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Meeting Report

WHO Study Group on Cell Substrates for Production of Biologicals

WHO Headquarters, Geneva,

11 - 12 June 2007*

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Contents

Abstract

For many years, the World Health Organization (WHO) has provided global leadership in defining technical specifications for quality assurance and safety of biological medicines produced in cell substrates. Current WHO requirements for the use of animal cells as substrates for production of vaccines and other biologicals were adopted by the WHO Expert Committee on Biological Standardization in October 1996 (WHO TRS 878). Since then, significant progress especially in the development of vaccines in novel continuous cell lines of mammalian origin as well as in insect cells has been made and consequently there is an increasing need for the re-evaluation of existing criteria for the acceptability of such cell lines. In addition there is also a need to consider new issues in cell substrate safety arising from these new cell types and developments in technology and scientific knowledge. In response to these demands, the WHO Study Group on Cell Substrates was formed in 2006 to initiate revision of WHO requirements and to address need for further research in this area. At its second meeting on 11-12 June 2007, the Study Group reviewed scientific data that would form the basis for new recommendations and made a number of proposals for further investigations. The Study Group is working on the preparation of a revised WHO document, and a broad consultation with regulators, manufacturers, and other relevant parties is planned for 2008.

Keywords: WHO Study Group, cell substrates, continuous cell lines, vaccines, recommendations

1. Introduction

Most biologicals are produced in living cells, and even though there may be purification steps involved in the manufacturing process, some residual cellular constituents are present in the final products. A number of continuous cell lines (CCLs), including insect cells (e.g., MDCK, 293, PER.C6, HeLa, *Trichoplusia ni* Hi-5), are now

being used for the development of new biologicals, and WHO was requested to take the lead in a global consultation process to set standards for the safety assessment of new cell substrates. Development of new criteria for the acceptability of such cell lines, and the improvement of those that already exist, has been identified as a priority by several WHO expert committees (Expert Committee on Biological Standardization, October 2005; Strategic Advisory Group of Experts, November 2005; and Global Advisory Committee on Vaccine Safety, June 2005).

Dr. David Wood, Coordinator, Quality, Safety and Standards Team of the Immunization, Vaccines and Biologicals Department of the World Health Organization, opened the second meeting of the WHO Study Group and welcomed all participants to WHO. He stressed the importance of the work that the Study Group has undertaken over the past year in facilitating further developments in the requirements for safe and effective biological products. Historically, WHO has played a key role in developing global norms and standards for cell substrates through its ongoing biological standardization programme. The Study Group was established in 2006 to review scientific advances in this area and the potential availability of several new cell substrates for production of biologicals. Despite the availability of a broad range of cell substrates that could be used for this purpose, the number of cell substrates widely accepted by regulatory authorities is still limited (e.g., chick embryo fibroblasts, MRC-5, WI-38, Vero, CHO). In this context, provision of scientifically sound, evidence-based, impartial guidance on the risks and benefits using novel cell substrates would facilitate development of new vaccines manufactured in cell lines. Furthermore, where risks exist, either real or perceived, a consensus on specifications and risk-reduction strategies to assure the safety of the products is of critical importance. This is especially relevant in the case of cell substrates for vaccine manufacture, where many of these products are intended for use in healthy children.

One of the goals of this Study Group is to contribute to a new version of the WHO Requirements for cell substrates by undertaking a revision of the current document [1] as well as by addressing new issues to be considered by regulators and manufacturers. This in turn will strongly influence the acceptance or non-acceptance of new cell substrates for production of biologicals, since WHO Requirements serve as a primary reference in many WHO Member States.

The Study Group appointed Dr. John Petricciani as Chairman and Dr. Glyn Stacey as Rapporteur.

2. *Remit of the Study Group and progress made since May 2006,* *Dr. Ivana Knezevic*

The first meeting of the Study Group, held in May 2006 in Geneva, was focused on the scope of the work that the Group is undertaking. The remit for this group of leading experts in the area of cell substrates was to review the latest knowledge and to coordinate studies to define risks imposed by residual cellular DNA in the context of vaccine production. Such studies are recognized as regulatory research intended to provide a scientific basis for new recommendations. In addition, the Study Group should assist National Regulatory Authorities (NRAs) in establishing standards for the evaluation of biologicals produced using cell substrates. This activity includes revision of the WHO Requirements [1] as well as proposals for the development of measurement standards for the purpose of cell-substrate evaluation. The discussion in 2006 was focused on CCLs, new cell substrates for vaccine development, and the WHO Vero reference cell bank 10-87. The Study Group agreed to provide scientific advice on the evaluation of cell banks as part of its remit. It was also recognized that there were a number of issues in terms of legal status of WHO banks, including open access to all countries and practical arrangements in undertaking appropriate monitoring of their use. Thus, there was a need for a separate forum on these issues and development of a long-term strategy for WHO cell banks. A detailed report of the meeting held in 2006 is available on request to WHO.

Following discussion of the Study Group in 2006, some additional studies on tumorigenicity of CCLs as well as on the oncogenicity of DNA and infectivity of viral DNA were completed. In addition, detailed analysis of the methods used for determination of residual cell DNA in biological products was undertaken by the group members. As a result, new guidance on tumorigenicity testing as well as a review of current technical approaches for testing residual cell DNA are being prepared for further consideration.

