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A Defined-Risks Approach to the Regulatory Assessment of the Use of Neoplastic Cells as Substrates for Viral Vaccine Manufacture

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1.0 Introduction

1.1 Background.

In 1954, the Armed Forces Epidemiology Board of the United States recommended the use of "normal cells" rather than cell lines established from human tumors for the development of adenovirus vaccines [1]. This decision was based on concerns over the possibility that cells from human tumors might be contaminated with occult oncogenic agents that would expose vaccine recipients to the risk of developing neoplastic diseases. As exhibited by current regulatory guidelines and activities of control authorities worldwide, the precedent established in 1954 by the Armed Forces Epidemiology Board against the use of tumor cells for vaccine development continues to be a factor in the approval of cell substrates for vaccine manufacture.

Over the past forty years, our understanding of neoplastic processes and viral carcinogenesis has improved dramatically. Furthermore, the ability to detect and identify infectious agents has improved. The molecular technology that has produced these advances has also provided opportunities to develop new vaccines and biologicals. In addition, diseases such as AIDS or the threat of emerging infectious agents such as the H5N1 or H9N2 influenza viruses require innovative strategies in immunoprophylaxis. The development of strategies and the vaccines necessary to ameliorate current and future epidemics may require, or could be greatly enhanced by, the use of neoplastic cells.

1.2 Purpose and Scope

The ICH Harmonized Tripartite Guideline (CPMP/ICH/295/95) developed in 1995 establishes procedures for the regulation of neoplastic cells for the manufacture of biotechnology products. However, this ICH Guideline specifically excludes from consideration the use of neoplastic cells for the manufacture of "inactivated vaccines, all live vaccines containing self-replicating agents, and genetically-engineered live vectors". To address the possible use of neoplastic cells for vaccine manufacture, the purpose of this current document is to initiate a re-examination of the precedent established in 1954 by the Armed Forces Epidemiology Board by outlining a CBER

approach to assess the possible risks of using neoplastic cells as vaccine substrates. The basic feature of this approach is the development of an algorithm (Defined-Risks Approach Algorithm [DRAA]) for defining and evaluating, if possible quantitatively, the potential risks associated with tumor cell substrates and thereby establishing defined (within the limits of current technology) levels of product safety that can be used for regulatory management.

The cell substrates covered in this document include all types of neoplastic (used in its broadest sense) cells and includes spontaneously-transformed cells, virus-transformed cells, or other types of immortalized cell lines that are either tumorigenic or nontumorigenic. The development of this approach is an extension of one that was developed in the 1980's to evaluate and develop regulatory mechanisms to manage the introduction and use of tumor cells and hybridomas to manufacture highly-purified biologicals, such as interferons and monoclonal antibodies, for human use [2, 3].

It is logical to assume that some of the potential risks addressed in this document could occur more frequently than others. However, as the likelihood of an occurrence will be influenced to a considerable degree by the vaccine-substrate combination, no attempt will be made in this discussion to establish a hierarchy of risks. Furthermore, as noted below, those categories of potential risk that are least likely or unlikely should be identified during the critique of this document and the scientific validation of the Defined-Risks Approach.

2.0 Identifying the issues related to the use of new cell lines as vaccine substrates.

The CBER approach to the possible concerns over the use of tumor cells as vaccine substrates is based on the premise that, when issues of vaccine safety and public health are debated by regulatory agencies in the United States, all issues and concerns need to be identified and openly discussed. During this process, those issues and concerns deemed to be irrelevant or inappropriate can be considered and dismissed. Therefore, the first step in the process of evaluating the use of neoplastic cells as substrates for viral vaccines is the inclusive identification

of those issues that may affect the safe use of these substrates. A detailed listing of these issues for cells derived from human or primate tumors (considered for the purposes of this document to represent the highest level of risk) is presented in Table 1.

As noted in Section 1.2 , immortalized, non-tumorigenic cells are considered in this document to be a subset of neoplastic cells. The issues of general concern related to the use of immortalized, non-tumorigenic cells as vaccine substrates are compared with the issues associated with the use of human tumor cells in a separate column in Table 1. Due to their lack of apparent tumor-forming capacity, these types of neoplastic cells would appear to represent less risk than immortalized cells that are tumorigenic. Additional discussion specific to neoplastic cell lines in this category is presented in Section 3.

2.1 Definitions

Within the context of this document, the term "risk" is used, depending upon the context, to imply or represent the possibility of either theoretical or actual adverse events. It is important to point out that, as far as we are aware, there have been no actual adverse events reported to date in humans that have been attributed to the manufacture of biologicals or vaccines in neoplastic cells. Thus, the concerns posed by the use of neoplastic cells as vaccine substrates in this document represent concerns over the theoretical possibility of the risk of adverse events. The long-standing precedent against the use of neoplastic cells as substrates for vaccine development necessitates, on the part of regulatory authorities, a methodical and "state of the art" scientific approach to assessing the issues posed by the introduction of neoplastic cells for vaccine development if public confidence in programs of immune prophylaxis are to be maintained.

The concept of risk posed by the use of any vaccine must be considered in the context of the benefit to be derived by the use of that vaccine. For the discussions in this document, the relationship of vaccine benefit to vaccine risk posed by cell substrate issues is addressed in detail in Appendix 1.

2.2 Contamination with viable tumor cells.

The first item listed under general concern is product contamination with viable tumor cells. At first glance, this concern may appear trivial, as viable substrate cells are always removed from viral vaccines. However, if cells from human tumors are used as the manufacturing substrate, the issue becomes more complex. On occasions, the inoculation of neoplastic cells into humans has produced tumor allografts [4, 5]. Thus, for regulatory purposes, not only will it be necessary to remove/eliminate the cell substrate, it will be necessary to document by validated procedures the level/efficiency with which the manufacturing process eliminates/removes tumor cells from the final product.

