WHO Technical Report Series, No. 878. 1998

Annex 1
Requirements for the use of animal cells as *in vitro* substrates for the production of biologicals
(Requirements for Biological Substances No. 50)

Introduction

Historically, the major concerns regarding the quality of biological products produced in animal cells have been related to the possible presence of adventitious contaminants and, in some cases, to the properties of the cells themselves. There are additional concerns regarding the quality of products prepared using recombinant DNA technology in relation to the expression construct contained in the cell substrates. It is well established that the properties of cell substrates and events linked to growth can affect the quality of the resultant biological products and, furthermore, that effective quality control of these products requires appropriate controls on all aspects of the handling of cell substrates.

General considerations

Types of animal cell substrates

Primary cell substrates

Primary cells obtained directly from the trypsinized tissues of normal animals have played a prominent role in the development of virology as a science, and of immunology in particular. Cultures of primary cells from different sources have been in worldwide use for the production of live and inactivated viral vaccines for human use for more than 40 years, and experience has indicated that these products are safe and effective.

Major successes in the control of viral diseases, such as poliomyelitis, measles, mumps and rubella, were made possible through the wide use of vaccines prepared in primary cell cultures, including those from chicken embryos and the kidneys of monkeys, dogs, rabbits and hamsters, as well as other tissues. Cultures of monkey kidney cells have been used for the production of inactivated and oral poliomyelitis vaccines for more than 40 years, and the same cell system continues to be used for the production of both vaccines.

Primary cell cultures have the following advantages: they are comparatively easy to prepare using simple media and bovine sera; and they possess a broad sensitivity to different viruses, some of which are cytopathogenic. In addition, primary cells can now be grown in bioreactors using the microcarrier method (*1*).

However, where suitable alternative cell substrates are available, primary cell cultures are less likely to be used in the future for the following reasons: contamination by infectious agents, such as viruses,

is a common problem; the quality and sensitivity of cultures obtained from different animals is variable; and it will become increasingly difficult to obtain cultures derived from nonhuman primates.

Primary cell cultures obtained from wild animals show a high frequency of viral contamination. For example, it is generally accepted that monkey-kidney cell cultures can be contaminated with one or more adventitious agents, including simian viruses. The number of viruses isolated and the frequency of isolation depend on many factors, including the method of isolation, test cell systems used, number of passages and duration of incubation and co-cultivation, and are directly proportional to the incubation period of the cultures. The frequency of contaminated cell cultures can be significantly reduced by careful screening of the source animals for the absence of antibodies to relevant viruses. The use of animals bred in a carefully controlled colony, especially those which are specific-pathogen free, is strongly recommended. The use of secondary or tertiary cells on which testing for adventitious agents can be performed will also reduce the frequency of contaminated production cell cultures.

Diploid cell substrates

The essential features of diploid cell lines of human (e.g. WI-38, MRC-5) or monkey (FRhL-2) origin are: they have a finite capacity for serial propagation, which ends in sensescence; and they are non-tumorigenic and display diploid cytogenetic characteristics with a low frequency of chromosomal abnormalities of number and structure. Substantial experience over the past 25 years has been accumulated on the karyology of WI-38 and MRC-5 diploid cell lines, and ranges of expected frequencies of chromosomal abnormalities have been published (*2*). More sophisticated cytogenetic techniques (e.g. banding) have demonstrated subtle chromosomal abnormalities that were previously undetectable, thus making the previously established ranges of abnormalities obsolete. Recent studies have shown that subpopulations of human diploid cells with such abnormalities may appear and disappear over time, and that they are non-tumorigenic and undergo senescence.

The possibility of using human diploid cell substrates for the production of viral vaccines was demonstrated more than 35 years ago. The experience gained with oral poliomyelitis and other viral vaccines in successfully immunizing millions of children in many countries has clearly demonstrated the safety of vaccines produced on such substrates (*3*).

The main advantage of diploid cell lines in comparison to primary cells is that they can be well characterized and standardized, and production can be based on a cell bank system. In addition, unlike the continuous cell lines discussed below, they possess a finite life and are not tumorigenic. The cell bank system usually consists of cell banks of defined passage levels and may include a master cell bank and a working cell bank.

However, diploid cell lines have the following disadvantages: they are not easy to use in large-scale production, such as bioreactor technology employing the microcarrier method; in general, they need a more demanding growth medium than other cell substrates; and they usually need larger quantities of bovine serum (either fetal or donor calf) for their growth than do continuous cell lines.

Continuous-cell-line substrates

Continuous cell lines have the potential for an infinite life span and can usually be cultivated as attached cells or in suspension in a bioreactor. They have been derived by the following methods: (a) serial subcultivation of a primary cell culture of a human or animal tumour cell, such as HeLa or Namalva cells; (b) transformation of a normal cell having a finite life span with an oncogenic virus, for example, a B lymphocyte transformed by the Epstein-Barr virus; (c) serial subcultivation of a normal cell population generating a new cell population having an infinite life span; or (d) fusion between a myeloma cell and an antibody-producing B lymphocyte.