Key issues discussed by the Study Group in 2006 were presented at several meetings with international participation, and an interest for new specifications expressed by regulators, manufacturers and academia was noted. Moreover, other consultative groups at WHO have also addressed a number of questions with regard to quality assurance of vaccines produced by novel technologies. The recent example of the human papillomavirus (HPV) vaccine developed in insect cells (*Trichoplusia ni* Hi-5 Rix 4446) illustrates this knowledge gap. It is clear that the absence of specific recommendations for the assessment of novel vaccines, in particular their safety, could cause unnecessary delay in vaccine availability and therefore a systematic approach in cell substrate investigations is needed. A number of issues need to be addressed in the evaluation of an insect cell bank as a substrate for vaccine production. These include: appropriate growth temperature; a need to introduce new or modify existing quality control procedures; extensive testing for potential adventitious agents; the interpretation of PERT assay results; the relevance of conventional tumorigenicity testing and risk assessment. These specific issues were considered by the Study Group at its meeting in May 2006. Meanwhile, WHO guidelines to assure the quality, safety and efficacy of recombinant HPV-like particle vaccines were adopted by the Expert Committee on Biological Standardization in 2006 [2].

The second meeting of the Study Group was set up as a continuation of the

scientific review initiated in 2006, with additional discussion on adventitious agents. The following issues were addressed:

1. The need for establishing new reference preparations and reagents (e.g., for the testing of residual cellular DNA and adventitious agents, cell banks of positive-control cells for tumorigenicity testing, etc.),
2. Validity criteria for methods proposed in the revised document and
3. Provision of testing protocols as an appendix of the document.

It was also emphasized that all WHO recommendations for currently licensed vaccines produced in cell substrates refer to the generic document on cell substrates [1]. It was recognized by the group that it could be counterproductive to attempt to apply new recommendations to existing products. Therefore, the implications of the proposed changes on existing vaccines should be carefully considered throughout the revision process.

3. Adventitious agents

3.1. Adventitious agents: Testing Procedures in the context of revision of WHO requirements, Dr. Rebecca Sheets

Given that this extensive topic will be the focus of a future meeting of the Study Group, this session served as an introduction to the issues to be considered in the revision of WHO requirements. In addition to the battery of tests for adventitious agents that already exists and is described in the present document, there is recognition that new viruses and other adventitious agents can emerge, and such agents could contaminate cell substrates, possibly through raw materials. Furthermore, new tests have been developed, or are in current development, since the date of the prior document. Also, in keeping with

the principles to reduce, refine, and replace the use of animals in testing, there is a desire to develop new in vitro tests that can fulfill these principles.

As discussed at the 2006 meeting of the Study Group, an international conference was held in 2004 [3] that produced consensus recommendations and identified research gaps on a number of vaccine cell substrate-related scientific issues, among which were sessions relevant to the current topic – specifically on adventitious agent test methods, bovine (and porcine) viruses, and transmissible spongiform encephalopathies. Funding mechanisms to address some of the identified research gaps have been identified and implemented by the U.S. National Institutes of Health; however, if the Study Group were to identify further research gaps, new sources of funding would be needed. These funds could be from existing health sciences' funding organizations or by other collaborative mechanisms to implement the research; the WHO might serve to facilitate such funding and collaborations.

The current funding mechanisms are, or will be, addressing the following research gaps:

- the development of certain new adventitious agent test methods;
- the development of a sensitive animal model(s) and appropriate positive controls to assess oncogenicity of cellular DNA to permit quantitative risk assessment of residual cellular DNA from continuous cell lines;
- an assessment of whether vaccine cell substrates can propagate transmissible spongiform encephalopathies whether exogenously introduced *via* animal-derived raw materials (specifically bovine in nature, such as bovine serum) or inherent to the cell substrate (particularly if human in origin);
- a determination of the breadth and sensitivity (approximate limit of detection) of the existing *in vivo* and tissue culture adventitious agent test methods;
- a determination of the continued appropriateness of applying existing veterinary standards for the use of animal-derived raw materials (particularly

bovine and porcine) in the production of human biologicals; and

- the development of a test method appropriate for use in validating clearance of biological activity of cellular DNA in manufacturing processes.

When the Study Group undertakes the process of updating the existing document with respect to adventitious agent testing, they will need to keep in mind the following:

1. New data on the emergence of new agents and of new tests and also the appropriateness of replacing or updating existing tests; 2. International harmonization of test methods; 3. New cell substrates for which the existing tests may not be adequate (e.g., insect cells that grow at lower temperatures than most currently used cell substrates and are subject to a different panoply of agents); and 4. Whether additional data are required to guide their decision-making.