2.3 Adventitious agent contamination

The possibility of adventitious agent contamination represents perhaps the most challenging of the issues of general concern. In the past, adventitious agents such as avian leukosis viruses (ALV) and hepatitis B virus (HBV) have been present in yellow fever vaccines [6, 7]. Simian virus 40 (SV40) was present in early poliovirus vaccines and adenovirus vaccines [8]. Adenovirus-SV40 recombinant viruses were also present in early adenovirus vaccines [9, 10]. The agent of Creutzfeldt-Jakob disease has been present in growth hormone and dura mater grafts [11]. Hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV) and parvovirus B19 have been present in blood and blood products [12]. Minute virus of mice (MVM) has turned up in long-term, fermentor cultures used to manufacture biologicals [13], and pestivirus RNA has been detected in preparations of interferons and certain measles vaccines [14, 15]. Reverse transcriptase (RT) activity, indicative of retrovirus gene expression, has been detected by very sensitive polymerase chain reaction (PCR)-based RT (PBRT) assays [16][17][18][19] in chick embryo fibroblasts (CEF) [20][21] and in vaccines manufactured in CEF and embryonated eggs [22][20][23][24]. Adventitious agents that might be present in cells derived from tumors that develop in animals or humans represent an expanding array of oncogenic and nononcogenic viral agents that may be either etiologically related to the original neoplasm or occur as non-etiologic passengers that resided in neoplastic tissues or in the blood, lymphatic or extracellular fluids of the patient or animal from which the tumor was removed. In addition, neoplastic cells that have been serially passaged in cell culture for prolonged periods may have been contaminated either with occult agents induced during serial passage [25, 26] or with a wide variety of viral agents that are propagated in institutional laboratories.

Extensive testing of neoplastic or other novel cells growing in tissue culture for known adventitious agents would be a part of the standard process of cell substrate evaluation. Testing for adventitious agents in neoplastic cell substrates would include standard tissue culture and animal tests that are routinely used to evaluate cell substrates currently in use for vaccine production. In many cases, it may be necessary to develop new tests specific for other agents. For example, over the last few years, to help address concerns that poliovirus vaccines grown in primary monkey cells might be contaminated with adventitious viruses, CBER established assays

for simian immunodeficiency virus (SIV), human immunodeficiency virus (HIV) [27], (A. Sierra-Honigmann and P. Krause, unpublished) and simian cytomegalovirus (CMV) (A. Sierra-Honigmann and P. Krause, unpublished). As discussed in Appendix 2, all methods used to detect adventitious agents are restricted by a definable threshold in their sensitivity to detect infectious agents. This threshold then defines the level of assurance the regulatory authorities must use to assess the absence of adventitious agent(s) from cell substrates. Thus, for regulatory purposes, the challenge is to define and examine these limits and determine their implications for vaccine safety.

While ensuring the absence of the extensive variety of agents that could be present in such cell substrates might be a challenge, even with current technology, the most difficult task for the regulatory process will be to assure the absence of unknown or unsuspected agents. Such agents might be present as exogenous viruses, or as latent or endogenous viruses that have been or could be induced during serial passage of the neoplastic cell substrate or during replication of the infecting vaccine, or generated by recombinational events between the vaccine and latent or endogenous viral genomes [28, 29]. Highly-sensitive assays, like the PCR-based assay for RT activity, would likely detect induced retroviruses. However, nonspecific molecular assays that can be used to detect low levels of occult non-retrovirus RNA viruses, DNA viruses, or unusual agents are not available.

2.4 Issues associated with the presence of residual cell substrate DNA.

An issue of perennial concern with the use of neoplastic cells or continuous cell lines, especially cell lines established from human tumors, is any risk that might be attributed to or associated with the presence of residual DNA from the cell substrate in viral vaccines. This issue was discussed extensively during the deliberations over the use of continuous cell lines in the manufacture of biologicals. The primary concern expressed during those discussions was the possibility that residual DNA from continuous cell lines, if present in sufficient concentration, might be capable, by the transfer of activated cellular oncogenes or insertional mutagenesis, of inducing neoplastic disease in vaccinees.

To avoid the risk of transfer of activated oncogenes in purified biologicals, a limit of 10 pg (CBER recommendation) to 100 pg (WHO limit) per dose on the amount of residual cellular DNA permitted in biologicals was established in the mid 1980s [3]. After more discussion and re-evaluation of available data, the permissible amount of cellular DNA in biological products was increased from 100 pg per dose to 10 ng per dose by the WHO in 1997 [30]. This limit is meant to apply to purified biologicals produced in continuous cell lines and not to products given orally or products derived from microbial, diploid, or primary cell culture systems. The 10 ng figure was derived by considering data and theoretical calculations regarding the tumorigenicity of injected DNA. However, it should be noted that, for live viral vaccines and other less purified products, especially those that consist of intact virions produced by lysis of the cell substrate, it may not be possible to limit the total DNA to 10 ng per dose. Thus, the question of the type of data that would be necessary to provide assurance regarding safe quantities of residual cellular DNA for vaccines produced in neoplastic cell substrates continues to be an issue.

Past discussions of the risks posed by residual DNA have focused on the possibility that such DNA might induce adverse neoplastic events, and considerable effort has been directed toward estimating the frequency with which such an event might occur provided activated oncogenes were transferred intact to vaccinated individuals [31]. The arguments that were presented against the possibility of such events were strongly supported by the published observations that, even though DNA from a few human tumors can transform NIH 3T3 cells in tissue culture, DNA from human tumor cells tested thus far has never been found to induce tumors when injected into experimental animals. In addition, there is evidence that a small number of human volunteers who were inoculated in the mid 1950's with an experimental adenovirus vaccine prepared in HeLa cells experienced no untoward effects over a 11-year follow up [32, 33] and that individuals receiving transfusions of blood from donors who subsequently developed hematogenous malignancies have experienced no discernible increase in neoplastic diseases compared with the general population [34, 35]. While germane, when considering the levels of risks that may be associated with the use of tumor cells for vaccine manufacture, these arguments are incomplete. Based on the limited data available on the transforming capacity and tumor inducing capacity of

DNA derived from a wide variety of tumors, it seems necessary to continue the discussion of the potential risks posed by tumor cell DNA (see Appendix 3).

Another issue associated with cell-substrate DNA that has not been discussed as thoroughly as the tumorigenicity risk is the potential for this DNA to be infectious. Viral genomic DNA is infectious in cell cultures and when injected into susceptible animals. Moreover, tumor cells and primary cells may harbor latent viruses and thus contain virus genomes. DNA from cells used to produce retroviral vaccines will contain retrovirus genomes as a byproduct of vaccine production. In fact, it appears that polyomavirus DNA is more infectious than it is tumorigenic [36]. This suggests that much of the effort in understanding the risk associated with DNA in biological products should be focused on understanding and minimizing the risk of infectivity [37].