While cell transformation can occur spontaneously in various animal cells grown *in vitro* (continuous cell lines from African green monkey kidney cells (Vero), baby hamster kidney cells (BHK21) and Chinese hamster ovary cells (CHO) were established in this way), it has not been reported with human cells derived from normal tissues.

Hybridoma cells express monoclonal antibodies and hybridoma cell lines have generally been established from rodent hybridomas. Human hybridomas are obtained by the transformation of a B lymphocyte with Epstein-Barr virus, usually followed by subsequent fusion with a murine myeloma cell.

Continuous cell lines are now considered to be suitable substrates for the production of many biological medicinal substances and possess distinct advantages over primary and diploid cell substrates (*4*). A cell bank system similar to that used for diploid cell lines provides a means for the production of biologicals for an indefinite period based on well characterized and standardized cells. Continuous cell lines tend to be less demanding than diploid cell lines; as a rule they grow

well using ordinary media and serum, and some do not require serum at all. They can also be used in microcarrier cultures and/or suspension cultures for large-scale production in bioreactors.

However, many continuous cell lines express endogenous viruses and are tumorigenic. Their theoretical disadvantages therefore include the risk of tumorigenicity associated with residual cellular DNA that may encode transforming proteins.

In 1986, a WHO Study Group considered a number of issues associated with the acceptability of new cell substrates for the production of biologicals (*5*) and concluded that, in general, continuous cell lines were acceptable for this purpose, but that differences in the nature and characteristics of the products and in manufacturing processes must be taken into account when making a decision on the use of a particular continuous cell line in the manufacture of a given product. WHO Requirements for Continuous Cell Lines used for Biologicals Production were published in 1987 (*6*).

In addition, the WHO Study Group recommended the establishment of well characterized cell lines that would be of value to national control authorities and manufacturers of biologicals. In following up this recommendation, WHO developed a WHO master cell bank for Vero cells, a continuous cell line established from the kidneys of African green monkeys. The reason for selecting this cell line was that it offered the immediate prospect of improving the quantity and quality of several vaccines being produced in other systems.

A master cell bank of Vero cells was donated to WHO, by a manufacturer, at the 134th passage. The maximum passage level recommended for production is 150. Studies of tumorigenicity in newborn rats suggest that cells in the passage range 134–150 are not tumorigenic. Collaborative studies in 10 laboratories with respect to sterility, adventitious agents, tumorigenicity, presence of reverse transcriptase and identity showed that the WHO Vero cell bank met the WHO Requirements for Continuous Cell Lines used for Biologicals Production (*6*).

The WHO master cell bank of Vero cells is stored at the European Collection of Animal Cell Cultures (ECACC), Porton Down, England and the American Type Culture Collection (ATCC), Rockville, MD, USA. Producers of biologicals and national control authorities can obtain cultures of these Vero cells (free of charge), as well as additional background information, from Biologicals, World Health Organization, 1211 Geneva 27, Switzerland (*7*).

Potential risks associated with biologicals produced in animal cells

The main potential risks associated with the use of biologicals produced in animal cells are directly related to contaminants from the cells, and they fall into three categories: viruses and other transmissible agents; cellular DNA; and growth-promoting proteins. A summary of the risk assessment for each follows. More comprehensive statements have been published elsewhere on the risks associated with contaminating DNA (*5*, *8–15*) and growth-promoting proteins (*5*).

Viruses and other transmissible agents

The 1986 WHO Study Group reviewed the potential risk to human recipients of products manufactured in cells containing viral agents. These may include complete viruses with known patterns of replication, such as simian virus 40 (SV40), virus particles such as type A retroviruses, which can be visualized by electron microscopy, and persisting viral genomes or parts of genomes, for example those of the hepatitis B and Epstein-Barr viruses. As described below, cells differ with respect to their potential for carrying viral agents pathogenic for human beings.

Primary monkey-kidney cells have been used to produce hundreds of millions of doses of poliomyelitis vaccines over the past 40 years, and although latent viruses, such as simian virus 40, were discovered in these cells, control measures were introduced to eliminate the risk associated with the manufacture of vaccines in cells containing those endogenous viruses. Additional controls may be needed as new viral agents and technologies are identified.

Human and nonhuman primate lymphocytes and macrophages may carry latent viruses, such as herpesvirus and retroviruses. Continuous lines of non-haematogenous cells from human and nonhuman primates may contain viruses or have viral genes integrated into their DNA. In either case, virus expression may occur under *in vitro* culture conditions.

Avian tissues and cells harbour exogenous and endogenous retroviruses, but there is no evidence for transmission of disease to humans from products prepared using these substrates. For example, large quantities of yellow fever, measles and live influenza vaccines have been produced for many years in eggs that contain avian leukosis viruses, but there is no evidence that these products have had any harmful effects in their long history of use for human immunization.

Rodents harbour exogenous and endogenous retroviruses. Lymphocytic choriomeningitis virus and haemorrhagic fever viruses from rodents have caused disease in humans by direct infection.