3.2. New Concerns and New Methods of Evaluating Cell Substrates for Adventitious Agents, Dr. David Onions

The development of cell substrates from animals such as the dog, Syrian hamster, and insects, and the use of tumorigenic cells, has raised new concerns of adventitious agent contamination. These contaminants could be novel, even undiscovered, viruses, or other types of novel agents in the species concerned as well as latent oncogenic viruses. For instance, the current European and US guidelines for testing canine cells [4-7] are largely based on veterinary concerns and do not take account of recently discovered viruses. Moreover, viruses that can have a low pathogenicity in their host species can be oncogenic when they infect a heterologous host.

Consequently, in testing MDCK cells, account should be taken of recently discovered viruses in the dog (e.g., canine circovirus) and viruses now thought to be capable of infecting dogs (e.g., hepatitis E virus and bornavirus). Insect cells harbour

many silent virus infections including those families of viruses normally considered to cause only lytic infections in mammalian cells. These viruses may be revealed after stress or superinfection with other viruses.

Fortunately, new testing methodologies are becoming available, and regulations or recommendations for testing should take these into account. For instance, the new guideline from the European Pharmacopoeia [8] now permits the use of PCR methods to detect mycoplasma under very specific validation criteria. PCR detection alone can have some disadvantages, but a hybrid culture and PCR-detection system have been shown to shorten the Points to Consider Mycoplasma test to 14 days without compromising the sensitivity of the conventional 28-day assay.

Safety-testing strategies are based on both broad and specific tests, but there is always concern that viruses, particularly latent or oncogenic viruses, may have been missed. In developing tests for MDCK cells, new degenerate PCR systems with the capability to detect all known herpesviruses or polyomaviruses have been introduced [9]. These systems have been designed to reveal new members of these potentially oncogenic virus families and therefore play an important role in evaluating any new cell substrate. This principle can be extended to develop either highly degenerate PCR for all virus families, with mass spectrometry or array-based detection of the amplicons. Alternatively and less sensitively, random RT-PCR/PCR priming can be employed with the array-based detection employing oligonucleotides derived from highly conserved regions from each virus family. These new approaches could provide additional assurance of the safety of the new generation of vaccines and biological products.

3.3. Way forward - discussion

The Study Group recognized that new cell substrates could themselves harbour currently undiscovered, novel infectious agents as inherent contaminants/elements. It was

agreed that investigation of adventitious agents should be streamlined towards new generic tests that could detect a broader range of viruses within specific virus families. This should be primarily validated for new products under development. In line with this, the Study Group agreed that a thorough analysis of current approaches in testing adventitious agents should be undertaken as part of the revision of the WHO Recommendations for cell substrates. This should result in a position paper summarizing current approaches in testing adventitious agents with some proposals for improvements. The availability of a range of new detection systems offers great promise for delivery of enhanced test systems that would enable broader and more sensitive detection of adventitious agents, and could replace some of the established tests. However, these new assays will require careful consideration and validation to provide appropriate testing platforms for the future.

The issue of prions was also considered, and a need for guiding principles in reducing risk of TSE was identified. Generic WHO guidance on this topic [10], as well as relevant documents issued by other bodies, should be considered in the revision of the document. The group emphasized the importance of formulating clear recommendations on this issue in the context of the use of cell substrates in addition to addressing generic issues, such as TSEs, and providing reference to appropriate documents and guidance.

4. Tumorigenicity of continuous cell lines

4.1. The Impact of Recent Paradigm Shifts in Cell Substrate Selection on Cell Substrate Tumorigenicity Testing, Dr. Andrew M. Lewis Jr.

Selection of continuous (immortalized) cell lines that frequently possess the capacity to form tumors *in vivo* in animal tests is changing the types of data needed from *in vivo* tumorigenicity assays for regulatory decision-making purposes. Since the proscription against the use of tumorigenic cell lines in 1954, tumorigenicity assays were

undertaken to document the absence of the expression of a tumorigenic phenotype by diploid cell lines and the single continuous cell line (VERO) that, until recently, have been the only cells, other than primary cultures, employed in vaccine manufacture. There has been an increasing demand to use immortalized cells that have the capacity to express a tumorigenic phenotype for vaccine production. This development has meant that the tumorigenicity-testing paradigm shifts from the need to document the absence of a tumorigenic phenotype to the need to characterize the tumorigenic phenotype that is expressed. This paradigm shift is also driven by the basic requirement to carefully characterize all of the components used in the vaccine manufacturing process. Furthermore, the current perception that the level of potential risk posed by immortalized cells that express highly tumorigenic phenotypes (i.e., require $\leq 10^5$ cells to form tumors; examples include HeLa and BHK-21 cells) is greater than the level of risk posed by cells that express non-tumorigenic or weakly tumorigenic phenotypes (i.e., require large numbers of cells – 10^6 to 10^7 – to form tumors; one example is 293 cells). Approaching *in vivo* tumorigenicity assays as a relationship between the dose of cells administered and the tumor-forming response of the inoculated animal host provides quantitative data that can be used to characterize the phenotype of the cell line being studied and develop testing algorithms for cell-substrate DNA and cell lysates that are appropriate for the level of possible risk that is perceived. These data include the tumor-forming capacity expressed as the dose that produces tumors in 50% of animals (tumor-producing dose at the 50% endpoint, i.e., TPD_{50}) and the time required for tumor development (tumor latency), each of which could be determined with reasonable accuracy in a variety of animal models. In addition to providing quantitative data that can be subjected to statistical analyses, data from dose-response assays can be evaluated for variables that might indicate the presence of a yet-to-be detected adventitious agent in the cell substrate. Furthermore, careful assessment of the histopathology of tumors that develop at both the site of inoculation and at a site distant to the inoculation site can be used to determine whether components of tumorigenic cell lines possess oncogenic activity. There are a variety of *in vivo* animal models that can be used for tumorigenicity assays that enable the

evaluation of dose-response data. However, at this time, the athymic nude mouse, due to its ability to accept allografts and xenografts, the extent of the experience with its use evaluating the tumorigenicity of cells from different species, and its wide availability, appears to be the model of choice for tumorigenicity testing.