Several factors may influence an assessment of the tumorigenicity and infectivity risks associated with residual DNA. These include the total quantity of DNA in the vaccine, the number of doses to be given, the size of the DNA (larger DNAs may not get into cells as well as smaller DNAs, and smaller DNA fragments might not encode complete genes or genomes), sequence-related properties of the DNA (for example, whether it encodes a virus), the number of copies of potentially infectious or tumorigenic sequences per cell, and the state of these DNAs. The state of a DNA includes such factors as whether it is associated with chromatin, whether it is integrated into a cellular genome, and whether it is linear or circular.

An improved understanding of the relative infectivity of different types of DNAs would assist in developing tests to ensure that quantities of DNA deemed to be infectious are not in biological products produced in neoplastic cell substrates. In evaluating new cell substrates, it is particularly important to understand the potential infectivity of DNAs from viruses that may be latent or resident within cells, including polyomaviruses, herpesviruses, retroviruses, and parvoviruses. This may require the development of non-specific (generic) assays to detect sequences of viruses in these families.

2.5 Viral-viral and viral-cellular interactions.

During the replication of viruses in infected mammalian cells, the complexities of interactions that can occur between infecting virions and occult viruses or between infecting virions and cells undergoing infection have become the topic of experimental analysis. In virus-infected cells or in cells simultaneously infected with two different viruses, recombination, genomic reassortments, pseudotyping, or sequestering of the DNA sequences of the infected cell by the infecting virus were initially considered to be unusual events. More recent studies have found that virus-virus genomic recombination-reassortment-pseudotyping or virus-cell gene recombination or encapsidation resulting in transduction appear to occur with sufficient frequency (from estimations of 1/7 virions [38] to 1 in 10^4-10^5 virions [39] to pose potential concerns when considering the use of neoplastic cells as substrates for vaccine development. These events may become even more of a concern when neoplastic cells to be used for vaccine manufacture are obtained from spontaneously developing tumors from adult humans or animals.

As specific examples of these events, defective murine retrovirus vector genomes can recombine with endogenous murine provirus genes that have been introduced into cells [38] or that occur naturally [40] to form replication-competent retroviruses. In a murine retrovirus vector tested in rhesus monkeys, the replication-competent retrovirus that was recovered from the monkeys was more infectious in tissues culture assays than the prototypic retrovirus [41]. Furthermore, T-cell lymphomas arose in animals inadvertently inoculated with this replication-competent retrovirus in protocols designed to evaluate the potential usefulness of retrovirus vectors for gene delivery [42]. Thus far, the versatility of defective murine retrovirus vectors in recombining with helper sequences in third-generation packaging cell lines has overcome the requirement for a minimum of three independent recombinational events during replication in the packaging cell line to generate replication-competent progeny [43].

In addition to the ability of certain retroviruses in certain cells to acquire, by recombination, those endogenous retroviral cellular sequences necessary for replication, retroviruses apparently possess the ability to insert themselves as proviruses into the genomes of large DNA viruses such as herpesviruses [44]. By this mechanism, unsuspected retroviruses might parasitize the genomes of

vaccine viruses that possess large DNA genomes (especially herpesviruses and herpesvirus vectors). Until such phenomena are better understood, the consequences for vaccine safety of retroviral genomes transduced by parasitized vaccines is difficult to predict.

The ability of viral-viral or viral-cell gene recombination events to alter the replication competence and the cross-species pathogenicity as well as the creation of novel viruses that do not exist in nature [41] pose questions for the regulation of vaccines manufactured in tumor cells that need to be addressed.

2.6 Biologically active cellular proteins.

There have been reports of biologically significant levels of cytokines (IL-1 and IL-6) in viral vaccines and in biologicals derived from a spontaneously transformed and tumorigenic Chinese hamster ovary cell line carrying recombinant transgenes [45]. It has been proposed that the presence of cytokines might contribute to adverse local and systemic reactions [45]. Tumor cells are known to secrete various cytokines and other regulatory proteins [46, 47], and infections with viruses such as rubella, mumps, and measles viruses have been shown to stimulate infected cells to secrete cytokines [45]. Based on these observations, it may be necessary to consider the possible consequences of the production of biologically-active proteins by tumor cell substrates used in the manufacture of viral vaccines.

In addition to the possible presence of biologically-active regulatory proteins, when considering the use of cells derived from human tumors, it is possible that the status of the PrP gene and its protein product will need to be evaluated. Based on current concepts regarding the role of aberrant PrP proteins in the etiology of Creutzfeldt Jakob disease, the nucleotide sequence of the PrP gene and the form of its protein product may be an issue for the safety of vaccines produced in neoplastic or other cells derived from humans.

2.7 Genomic instability.

Spontaneous tumors that arise from single cells as well as laboratory-derived clonal populations of neoplastic cells have been shown to consist of heterogeneous populations of cells that express or exhibit a variety of genotypes and phenotypes. The origin of these heterogeneities can be due either to genetic (mutational) alterations or epigenetic alterations of the neoplastic cell genome. There have been few systematic evaluations of the rate of spontaneous mutations in diploid non-neoplastic cells and their neoplastic derivatives. However, those studies that have been done found the rate of spontaneous mutations was the same in both cell populations [48]. Another study found that the epigenetic changes of hypomethylation followed by *de novo* methylation induced by 5-azacytidine could also produce phenotypic heterogeneity in clonal populations of tumor cells [49]. As changes in either the genotype or phenotype might result in the induction of occult agents lying dormant within the cell, the types of viral-viral or viral-cellular interactions that occur, or in the nature of the biologically active proteins being produced, the implications of genotypic and/or phenotypic instability in tumor cell substrates used for the production of viral vaccines may need to be considered.

3.0 Issues associated with the use of non-tumorigenic neoplastic cell lines for vaccine manufacture.

As noted in Section 1.2., immortalized non-tumorigenic cells are considered in this document to be a subset of neoplastic cells. The issues of general concern related to the use of immortalized non-tumorigenic cells as vaccine substrates are compared with the issues associated with the use of human tumor cells in Table 1. With the exception of tumor cell contamination and genomic instability, the issues and concerns remain the same as for cells derived from tumors. For purposes of the discussion of this document, we propose that the Defined-Risks Approach applied to tumor cells also be applied to issues related to adventitious agent contamination, cell DNA contamination, viral-viral interactions and viral-cell interactions associated with the use of immortalized nontumorigenic cell substrates for vaccine manufacture. The application of the Defined-Risks Approach to these issues will be discussed in more detail in the next Sections.

