally, the panel recommended that the World Health Organization (WHO) convene a working group to define the types of studies that need to be conducted in order to resolve the unanswered questions regarding continuous cell line DNA oncogenicity. Research gaps identified during the session included the following:

- The need for development of sensitive animal models
- The need to develop appropriate positive controls which will not contaminate an animal facility
- To address whether size, amount, route of administration, or form of the DNA (linear fragments, closed circular or chromatin) change the risk (which currently remains theoretical)
- Standardized methods to validate inactivation of the biologic activity of residual cellular DNA

Oncogenicity of viruses that may be latent in continuous cell lines

Information disseminated in this session included: new techniques for detecting specific oncogenic viruses or virus families that are available including PCR with degenerate primer sets, representational difference analysis and a PCR-based assay (referred to as PERT) to detect reverse transcriptase activity of retroviral elements. Panel recommendations on these new assays were that, with the exception of the PERT assay which is now routinely used, the newer technologies remain insufficiently robust to use in cell substrate characterization at this stage. The issue of whether infectivity in newborn rodents is useful was discussed and it was agreed that it is important to know what virus or virus family is being tested for, in order to optimize a test in the appropriate species. As with the reduction of theoretical risk from residual cellular DNA, it was agreed that reduction of the size of residual cellular DNA could also reduce the size to below that capable of inducing infection or oncogenicity and thus the risk of infectious viral genomes. Research gaps identified during the session included the following:

- A need for continued effort to develop robust assays for oncogenic viruses
- If infectivity or oncogenicity in newborn rodents is to be used as a test for oncogenic viruses (known or unknown), then there is a need to define which animal models are optimal and which positive control(s), that will not contaminate animal facilities, should be used
- A need to develop methods for long-term follow-up of vaccinees for safety events that may emerge years after vaccination (e.g., cancer or other latent diseases)

Adventitious agent test methods

Information disseminated included the following: the routine methods used for testing cell substrates, viral seeds and vaccine lots for adventitious agents are based on methods developed in the early days of cell culture-based vaccine manufacture and clinical viral diagnostics [7,8]. Originally, individual tests were implemented to detect specific agents of concern for specific products. Over the years, they have emerged as broad general screening assays. While each of these routine tests has its limitations, overall, they have served industry and regulatory agencies well to recognize contamination and release safe and pure products. It was recognized that the routine tests are intended to detect gross levels of contamination, not low-levels. However, the redundancy of testing at the levels of the master and working cell banks and viral seeds, raw materials, and lot-by-lot, as well as other quality control measures (facilities controls, personnel controls, environment controls, process control and product control, i.e., good manufacturing practices) overcome the limitations of reliance on any single test. It was reported that contamination is only found infrequently and more predominately with rodent substrates, such as CHO cells. Finally, it was accepted that newer detection methods are inadequately robust to replace the broad general screening tests routinely implemented for decades. Thus, the *in vivo* adventitious agent tests continue to add value to the overall testing schema and should not be eliminated at present. It was also acknowledged that it may be easier to introduce new adventitious agent test methods for newer products than to replace tests for currently licensed vaccines. Research gaps identified in this session included the need to:

- Establish methods for long-term follow-up of vaccinees for safety events
- Develop broad general screening assays that are equally or more sensitive and robust at detecting infectious viruses as the routine tests currently used
- Systematically determine the breadth and sensitivity of the existing tests in order to compare newer tests

Level of assurance

During this session it was disseminated that assurance of product safety is provided by a combination of factors including manufacturing consistency and redundancy of quality control tests. It was acknowledged that the sample sizes tested were not chosen so much on the basis of statistical sampling principles as on historic use. However, the track record for the samples tested in the routine tests has been proven by the release of products that have been safely used in millions of people over decades and by preventing contaminated products from being distributed. The panel recommended that the sample sizes currently used are adequate and should not

be changed. It was acknowledged that the level of assurance achieved is a result of the combination of redundancy of testing, manufacturing consistency, compliance with good manufacturing practices and adequate quality control. No research gaps were identified in this session.