4.2. Tumorigenicity of CCLs – new *in vivo* and *in vitro* data, Dr. Yeowon Sohn

The range of cell substrates used in currently licensed biologicals is limited and the emergence of new vaccines and therapeutics necessitates the development of new cell substrates. Selection of a particular cell substrate influences the safety and required purity of the biological product manufactured in it. Continuous cell lines usually have biochemical, biological and genetic characteristics that differ from primary or diploid cells. Production of biologicals from continuous cell lines should be based on well-characterized master and working cell banks. Evidence that the cell line is free from cultivable bacteria, mycoplasma, fungi and infectious viruses, and, where appropriate, potentially oncogenic contaminants, should be provided. Continuous cell lines should be characterized so that appropriate controls for the purity and safety of the final products can be included.

To check the tumorigenicity potential of cell substrates or the oncogenic potential of residual DNA, tumorigenicity and oncogenicity assays were performed on several continuous cell lines. Vero and NIH 3T3 did not show any tumorigenic potential in BALB/c nude mice. CHO and 293T were highly tumorigenic in *in vivo* tumorigenicity assays using BALB/c nude mice, while HeLa cells showed only "moderate" tumorigenicity in this assay. NIH 3T3 cells transfected with expression plasmids for c-Myc and H-ras showed tumorigenic potential in the *in vivo* tumorigenicity model. Using colony formation in soft agar gels, an assay that has been considered as an *in vitro* surrogate for tumorigenicity in immunocompromised animals, several CCLs, such as

T98G, NCI-H128, SiHa, and 293T, showed different results in two laboratories. It is clear that validation criteria for this assay are needed.

DNA microarray technology was used for the development of biomarkers for immortalization and transformation. NIH 3T3 cells transfected with expression plasmids for c-myc and H-ras^{G12V} were used as model system. Thirteen genes, including Thbs1, Dusp1, Gadd45a, and Mina, were selected as biomarkers associated with immortalized and transformed cells, and these genes could provide potential markers to predict the transformed and possibly tumorigenic characteristics of cell substrates [11].

4.3. Tumorigenicity of CCLs – Discussion

***In vivo* testing**

Although the biology of the expression of a tumorigenic phenotype by some CCLs in animal species is not yet fully understood, the Study Group concluded that tumorigenicity should be considered as just one of the biological characteristics of a cell line. It was emphasized that tumorigenicity of CCLs is not a direct indicator of the risk of biologicals produced in such cells. The message to be conveyed is that a tumorigenic CCL should not be precluded from use in the production of biologicals. However, the manufacturing process and the testing of products derived from CCLs should demonstrate that the risks that might have been associated with the CCL have been reduced to levels below the level of detection by currently available technology. Other characteristics of tumorigenic cell lines should be assessed in order to evaluate those risks. In particular, further investigations of oncogenicity and infectivity of components (lysates and DNA) derived from those cells (e.g., to assess the risk of transmission of oncogenes or infectious genomes) should be undertaken, and the results should be carefully considered in the decision on the appropriateness of a cell line for a given product.

Quantification of tumorigenicity is of interest, but the interpretation of such data

is still a subject of discussion. Parameters for defining the tumorigenicity profile of a CCL, as a biological characteristic, were considered, and several endpoints were proposed. These include the TPD_{50} and the average tumor latency. Such data might provide valuable information on the master cell bank, and they could serve as a basis for monitoring the consistency of cell banks. Data indicate that the tumorigenic profile can change between related cell lines, possibly due to genetic or epigenetic changes, potential presence of viral contaminants, adaptation of cells to growth in serum-free medium or other growth conditions, and other factors. The Study Group agreed that when a CCL is observed to change from a "non-tumorigenic" to "tumorigenic" phenotype, this should always be considered as a signal that important changes might have occurred, and the potential implications of this change in phenotype will require further study and discussion with the appropriate NRA.

The Study Group also discussed testing methodologies, advantages and disadvantages of different animal models, use of control cells and the length of test observation period. A number of proposals for further improvements of a revised section in the new WHO Recommendations on tumorigenicity testing were made. It was agreed that inclusion of a negative-control cell, such as MRC-5, does not provide added value and therefore should not be recommended in the revised document. With respect to positive controls discussed by the Study Group in May 2006, establishment of qualified WHO bank of HeLa cells would make a useful contribution to the standardization of tumorigenicity testing. Regarding the observation period, this will depend on the animal model used and the endpoints to be measured. Nevertheless, the Study Group considered three months to be a reasonable minimum observation period. In addition, it was agreed that any plan for assessing tumorigenicity should be discussed with and approved by the respective NRA before initiating such testing.