4.0 Defined-Risks Approach Algorithm (DRAA)

The algorithm that CBER proposes for the defined risks approach consists of five steps. 1. Identifying the possible risk event ; 2. Estimating or determining the frequency with which the risk event may occur or has been observed to occur either in nature or under experimental conditions; 3. Estimating the possible frequency of the risk event per dose of vaccine; 4. Developing and determining the sensitivity (with respect to lower limits of the assay's ability to detect the risk event) of one or more assays that can be use to detect the risk event; 5. Developing and validating one or more processes that can be used to establish a product-specific safety factor at a level of </= 1 risk event per 10^6 doses (see Appendix 1) or determine the level at which current technology can be used to establish a safety factor/limit.

4.1 Application of the DRAA to the issue of tumor cell clearance

Applying the DRAA to the issue of residual viable tumor cells in the final product represents a straightforward determination of the number of viable substrate cells required per vaccine batch, the number of cells required to produce a single dose of vaccine, and the total number of cells removed during the processing of a batch of vaccine. Based on these numbers, the lower limit of the risk per dose of product contamination by a single, viable tumor cell can be established. Using the assumed safety factor from Appendix 1, the risk per dose should be </= $1/10^6$.

4.2 Application of the DRAA to the issue of adventitious agent clearance

The application of DRAA to the issues associated with the possible presence of adventitious agents, especially unsuspected or unknown agents, in neoplastic cell substrates appears to represent a challenge to scientific ingenuity. With regard to the absence of known viruses, it will be necessary to determine the sensitivity of the assays in terms of virions per unit volume used to demonstrate the absence of a possible agent, the number of cells per product dose, and the number of cells that can be demonstrated to be agent free. From these data, the lower limit of the risk of contamination by a known virus can be established. The problem is more difficult for unknown (or unsuspected) viruses. It will be necessary to develop generic assays that can detect novel viruses, and it will be necessary to establish the limits of the sensitivity of these assays. It

is in the development and characterization of such generic assays that further research will improve our ability to detect adventitious agents in cell substrates.

An example of a generic assay is the reverse transcriptase (RT) assay. The RT assay detects RT activity produced by all (known or unknown) retroviruses, and even very low levels of RT can be detected by PCR-based RT (PBRT) assays [16][17][18][19]. These assays may also be combined with tissue culture methods. The DRAA highlights the need to understand the sensitivity of existing non-specific assays, including PBRT assays. In order to interpret the meaning of a negative test in assessing risks associated with a particular cell substrate, it will be necessary to know the test's sensitivity to detect a specific number of virus particles against a background of a fixed number of uninfected cells.

Other nonspecific molecular assays for detection of adventitious agents in cell substrates need to be considered. One approach is to use degenerate primers for various virus species to detect their nucleic acids. For example, in each of the past few years, new herpesviruses have been discovered using degenerate PCR primers derived from herpesvirus polymerase sequences [50-52]. It may be possible to develop degenerate PCR primers to amplify and detect nucleic acids from viruses in different families. The possibility that these techniques could yield false-positive results will also need to be considered. Specific or degenerate (non-specific) oligonucleotide sequences could be used in oligonucleotide arrays to detect viral nucleic acids. The high throughput made possible by this technology could permit screening of large numbers of specimens.

PCR-based subtraction methods, such as those that were used to find viral sequences in diseased human tissues (e.g., representational difference analysis), could be used to examine vaccine or cell substrate samples for the presence of nucleic acids that could represent adventitious viruses [53]. This technique would require identification of two otherwise comparable samples, one of which is less likely to contain adventitious agents.

Another molecular approach to the development of non-specific assays for adventitious viruses is to purify nuclease-resistant nucleic acids from a vaccine or cell substrate sample and use generic primers to amplify non-specifically those nucleic acids that are resistant to nuclease digestion. The protein capsids of many viruses protect their nucleic acids from such digestion, so that this method might be able to identify contaminating encapsidated nucleic acids.

As currently performed, tissue culture assays are nonspecific. It is possible that broadened tissue culture assays (e.g., transfection of cell-substrate-derived nucleic acids into susceptible cell lines or culture for longer periods) may provide better sensitivity to detect adventitious agents in novel cell substrates. These assays depend on the use of some method to monitor infection in the cells, such as detection of cytopathic effect or reverse transcriptase production.

In addition, improved animal models that incorporate the use of immunocompromised animals might improve the sensitivity of testing for unknown agents. However, limits on the number of animals and amount of product that can practically be injected may also limit the sensitivity of animal assays.

For any of these methods, it would be necessary to understand the method's sensitivity to detect known viruses before its ability to detect unknown viruses can be evaluated (see Appendix 2).

4.3 Application of the DRAA to the issue of residual cell substrate DNA

The application of the DRAA to estimating risks associated with residual cell DNA involves the development of assays of defined sensitivity that can be used to ensure the absence of neoplastic or infectious risk-events attributable to residual cell substrate DNA at levels of </= 1 /10^6 per vaccine dose. The development of such assays depends upon estimations of the quantities of neoplastic cell DNA that must be documented to be unable to induce such risk-events.

The neoplastic risk potential associated with chromosomal insertion of foreign DNA has been estimated [31]. Based on the likelihood of injected DNA entering a cell and integrating into

genomic DNA, the probability that a single intracellular DNA molecule would activate a single proto-oncogene was estimated at 3×10^{-10}, and the probability of two independent such events was estimated at 10^{-19}.

Previous consideration of the neoplastic risk potential of injected DNA did not consider the possibility that the risk of neoplastic events could depend upon the relative tumorigenicity (i.e. tumor forming capacity in terms of number of cells required to produce tumors in injected animals) of the candidate substrate (see Appendix 3). The ability of different types of human tumor cells to form tumors in animal models varies substantially, with some cells unable to induce a tumor after injection into nude mice and others requiring from tens of cells to millions of cells to induce a tumor. It is possible that the DNA from highly tumorigenic cells (those requiring 1 to a few 10's of cells for tumor formation) could represent a greater risk for the induction of a neoplastic event than the DNA from neoplastic cells that require thousands to millions of cells to form tumors or those than do not form tumors.