Bovine (& porcine) viruses

Information disseminated included the following: it was acknowledged that the basis for the tests for bovine and porcine viruses is the requirement for veterinary biologics [6] without consideration of the human susceptibilities to these viruses but rather in consideration of preventing veterinary vaccines from becoming a source of veterinary epidemics. Considerable discussion centered on the evolving recognition of the need to include testing, beyond that described in the 9 CFR Part 113 tests, for infectious bovine polyomavirus in lots of bovine serum used as a raw material in biologics manufacturing (or in materials, including cell substrates, that may have been exposed to bovine serum). It was also reported that bovine serum of suitable quality for biologics manufacturing is in increasingly short supply. While converting to serum-free culture is feasible in many cases, with the increasing availability of well-defined animal material-free culture media for many cell lines, which may in fact already be implemented approximately 50% of the time, it may prove difficult to implement for already licensed products, as the validation of the lack of adverse impact of such a manufacturing change is not straightforward. Recommendations from the panel included the following: despite recent legislative changes in the EU, it was agreed that serum lots need not be free of antibodies to bovine viral diarrhea virus. Tests for bovine polyomavirus should not simply assess presence of nucleic acids (i.e., PCR tests) but actually test for infectious bovine polyomaviruses. Finally, alternative geographical sourcing for bovine serum or for use of specific donor herds should be considered to ensure continued supply of high-quality bovine serum for biologics manufacturing. However, there was acknowledgement that one must also consider the risk of introduction of diseases, such as that caused by the foot-and-mouth disease virus, from new geographical regions. The feasibility of sourcing from donor herds for fetal bovine serum was also questioned. Research gaps identified in this session included the following:

- The need for a systematic consideration to be applied to the agents listed in 9 CFR Part 113 with regard to their relevance when fetal bovine serum is used for production of biologics for human use, including considering which agents are present in fetal serum, which are zoonotic for humans and whether there are other bovine or porcine viruses that may be of equal or greater concern in humans
- The need to develop and implement robust infectivity assays for detection of bovine polyomavirus
- The need to survey the prevalence of infectious bovine polyomavirus in lots of bovine serum

Bovine spongiform encephalopathy

Information disseminated included the following: the current tests for detecting the agent that causes BSE remain inadequate as screening tests for cell substrate or viral seed characterization, raw materials control or biologic product control. Specific strains of murine-adapted scrapie can be propagated in certain murine cell lines, however, the generalizability of these data to other species' TSE and other species' cell lines has not been studied systematically. Risk assessment and management remain the best means for preventing contamination of biologic products with the agents that cause TSE. The panel recommended that such risk assessments may be periodically reassessed when data evolve that may impact on the underlying assumptions of those assessments. Research gaps identified in this session included the following:

- The need to develop and implement adequately robust, sensitive, rapid (i.e., cell culture-based) tests for detecting TSEs of various species (including bovine and human) in biologic products, raw materials, cell banks and viral seeds
- The need to determine whether TSE agents of other species (other than mice – particularly bovine and human) can propagate in the cell substrates used for vaccine production

Conclusions

Progress in the field of vaccine cell substrates was clearly demonstrated as newer continuous cell lines are being used for investigational vaccine production (and in the case of one European licensed vaccine). Regarding the level of assurance provided by the sample sizes tested in the routine tests currently used to characterize cell substrates, consensus was reached that while not based on principles of statistical sampling, there is a proven track record demonstrating that the combination of quality control measures, compliance with good manufacturing practices, consistency of manufacturing and redundancy of testing at various stages of production assures the safety of biologic products, including vaccines. However, several research gaps remain in the areas of oncogenicity of cell line DNA, oncogenicity of viruses that may be latent in cell lines, improved methods for adventitious agent testing (including bovine and porcine viruses) and assuring the freedom of biologic raw materials and cell lines from BSE and other species' TSE agents (including human). This conference served to disseminate up-to-date information relevant to these questions, to develop consensus recommendations and identify research gaps that preclude decision making.

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