***In vitro* testing**

Data presented by Dr Sohn led to a discussion of validity criteria and the need for further guidance. However, the relevance of the test performed in soft agar as a surrogate for tumorigenicity was considered questionable, and the group proposed discontinuation of this test as a requirement. While this test is a measure of biological characteristics of the cell line, such as loss of anchorage-dependent growth, it is not a reliable measure of *in vivo* tumorigenicity, i.e., the ability of a cell line to grow and form tumors in a xenogeneic animal. It is therefore considered as an *in vitro* transformation assay rather than tumorigenicity test. For future investigations, a qualified bank of NIH 3T3 cells would be a valuable source of cells to assist international standardization of the *in vitro* trans-formation assay.

5. Oncogenicity and infectivity of cell DNA

5.1. DNA Oncogenicity – review of new data, Dr. Keith Peden

Cell substrates derived from tumors, or cells that are tumorigenic in immunocompromised rodents have not been considered suitable for vaccine manufacture in the past, as the components of such cells could be oncogenic and induce cancer in vaccine recipients. To date, this concern remains theoretical. Although the DNA from such cells has been considered one risk factor, all the data that currently exist to address this issue have been negative [3,12]. However, data from studies with negative outcomes are less persuasive than data from studies with positive outcomes, and so an effort to generate a system to develop positive data has been undertaken. As a first step in the development of *in vivo* assays to measure and quantify the oncogenic activity of DNA, we have generated expression plasmids for the dominant cellular oncogenes T24-H-*ras* and c-*myc* and evaluated their activity in a number of mouse strains, both immune competent and immune deficient. Our studies have demonstrated that these oncogene-expression plasmids are active *in vivo* in both immune-competent and immune-deficient mice, with newborn animals being more sensitive to oncogenic insult than adults. Placing both oncogenes on the same plasmid molecule increased the assay sensitivity, as expected

if neoplastic conversion required a single cell to take up both plasmids. A strain of mouse deficient in both T cells and NK cells has been identified as the most sensitive indicator strain to date; the data suggest that levels of the dual oncogene expression plasmid DNA as low as 25 ng can be oncogenic. Current work is directed towards determining the sensitivity and variability of the assay with this strain in order to establish a standardized assay. With such an assay, it should be possible to quantify the oncogenic risk of cellular DNA containing activated dominant oncogenes.

5.2. DNA Infectivity – detection of biological activity, Dr. Keith Peden

The potential risk of cell-substrate DNA could arise through either its infectivity or its oncogenic activities. Of the two, infectivity appears to be a more sensitive indicator than oncogenicity both *in vitro*, where the difference is about 100 fold [13], and *in vivo*, where polyoma virus DNA was shown to be about 1000-fold more infectious in mice than it was oncogenic in hamsters [13]. To assess the effectiveness of various procedures to eliminate the biological activity of DNA, we have used an *in vitro* infectivity assay where a cloned proviral copy of a retrovirus genome is transfected into monolayer cultures, and infectious virus produced from the transfected cells is amplified by co-culture with permissive cells. This system can detect the infectivity of 1 pg of the cloned DNA, an amount of DNA that corresponds to about 100 000 molecules. To evaluate methods that eliminate the biological activity of DNA, we reasoned that the use of the more sensitive *in vitro* system would be preferable to an *in vivo* DNA oncogenicity assay, and that methods that eliminated DNA infectivity would also eliminate DNA oncogenicity. We have evaluated two methods commonly used in vaccine manufacture for their ability to reduce the biological activity of DNA. Benzonase is frequently used during the production of vaccines, and beta-propiolactone (BPL) is commonly used to inactivate virus vaccines. Under the appropriate conditions, both methods were capable of reducing the biological activity of DNA and, when an amount of residual cellular DNA per vaccine dose of 10 ng is factored in, obtaining clearance values (or safety factors) of 10 million.

5.3. Approaches in reducing risk of cell DNA - Discussion

The Study Group agreed that the data generated on oncogenicity and infectivity of cell DNA were important in defining potential risk for vaccinees. Quantifying the risk for a specific CCL in the context of a specific product was considered essential for selection of appropriate strategies for risk reduction. It was also considered highly likely that reduction of DNA fragment size reduces the risk from DNA and increases the safety margin, as the smaller the DNA fragments are the lower would be the probability that intact oncogenes and other functional sequences would be present. Studies performed at CBER suggest that DNA fragments smaller than 200 bp will give substantial safety margins for products that meet the 10 ng per dose limit. This raised some questions concerning the feasibility of detecting small DNA fragments and of quantifying the proportion of the digested DNA of different sizes. Several methods of reducing or eliminating DNA activity (i.e., infectivity and oncogenicity) at different stages of manufacturing were considered, such as by beta-propiolactone and benzonase treatment; these reagents had been shown to reduce DNA activity in the *in vitro* transfection/co-culture system.