For purposes of regulatory management, it may be necessary to consider the possibility that a neoplastic risk event associated with residual tumor cell DNA could be related to the efficiency with which the cells from a particular tumor form tumors *in vivo*. Based on this concept, the fewer cells of a particular cell substrate that are required to establish the cell line's tumor-producing dose 50% endpoint (TPD_{50}) value (see Appendix 3), the larger the amount of cell DNA from that cell line that must be shown to be risk-event free. However, it should be noted that the relevance of the ability of a cell to form tumors after injection into animals has not been correlated with the oncogenicity of its DNA. Data comparing the tumorigenicity of DNA derived from highly tumorigenic cells (i.e., those requiring 1-10 cells for tumor formation) with DNA derived from weakly tumorigenic cells (i.e., those requiring 10^4 -10^7 cells for tumor formation) would help to resolve this issue.

Because cellular genomic DNA obtained from tumor cells has not been shown to induce tumors in those animal models (syngeneic animals and nude mice) in which it has been tested, a careful evaluation of any possible relationship between the tumorigenicity of neoplastic cell lines and the

purified cellular DNA obtained from those cells is likely to require a better understanding of the sensitivity of the animal models with respect to their susceptibility to tumor development. These models include highly immunosuppressed animals, transgenic mice that are deficient in p53, and transgenic mice that constitutively express oncogenes such as *ras* [54]. At this time, it is not clear whether such models might detect oncogenic DNA that has proven to be nononcogenic in other animal models. As the injection of genomic DNA from oncogenic viruses can lead to tumors in some currently-used animal models, but injection of DNA extracted from tumor cells does not, further investigation of tissue culture transformation assays, like NIH 3T3 cells, either alone [55, 56] or in combination with animal models in which transformed cells are injected into animals to look for tumors [57] might be worthwhile. One also could consider assessing the tumorigenicity or infectivity of cell-substrate extracts, which could detect occult unknown tumor viruses. These methods could be developed in a quantitative fashion for optimal use in risk assessment.

Based on published data, the ID_{50} of injected virus genomic DNA may range from 4 ng to 38 µg [37]. In limited studies, the infectivity of ingested DNA was considerably lower [36]. Accounting for the dilution effect in cellular DNA, residual DNA from a cell containing one copy of polyomavirus DNA per cell would carry a risk of 1.2×10^{-4} infections per µg of residual DNA [37]. Using the same logic, residual DNA containing one copy of a retrovirus genome per cell would carry a risk of $2.5\text{-}38 \times 10^{-8}$ infections per µg. Assuming a desired risk of less than 10^{-6} per dose, for polyomavirus DNA (see Appendix 1), this risk would correspond to 8 ng of DNA. For a cell line that has 50 copies per cell of a polyomavirus genome, this would correspond to a limit of 160 pg per dose. While this may be achievable for purified products, unpurified virus vaccines and virus vectors generally contain significantly greater quantities of residual cellular DNA.

These calculations assume that viral genomic DNA is as infectious or as tumorigenic when incorporated in cell substrate DNA as it is when it is linearized and injected directly. This risk estimation also assumes that the total risk is directly proportional to the amount of DNA injected. As one or both assumptions may be incorrect, additional work will be necessary to determine how

integration changes the infectivity or tumorigenicity of viral genomic DNA. For most considerations related to the state of injected viral and cellular DNA, studies thus far have not been performed in a quantitative fashion that permits the application of quantitative risk assessment models.

There are several approaches to assuring the absence of potentially dangerous quantities of infectious DNA. One is to limit the total residual DNA in the product based on a worst-case scenario of what types and quantities of infectious DNAs could be present. The other is to demonstrate the absence of integrated copies of viral genomes for those types of viruses that are known to establish latent infections. A third approach may be to reduce the size of the DNA such that a single DNA fragment can no longer encompass an infectious entity or encode a functional oncogene. The absence of DNA viruses could be demonstrated using generic PCR assays that are based on shared sequences within the virus families that establish latency (e.g., herpesviruses, parvoviruses, and perhaps polyomaviruses). For clonal cell lines, it should be possible to develop non-specific assays that demonstrate the complete absence of latent DNA virus genomes. For non-clonal cell lines, it would be necessary to define the sensitivity of such assays. Demonstrating the absence of latent infectious retrovirus sequences is more difficult because of the presence of endogenous retrovirus sequences and may depend on attempting to induce replication of potentially latent retroviruses. Study of the infectivity of genomic DNA from potentially latent DNA viruses relative to that of retroviruses may be helpful in the context of the DRAA, because assurance of the absence of DNA from viruses that are more infectious than retroviruses could permit limitation of residual DNA based on studies of the relatively less infectious retrovirus genomic DNA. Thus, an improved understanding of the infectivity of DNA from different virus families, and/or an improved ability to assure the absence of DNA from different virus families, may be necessary to completely evaluate novel/neoplastic cell substrates with respect to this issue.

If it can be established that the ID_{50}'s of tumor-virus DNAs are substantially less than the quantity required for tumor formation, then the question of a risk event for residual cellular DNA can be evaluated by assays for DNA infectivity. A possible alternative to determining and using risk-

event limits for the regulatory management of neoplastic cell substrates is the introduction of some form of DNA clearance into the manufacturing process. As the clearance of spiked model viruses has proved useful in regulating the safety of purified biologicals produced in tumor cells, the demonstrable clearance of model DNAs containing an infectious marker to below a defined limit during vaccine manufacture might be a suitable alternative to assessing large quantities of cell substrate DNA for the absence of neoplastic or infectious risk-events.

4.4 Application of the DRAA to issues of viral-viral and viral-cellular interactions

The application of the DRAA to issues of viral-viral or viral-cellular interactions will involve the development of assays to determine the rates at which various viral-viral and viral-cellular interactions occur in the vaccine strain – cell substrate combination proposed for vaccine manufacture. Once these rates are determined, the number of substrate cells required per vaccine batch and per vaccine dose can be used to calculate the expected number of risk events per dose. These data can then be used to determine the impact of these events upon vaccine safety. This approach will need to be tailored to specific types of vaccines, because the vaccine virus itself represents one of the constituents of these types of interactions. This approach permits the design of assays that could rule out such interactions in a way that would provide a rational scientific basis for estimating the level of possible risk associated with any new cell substrate.