In order to determine what safety margins with respect to DNA activity can be achieved by DNA inactivation with BPL or digestion with benzonase, Dr. Peden has made calculations based on his data. This approach was discussed and considered a critical step in developing scientific evidence for new recommendations. Peer review and publication of these data was seen as an absolute priority.

With respect to the final biological products, and vaccines in particular, the definition of potential risk that cell DNA might impose is a critical issue in applying risk-reduction strategies that would lead to the consistent production of safe vaccines. The Study Group concluded that the theoretical risk of CCL DNA applies to both non-

tumorigenic and tumorigenic cell lines, and that the focus of concern should be on the biological activity of cell DNA in terms of its potential oncogenicity and infectivity. This feature of cell substrates should be addressed in calculating the risk that a CCL may pose in the context of a specific product.

6. Residual cell DNA

6.1. Determination of residual cell DNA, Dr. Laurent Mallet

Historically, the specification for DNA content was initially established at 100 pg/ dose or less for parenteral products [14]. In 1998, this specification was modified to 10 ng/ dose or less for parenteral products, based on further evaluations performed in the intervening time. Neither limit applies to products derived from microbial, diploid or primary-cell-culture systems nor for products prepared on continuous cell lines but given orally [1].

Various issues that have an impact on the measurement of residual cell DNA were reviewed, including DNase treatment, reduction of DNA fragment size, and inactivation by beta-propiolactone (BPL) and other agents. Several analytical methods available for DNA quantification were also reviewed, and it was proposed that only a few of them are appropriate for residual DNA quantification in the context of biological products. This is due to the requirement to detect residual amounts in the biological products. Moreover, attempts made to detect DNA fragments of certain sizes revealed a need for careful consideration of appropriate assays for this purpose. Analytical methods based on hybridization, an immuno-enzymatic method (Threshold®), and quantitative PCR were considered to provide suitable performance, in particular by providing appropriate levels of sensitivity, for assessing residual DNA content. However, selection of an analytical method should also be based on the characteristics of the vaccine or biological in question. Data presented indicate that the Threshold® method might be more appropriate

for vaccines that are not treated with DNase, since this is a standardized method using qualified kits with a positive control. It is likely that qPCR is more appropriate for vaccines treated with DNase, because of the sensitivity and linear range of the assay to detect short fragments. Additionally, DNA sizing evaluation is a very attractive feature of this technology and could provide valuable information for the assessment of risk. Finally, hybridization, which had been considered as the method of choice by manufacturers, has been shown through a WHO collaborative study [15] to be highly variable between laboratories. Lack of standardization as well as poor performance with small fragments pose serious disadvantages for this method compared with the techniques described above.

With respect to the specification for residual cellular (rc) DNA in the final products, it has been considered that a 10 ng/dose was appropriate for biologicals produced in continuous cell lines. This specification is not associated with an analytical method, and thus additional points should be taken into consideration. Our experience showed that a qPCR method might result in higher levels of DNA than the Threshold® method. Moreover data suggest that BPL, a viral inactivation agent, might also reduce or destroy the biological activity of DNA [16, 17]. It has been shown recently that BPL will have an impact on DNA infectivity in an *in vitro* model [18, Section 6.2]. Fragment size reduction of DNA should also reduce the potential risk associated to residual DNA. A risk assessment should be done in order to define the DNA upper limit for a particular vaccine or biological product, based on the following parameters: nature of the cell substrate, inactivation process, the method used to assess DNA content, and the size distribution of DNA fragments.

6.2. Residual cell DNA in the context of cell substrate safety - discussion

Discussion on residual DNA detection was focused on the sensitivity of methods,

detection of small DNA fragments, the practical application of qPCR, and potential approaches for the development of a reference preparation for rcDNA. With respect to the sensitivity of different methods, it seems that this should not be an obstacle in the future. It was proposed that a potential approach for increasing sensitivity for the detection of cell DNA in the sample is the use of methods that can amplify conserved multicopy genes such as the rRNA genes. Discussion on the determination of small DNA fragments led to the conclusion that quantitative amplification methods can detect DNA fragments with sufficient sensitivity. In addition, it was concluded that in order to determine the percentage of fragments within a certain size range, both fragments smaller than 200 bp and those larger than this value should be measured and compared, adjusting results for length of the amplicon (and thus amplification efficiency). It was recognized that qPCR is a highly sensitive and reproducible technique. However, there was concern on how residual cell DNA could be determined where such a methodology is not available to certain manufacturers, particularly in developing countries. Consideration was given to the need for a reference material, and the Study Group concluded that such material would improve inter-laboratory comparisons. It is also seen as a step towards standardization of the methodologies. If the reference is desired to be cell line-specific, Vero cell DNA was considered to be a priority. However, if a conserved gene, such as the rRNA genes, proved to be suitable for this purpose, then cell line-specific reagents may not be required, and a single reference might serve for all cell substrates.

Discussion on the development of guidance in the form of a decision tree for the safety assessment of cell substrates led to the analysis of several parameters that might be considered for this purpose.

It was agreed that tumorigenicity is one of the characteristics of a cell substrate and would not preclude use of these cells for production of biologicals. However, a broad range of characteristics should be assessed in order to evaluate potential risk associated with the specific CCL in question and its intended use (discussed below). In particular,

further investigations such as detection of oncogenic viruses should be undertaken and carefully considered in making a decision on the appropriateness of a cell line for the product in question.

Another important aspect in evaluating potential risk for product recipients is a consideration of risk reduction approaches for the production process for each individual biological. In line with this, safety evaluation of a final biological product should be based on a thorough analysis of the biological characteristics of the cell substrate as well as those relating to the production process, which include but are not limited to: the impact of inactivating agents, reduction of total amount of cell DNA through purification process, and reduction of DNA fragment size. Furthermore, the clinical use of the product should be considered, as the risk/benefit evaluation will differ depending on the target recipient population and other factors such as dose size, frequency, and route of administration. The Study Group emphasized that best manufacturing practices play an important role in reducing risk of cell DNA and this aspect should be described in the revised document.

The level of risk associated with the use of different products derived using the same cells may differ; for example in the case of Vero-based inactivated rabies vaccine for parenteral use vs. Vero-based live attenuated polio vaccine for oral use. Moreover, within the same type of product (e.g., inactivated rabies vaccine), different manufacturing processes may lead to final products with different residual levels of risk. The group agreed that an enhanced safety evaluation should be recommended, and guiding principles for assessment of risk for a particular product should be addressed in the revision of the document.

7. New cell substrates

7.1. Insect cells, Dr. Peter Christian

There is a relatively small number but broad range of insect cells that could be used for production of biologicals in future. Many of these cell lines are derived from ovarian tissue and growth is generally optimal at 25-27ºC. Most insect cells go into heat-shock at temperatures above 30ºC. Good serum-free media are available for many lines and many lines are not substrate-dependent and will grow readily in suspension (optimal for production). With respect to characterization of cell seed, MCB, WCB, and cells at or beyond production PDL, the same general principles should apply as for other cells. For identity, similar methods to those used for mammalian cells should apply to insect cells, taking into account that chromosomal analysis is not easy with insect cells. Conventional methods used for sterility testing for detection of bacteria and fungi in mammalian cells could be used for insect cells with some alteration/additions to the incubation conditions. Detection of mycoplasma/spiroplasma may require different growth conditions from methods used for mammalian cells - although at least one, Spiroplasma, can be cultivated at 30ºC. Positive controls for these tests (particularly for Spiroplasmas) are an issue that needs to be resolved. Tumorigenicity as described for mammalian cells is difficult to perform and may be meaningless, since most insect cells die at 37ºC. *In vitro* tests for tumorigenicity may also not be appropriate.

With respect to adventitious agents, many insect viruses are poorly characterized and the availability and utility of general primers for some virus families is questionable. Additional complexity is introduced by absence of reference viruses for any insect virus, including baculovirus. In the context of infectivity assays, some persistent viruses will replicate in non-homologous cell lines and yet fail to induce CPE in non-homologous cells, but the presence of persistent viruses in the testing lines may well complicate the interpretation of data. Many insect cell lines have endogenous transposons and retrovirus-like particles. Some insect cell lines are positive in PERT assays but it is not easy to

interpret these data, as the activity/infectivity of such elements and viruses has not been studied in mammalian systems.

In conclusion, many of the quality issues relating to insect cells are similar to those for mammalian cells - albeit that in some cases changes may need to be made in specific testing methodologies. However, the insect viruses that may be present in any given cell line are generally unknown and/or uncharacterised. In addition, viruses tend to be ubiquitous in many insect cell lines. Stringent characterization of the working and master cell banks for an insect cell line is crucial and should involve a range of techniques and approaches. It is also essential to demonstrate that the manufacturing process is capable of eliminating and/or inactivating potentially present infectious agents. Strong regulatory input is needed on a case-by-case basis, but a lack of expertise within the scientific and regulatory communities is a fact that requires further consideration.

7.2 Other new cell lines - discussion

The Study Group considered the breadth of the groups remit and whether it should include other eukaryotic organisms such as plants, yeast, and algae. It was concluded that the scope should be limited to animal cells alone, and the Study Group would not at this stage deal with these related yet distinct areas. However, the revised cell-substrate recommendations should address novel animal substrates, in particular cells of avian, canine, and insect origin. Issues related to specialized cell types, such as stem cell lines, should also be considered in the context of new technologies, and other relevant WHO guidance should be taken into consideration.

8. Conclusions and next steps

Development of scientific evidence, knowledge management, and translation of scientific data into recommendations were recognized as some of the challenging tasks that the Study Group is facing. Publication of recent data in peer reviewed journals is

seen as a priority, and WHO was requested to facilitate this process.

The importance of defining a single standard for the evaluation of animal cells as substrates for production of biologicals that could be followed as minimal standards by all countries was emphasized. However, a number of issues related to testing approaches, access to new technologies and difficulties in meeting certain specifications need to be resolved for that purpose. Reference to other international consensus guidance on best practice in cell culture, such as Good Cell Culture Practice [19], was also considered important.