One approach is to quantitatively assess recombination or packaging rates in fixed numbers of cells (based on the number of cells per dose and the reliability with which the event needs to be ruled out) with or without selective pressure by examining the packaging of reporter genes such as antibiotic resistance genes that are expressed within a cell substrate. Another approach is to directly quantify cellular DNA or RNA (using probes that are specific for cellular genes) that may be packaged within viral particles. A full assessment of the nature and extent of those risks that might evolve from viral-viral or viral-cellular interactions will require further study to understand the rates at which such events occur under the wide variety of circumstances that are involved in vaccine development and manufacture.

4.5 Application of the DRAA to issues of biologically-active cellular proteins

The application of the DRAA to issues related to the presence of biologically-active cellular proteins produced by neoplastic cell substrates will involve determining the presence and concentration of the protein in the vaccine. Assuming that the threshold concentration for protein activity is known, it should be possible to establish appropriate limits on the per dose concentration to avoid a protein-associated risk event.

Application of the DRAA to the case of PrP proteins is more difficult. At this time, it is not known whether mutations in PrP expressed in tissue culture cells can induce spongiform encephalopathies in exposed animals or humans. Due to the absence of experimental data pertaining to this issue, any cell substrate that expresses a mutated PrP that is potentially pathogenic may be an inappropriate candidate for vaccine manufacture. With further regard to the use of neoplastic cell substrates, which are subjected to spontaneous mutations and genomic rearrangement over time, for the manufacture of viral vaccines, the question of monitoring such cell substrates during the manufacturing process or testing post-production batch preparations for mutated PrP proteins may need to be considered. Alternatively, it may be possible to estimate the risk of such events based on other data.

4.6 Application of the DRAA to issues of genomic stability

It is perhaps premature to consider the application of the DRAA to issues related to the genomic stability of neoplastic cell substrates. Other than the induced expression of latent viral genomes during serial tissue culture passage, it has not been established that acquired or induced heterogeneity in a clonal population of tumor cells can result in a risk event of any type. Until evidence for such a risk event is forthcoming, it is not necessary to develop methods to estimate its frequency. Without the ability to estimate possible frequencies of risk events, the DRAA cannot be directly applied.

5.0 Discussion

This document has attempted to outline the concerns that can be envisioned to attend the use of neoplastic cells as vaccine substrates. Within this outline, a Defined-Risks Approach has been developed to permit, where possible, a quantitative estimation of the theoretical risks that contribute to the concerns with the safety of vaccines manufactured in neoplastic cell substrates. Within the context of the Defined-Risks Approach, the potential risks of different types of cells can be considered.

Regulatory precedents suggest that it should be possible to compare the potential risks of different types of cell substrates with cell substrates that already have been tested in many individuals and are deemed safe. Years of experience with human diploid cell lines have led to the conclusion that they do not contain adventitious agents and are generally safe cell substrates. In particular, their finite lifespan has contributed to the perception that these cell lines do not contain transforming or otherwise harmful viruses. Moreover, injection of their DNA is generally considered to be an acceptable risk.

With respect to the question of known versus unknown viruses, based on many years of experience with human diploid cells, regulatory authorities have already set a precedent in concluding that these cells do not contain adventitious agents. This would imply that other cell lines derived from human diploid cells (for example, 293 cells, a cell line developed by transforming human embyronic kidney (HEK) cells with the E1 region of adenovirus 5 containing the E1A and E1B oncogenes) might also be considered safe with respect to this issue, because there is a known mechanism of transformation of a cell type that is originally deemed safe, as compared with continuous cell lines or cell lines derived from tumors, where the mechanism of transformation is unknown. While the adenovirus sequences introduced into 293 cells might have induced a process that could theoretically lead to a risk event, the same could be said of the unknown transforming event in continuous cell lines, which are often developed by serial tissue culture passage of primary cells. For tumor cell lines, one could argue that the fact that they are transformed supports the suspicion that they may contain adventitious agents, including unknown agents. Therefore, the risk of unknown adventitious agents might be less for 293 cells and other

types of neoplastic cells derived from human diploid fibroblasts transformed by defined viral or cellular oncogenes than for other neoplastic cell lines transformed by unknown factors. However, this assumes that 293 cells truly represent transformed diploid HEK cells, that there were no tumor cells in the HEK cells before transformation, and that the HEK cells were not contaminated during shipment, propagation, etc. Adequate records to prove all these things may not be available for 293 cells. Nevertheless, the 293 cell model provides a useful framework for assessing the issues associated with neoplastic cell substrates that can be developed by readily available technology.

Based on the assumption that DNA from human diploid cell lines is free of risk, the DNA risk associated with in vitro transformed human diploid cells also might be considered lower than that of cells from spontaneously arising tumors, which might be more likely to contain a latent virus.

Primary monkey kidney cells, which have been used for production of the live oral polio vaccine (OPV) for many years, may spontaneously transform into neoplastic cells for unknown reasons. The oral administration of this vaccine may reduce the level of concern as compared with parenteral administration of an unpurified vaccine produced in a similar cell substrate. Nonetheless, based on current data, the potential risk of adventitious agent contamination of primary cells appears to be higher than that of neoplastic cells, and due to their non-clonal origin, they are more difficult to test for the presence of latent viral nucleic acids.

Thus, a fundamental question related to the use of neoplastic cells is whether the history of the cell line, or the mechanism of transformation if it is known, should influence a regulatory assessment of risk. The possibility that a neoplastic cell contains an exogenous agent or has undergone some event that may either activate an unknown adventitious agent, provide an opportunity for the vaccine to acquire a risk-inducing activity, or render its DNA risky is at the root of the concerns about their use as vaccine cell substrates. The DRAA is based on quantitative assessment of the ability of various tests to assure that these events are unlikely within limits deemed necessary for the safe use of vaccines manufactured in these types of cells.

Considering the complexities of the issues involved, in some cases it may be simpler to design specific cell substrates with desired properties starting with cell substrates that are known to be safe. Under these conditions, the safety of a new neoplastic cell substrate could be considered relative to its progenitor. Thus, one approach could be to develop new cell lines based on human diploid cells that have been immortalized and engineered to permit the growth of different viruses, or express other desired characteristics. While this "designer cell" might be simpler from the perspective of defining cell substrate risk, it would also require considerable attention early in the vaccine development process to the selection, development and testing of the most appropriate cell substrate.