It was agreed that the document should be written in the style of recommendations rather than guidelines. The latter offers more flexibility and the Study Group found that WHO should continue providing specific advice for use of cell substrates wherever possible in the format of recommendations. However, it was recognized that provision of criteria for assessment of a cell substrate would be more helpful than defining its acceptability as a general principle. The concept of acceptability of cell substrates, described in 1986 [14], was intended by WHO to facilitate use of cell substrates and recognition of "acceptable" cell lines from a safety perspective and led to acceptance of certain cell lines by Regulatory Authorities. However, the regulatory environment has evolved over time and deciding acceptability based on tumorigenicity of CCLs as a primary parameter is no longer considered appropriate. It was emphasized that tumorigenicity is but one of the relevant biological characteristics of a cell line and as such should not serve as a direct indicator of safety.

A number of specific issues relating to the use of cell substrates for the production of biologicals were discussed by the Study Group in the context of current regulatory and manufacturing practices, and proposals for further improvements were made. Definition of risk and tools for quantifying risk for individual products was considered a prerequisite for appropriate evaluation of cell substrates and application of risk-reduction strategies

during the manufacturing process.

In line with the WHO initiative in promoting use of vaccines of assured quality [20], globally applicable criteria for the safety assessment of cell substrates, as well as of final biological products, are recognized as essential tools in establishing science-based regulation worldwide. The Study Group recognized that whilst new technologies might provide enhanced safety testing and replace established technologies, implementation for established biologicals might be inappropriate. Thus, consideration of new techniques and approaches in the revised WHO recommendation should be directed at new products and not necessarily at well established products. The feasibility of implementing new recommendations into regulatory and manufacturing practice world-wide has to be considered. The current status of cell substrate evaluation in developing countries is particularly important and should be addressed in the broad consultation.

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Dr I. Knezevic [a]**, Dr G. Stacey [b], Dr J. Petricciani [c] on behalf of the WHO Study Group on Cell Substrates[1]

[a] World Health Organization, Department of Immunization, Vaccines and Biologicals, Quality, Safety and Standards Team, Avenue Appia, CH-1211 Geneva, Switzerland

[b] National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, EN6 3QG,UK

[c] Palm Springs, CA, USA

** Corresponding author. Tel : +41 22 791 3136
Fax : +41 22 791 4971
E-mail : knezevici@who.int

[1] The WHO Study Group on Cell Substrates:

Dr. P. Christian, Division of Virology, National Institute of Biological Standards and Control (NIBSC), Potters Bar, London, UK; Dr. R. M. Dhere**,** Vaccine Production, Serum Institute of India Ltd, Pune, India, Representative of the

Developing Country Vaccine Manufacturing Network (DCVMN); Dr. S. Hughes, Frederick Cancer Research and Development Center National Cancer Institute, Frederick, USA; Dr. C. Hutchens, Pfizer, St Louis, MI, USA, Representative of the International Federation of Pharmaceutical Manufacturers and Associations (IFPMA); Dr. J. Joung, Scientist, Quality, Safety and Standards Team, Immunization, Vaccines & Biologicals Department, World Health Organization, Geneva, Switzerland; Dr I. Knezevic, Scientist, Quality, Safety and Standards Team, Immunization, Vaccines & Biologicals Department, World Health Organization, Geneva, Switzerland; Dr. S. Lambert, Scientist, Quality, Safety and Standards Team, Immunization, Vaccines & Biologicals Department, World Health Organization, Geneva, Switzerland; Dr. J. A. Lebron, Merck Research Laboratories, West Point, PA, USA, Representative of the International Federation of Pharmaceutical Manufacturers and Associations (IFPMA); Dr. A. Lewis, Office of Vaccines Review and Research, Center for Biologics Evaluation and Research (CBER), Bethesda, MD, USA; Dr. L. Mallet, Analytical Science & Assay Development, Sanofi Pasteur, France, Representative of the International Federation of Pharmaceutical Manufacturers and Associations (IFPMA); Dr. P. Nandapalan, Immunobiology Section, Therapeutic Goods Administration Ltd., Woden, Australia; Dr. D. Onions, Invitrogen, Glasgow, UK; Dr. K. Peden, Office of Vaccines Review and Research, Center for Biologics Evaluation and Research (CBER), Bethesda, MD, USA; Dr J. Petricciani, Palm Springs, CA, USA; Dr. R. Sheets, National Institutes of Health (NIH), Bethesda, MD, USA; Dr. J. Shin, Scientist, Quality, Safety and Standards Team, Immunization, Vaccines & Biologicals Department, World Health Organization, Geneva, Switzerland; Dr. Y. Sohn, Biologics Division, Korean Food and Drug Administration (KFDA), Seoul, Republic of Korea; Dr G. Stacey, National Institute for Biological Standards and Control, Potters Bar, Herts, UK; Mrs C.A.M van der Velden, Groenekansweg, Groenekan, Netherlands; Dr. R. Wagner, Department of Viral Vaccines, Paul-Ehrlich-Institut, Langen, Germany and Dr D. Wood, Coordinator, Quality, Safety

and Standards, Immunization, Vaccines & Biologicals Department, World Health Organization, Geneva, Switzerland.