Appendix 1. Thresholds for cell substrate risk

In developing the Defined-Risks Approach to evaluating the safety of neoplastic cell substrates (or any novel cell substrate), it is necessary to establish the level of risk that is considered acceptable. The acceptable maximum risk must be determined in the context of the need for the product. In all cases, the benefits of the product should exceed the maximum risk associated with cell substrate issues. The risks associated with cell substrate issues are difficult to define, because the endpoints are often theoretical and cannot always be measured directly in vaccinees or even in animals. For the purpose of this discussion, a cell substrate-associated risk event is defined as any potential event that poses a safety concern whose origin can be traced or attributed to the cell substrate. For example, the presence in the cell substrate or in the manufactured product of a naturally occurring or induced, replication-competent or replication-incompetent adventitious agent would be defined as a risk event.

Due to the need for public confidence in vaccines, it might be argued that for newer products, regardless of the benefit of the product, it would be advantageous to show that cell substrate-associated risks are highly unlikely to occur in vaccine recipients. This is an important issue with vaccines, which are often administered to healthy children rather than to adults or individuals who have already contracted a disease. Moreover, while for new vaccines it might be fairly simple to show that the maximum number of possible cell substrate-associated events will be below the morbidity of the disease, this estimation is likely to change after the vaccine has been in use and the incidence or morbidity of the disease being vaccinated against has been reduced. Under these circumstances, it is necessary to consider the potential continued use of the product with respect to cell-substrate issues, even after the disease is eliminated or nearly eliminated .

For purposes of discussion, this document uses an absolute approach to the issue of risk, in which the risk per dose of a product that is administered to 4 million healthy children per year (the approximate number of children immunized in the US per year) should have an estimated risk of a cell substrate-related adverse event of less than one in 10^7 or so. Even then, there might be a

risk event every few years, so that this might be too high. For a product administered several times to each child, it might be deemed necessary to show that the risk is less than one in 10^8. If the product saved 100 lives per year, one could argue that a risk of one in 10^6 might be acceptable, still providing at least a 10:1 margin of benefit:risk in favor of the product.

For any discussion of cell substrate risk, the model applied to the regulation of tumorigenic rodent cells to manufacture therapeutic biological products provides an informative example. These products require high levels of purification and demonstration of virus removal. As one example, if one assumes that there are 10^7 retrovirus particles per dose, and the particle:infectivity ratio of these particles is at most 1:10, 10^6 represents an upper bound on the number of infectious particles that would be present per dose. In order to assure a risk less than 10^{-6}, demonstration that the purification process removes or inactivates 10^{12} virus infectious units would be required. In reality, even higher rates (10^{13} - 10^{15}) of virus clearance are routinely requested of manufacturers of purified therapeutic biological products, including those with much lower particle:infectivity ratios, implying the perceived need for very high margins of safety with respect to this issue.

In contrast, the unpurified polio vaccine, which is produced in primary monkey kidney cells, includes a control cell testing scheme in which 25% of all cells are devoted to detection of potential adventitious agents. Using as a hypothetical example a lot size of around several hundred thousand doses, this implies that regulatory authorities are satisfied with a demonstrated adventitious agent contamination risk that is about one in 10^5 doses. Based on evaluations by the Institute of Medicine, society currently is unwilling to accept the 1-2 x 10^{-6} risk of vaccine-induced paralytic polio. In contrast, as documented by evaluation of this risk for currently-marketed vaccines, society appears to be willing to accept an anaphylaxis risk of between 10^{-7} and 10^{-6} events per dose.

Based on this discussion, the examples in this document present calculations based on the assumption that a risk of 1 per 10^6 of a cell substrate-related risk event for a hypothetical product is acceptable. Further discussion may lead to changes in this assumption, and it is

currently proposed solely for the purpose of creating a starting point for discussion of cell substrate risk. It is anticipated that the methods developed in response to the DRAA would be applicable regardless of this specific assumption.

Appendix 2. General factors involved in testing cell substrates for theoretical risks

In evaluating specific data relative to neoplastic or other cell substrates, the sensitivity of the test is particularly important. The ability of the test to demonstrate the absence of a specific cell substrate-related risk (as defined in Appendix 1) to the limit of detection accepted as the maximum permissible risk must be evaluated. For purposes of discussion and calculation, this risk is assumed to be one cell substrate-associated event per million doses. Many tests may only be sensitive to a much lower level. For example, PCR for specific viral contaminants (which generally can detect 1 copy per 10,000 cells per reaction) is seldom able to detect more than one copy of a cell-associated contaminant per 1 to 10 doses, because the products themselves contain DNA and RNA representing hundreds to thousands of cells, limiting the total number of doses that can be tested in a single PCR. Thus, for a product that contains the nucleic acids from 1,000 cells per dose, demonstrating that an adventitious agent is present in at most 1 in a million doses would require a test that can scan 10^9 cells for an infectious unit. For some products, it may be possible to test concentrated supernatants or to use additional amplification techniques to improve this sensitivity. However, it may currently be feasible to achieve this level of sensitivity for many types of cell substrate only with tissue culture assays, and even these may be cumbersome and difficult to implement for some products.

In general, an upper bound on the risk per dose of a cell substrate can be calculated as the number of cells per dose of the product divided by the number of cells that are tested for the absence of the risk. This latter number depends on the sensitivity of the various tests used to screen the cell line. Thus, higher numbers of cells per dose decrease the possible assurance of safety based on any given safety test, and larger numbers of cells documented to be free of the risk increase this assurance of safety.

Direct assessment of the tumorigenicity and infectivity of residual DNA associated with some products may be impractical. This is because in order to achieve meaningful safety margins, very

large quantities of DNA would need to be purified and tested in multiple animal models for infectivity and tumorigenicity. If a dose of a product contained one microgram of residual cellular DNA, assessment of a million doses in a single type of assay would require testing of one gram of DNA, whereas if a dose contained only 1 ng of residual DNA, then 1 mg of DNA would need testing. This approach may sometimes also require use of more animals than is practical.

Appendix 3. Possible impact of the tumor inducing capacity of neoplastic cells on assessing the risk posed by residual tumor cell DNA.

An estimation of the capacity of neoplastic cells to form tumors *in vivo* can be obtained by quantitative tumorigenicity assays in which the number of cells required for tumor formation at the 50% endpoint (tumor producing dose 50% or TPD_{50}) is determined [58]. Since cells from both animal and human tumors can exhibit TPD_{50} values ranging from 10^1 to 10^7 (Table 2), the mechanisms by which cells from highly tumorigenic lines (i.e. ,TPD_{50} values 10^0-10^2) form tumors *in vivo* may differ from cells representing less tumorigenic cell lines (i.e., TPD_{50} values of 10^4 -10^7) (see below).

If a single neoplastic cell has the capacity to establish a tumor in a fully immunocompetent, syngeneic adult host, the phenotype expressed by that cell must be capable of establishing all of the processes required for tumor development. Current evidence indicates that the cell's phenotype is determined by the organization and expression of its genome; thus, we will also assume that the tumor-inducing capacity of such a tumor cell is determined by its genome. The question is whether those organizational and functional changes in the DNA of highly tumorigenic cells that allow the cell to form a tumor very efficiently *in vivo* could be transferred by DNA obtained from such cells during the process of vaccine manufacture.

Current data support the concept that the immune response of the tumor-challenged animal plays an important role in determining the number of neoplastically-transformed rodent cells required for tumor formation. It is reasonable to assume that the tumor-inducing capacity of a tumor cell line with a TPD_{50} of 10^0-10^1 is unlikely to be influenced by the antitumor immunity available to a naïve host. Where in the scale of TPD_{50} values from 10^0 to 10^7 the impact of the host immune response begins to play a role has not been rigorously defined. However, there are data indicating that SV40-transformed Syrian hamster cells with TPD_{50} value of $10^{4.2}$ are resistant to lysis in vitro by NK cells and activated macrophages [59] and are not influenced by the immune responses available to nude mice, syngeneic newborn hamsters, syngeneic adult hamsters, or even

allogeneic adult hamsters [60]. Other data indicate that the host's immune system plays an important role in tumor formation for those cell lines that require large numbers of cells for tumor induction [61]. These processes could be due to artifacts of *in vivo* tumorigenicity assays. Alternatively, data from *in vivo* tumorigenicity assays could be indicative of unrecognized, genetically-controlled processes associated with the different tumorigenic phenotypes expressed by neoplastic cells. Irrespective of these considerations, until the mechanisms of tumor formation in animals by neoplastic cells growing in tissue culture are better understood, the DNA from highly-tumorigenic cells may need to be considered with greater caution than the DNA from less-tumorigenic cells until data show that such DNA represents no higher level of risk. From the perspective of estimating the potential risk associated with residual cell substrate DNA, the fewer tumor cells required to establish the TPD_{50}, the larger the amount of the DNA from such cells that must be shown to be risk-event free.

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Table 1.

Possible Issues Associated with the Use of Tumorigenic and Non-tumorigenic Neoplastic Cells as Vaccine Substrates

General Issues	Issues Specific for Tumorigenic Cell Substrates	Issues Specific for Nontumorigenic Cell Substrates	Documentation of Possible Risk
Tumor cell contamination	Induction of tumor allograft	Not applicable	Southam Science 125: 158, 1957; Gross, Oncogenic Viruses p 848-859, 1970
Adventitious agent contamination	Transfer of known and unknown viruses	same	Minor, Dev Biol Stand. 88:25 1996; Horaud, Dev Biol Stand. 88:19, 1996
	Formation of recombinant viruses	same	Lewis, Proc Soc Exp Biol Med 122: 214, 1966; Pryciak, PNAS 89: 9237, 1992; Jones, J Virol 70: 2460, 1996; Burke, Emer Inf Dis 3:253, 1997
	Activation and transfer of occult viruses; Presence of unusual agents	same same	Leiber, Science 182: 56, 1973; Heilbronn, PNAS 90:11410, 1993; Coggins, Nature 290:336, 1981
Cell DNA contamination	Transfer of: 1- activated oncogenes-mutant tumor suppressor genes; 2- infectious integrated-latent virus / provirus DNA	same	1- Blair, Science 218:1122, 1982 2- Boyd, J Virol 10:399, 1972; Letvin, Nature 349: 573, 1991; Willems, Virol 189: 776, 1992

Cell protein contamination	Transfer of 1-cytokines, 2-growth factors, 3-PrP^{sc}	same	1-Gearing, Lnct, 333: 1011,1989; 2-Undocumented, theoretical issues
Viral-viral; viral cell interactions	1- Generation of replication competent retroviruses; 2- Generation of new viruses or recombinant viruses 3-Generation of viruses capable of cross-species pathogenesis 4- Generation of pathogenic defective viruses 5- Viral-viral parasitism 6- Induction of latent virus replication; 7- Transduction of cell genes; 7- Transduction of viral genes	same	1- Chong, Gene Therapy 3: 624, 1996 2- Burke, Emerg Inf Dis, 3: 253, 1997; Purcell, J Virol, 70: 887, 1996; Berkhout, J Virol, 73:1138, 1999 3- Donahue J Exp Med 176: 1125, 1992 4- Jolicoeur Nature, 338: 505, 1989; Overbaugh, Science, 239: 906, 1988; 5-Isofort PNAS, 89: 991, 1992 6- Heilbronn, PNAS 90;11406, 1993 7- Linial Cell 49: 93, 1987; Stuhlmann J Virol, 64: 5783, 1990; 7- Patience, J Virol, 72: 2671, 1998; Scadden, J Virol: 424, 1990; Howk, J Virol: 115, 1978;
Genomic instability	Induced replication of endogenous agents; changes in configuration of cell genome leading to increased possibility of above events.	Not applicable	Elmore, Can Res 43: 1650, 1983; Kerbel, J Cell Phys Sup, 3: 87, 1984 Undocumented theoretical issue

Table 2.

Tumor producing efficiency of human tumor cells in nude mice

Cell line	Tumor type	TPD50 (log10)	Reference
293	Ad5 trans human kidney cells	6.5	Lewis unpubl.
SW480	Ca colon	5.2	"
HeLa	Ca cervix	4.9	"
A549	Ca lung	3.5	"
Fibrosarcoma	Fibrosarcoma	4.0	Gerswin JNCI 58: 1455, 1977
Melanoma	Malignant melanoma	3.0	"
Endometrial carcinoma	Endometrial carcinoma	1.0	"