Human diploid fibroblasts have been used for vaccine production for over 30 years, and although concern was initially expressed about the possibility of the cells containing a latent human virus, no evidence for such an agent has been found, and vaccines produced from this class of cell have proved to be free from viral contaminants.

In light of the differing potential of the various types of cells mentioned above for transmitting viruses pathogenic in humans, different types of testing are appropriate for products manufactured using these cells.

When either diploid cell lines or continuous cell lines are used for production, a cell bank system is used and the cell bank is characterized as specified in the appropriate requirements published by WHO. Additional methods such as testing for viral sequences or other viral markers should also be considered. Efforts to identify viruses constitute an important part of the characterization of cell banks.

When cell lines of rodent or avian origin are examined for the presence of viruses, the major emphasis in risk assessment is placed on the results of studies in which transmission to target cells or animals is attempted. Risk to human recipients should not be assessed solely on ultrastructural evidence of the presence of viral agents in the cells.

The overall manufacturing process, including the selection and testing of cells and source materials, any purification procedures used and tests on intermediate or final products, has to be such as to ensure the absence of detectable infectious virus in the final product.

There may be as yet undiscovered microbial agents for which there is no current evidence or means of detection. As such agents become identified, it will be important to re-examine cell systems for their presence. Positive findings will have to be discussed with the national control authority.

Cellular DNA

Primary and diploid cells have been used successfully and safely for many years for the production of viral vaccines, and the residual cellular DNA deriving from these cells has not been (and is not) considered to pose any risk. Continuous cell lines have an infinite life span due to the deregulation of genes that control growth. The DNA deriving from such cell lines is therefore considered to have the

potential to confer the capacity for unregulated cell growth, or tumorigenic activity, upon other cells.

The 1986 WHO Study Group advised on the levels of contaminating DNA deriving from continuous cell lines used in the production of biologicals for human use (*5*). Risk assessment based on an animal oncogene model suggested that *in vivo* exposure to one nanogram (ng) of cellular DNA, where 100 copies of an activated oncogene were present in the genome, would give rise to a transformational event once in 10^9 recipients (*13*). On the basis of this and other available evidence, the Study Group concluded that the risk associated with residual continuous-cell-line DNA in a product is negligible when the amount of such DNA is 100 picograms (pg) or less per parenteral dose. In determining this limit, the perceived problem was not the DNA itself but rather minimizing the presence of specific DNA sequences coding for activated oncogenes.

Additional calculations suggest that the risk of insertional mutagenesis that could lead to a neoplastic event is extremely small. In one recent report, it was predicted that a 10-μg dose of DNA would result in the inactivation of two independent tumour-suppressor genes, by insertional mutagenesis, within a single cell of a vaccine recipient in only one of 10^7 recipients (*9*). These very low calculated levels of risk are consistent with the limited human and animal experience to date (*10*, *16–18*).

Additional data published recently have shown that milligram amounts of DNA containing an activated oncogene from human tumour cells have not caused tumours in nonhuman primates during an evaluation period of 10 years (*16*). Also, human blood contains substantial amounts of DNA in plasma (75–450 μg per unit of blood) (*19*, *20*). Furthermore, contaminating DNA in a biological product generally occurs as small fragments unlikely to encode a functional gene.

The assessment of the safety of a product with respect to residual cellular DNA has to take into account: (a) the low levels of risk implied by the considerations described above; (b) the possible inactivation of any biological activity of contaminating DNA during processing; and (c) any reduction in the level of contaminating DNA during the purification process. A product may be considered safe on the basis of (b) and/or (c).

The current state of knowledge suggests that continuous-cell-line DNA can be considered as a cellular contaminant, rather than as a significant risk factor requiring removal to extremely low levels. On the basis of this reassessment, the Expert Committee concluded that

levels of up to 10 ng per purified dose can now be considered acceptable. The purification process has to be validated by appropriate methods, including spiking studies, to demonstrate its capability to remove DNA to an acceptable level. In addition, batch-to-batch consistency needs to be shown for clinical trial batches and for three or more consecutive production batches. Subsequently, routine release testing for continuous-cell-line DNA in the final purified batch may not be needed. Any exceptions need to be agreed with the national control authority. For example, data suggest that β-propiolactone, a viral inactivating agent, may also destroy the biological activity of DNA; use of this agent therefore provides an additional level of confidence even when the amount of DNA per parenteral dose may be substantial (*21*). Data should be obtained on the effects of such inactivating agents under specific manufacturing conditions so that firm conclusions on their DNA-inactivating potential for a given product can be drawn.

There may be instances where continuous-cell-line DNA is considered to pose a greater risk, e.g. where it could include infectious retroviral provirion sequences. Under these circumstances, acceptable limits should be set in consultation with the national control authority.

The new upper limit of 10 ng of residual DNA per dose does not apply to products derived from microbial, diploid or primary-cell-culture systems. The 1986 WHO Study Group stated that the risks for continuous-cell-line DNA should be considered negligible for preparations given orally; for such products, the principal requirement is the elimination of potentially contaminating viruses and toxic proteins. The upper limit of 10 ng of residual continuous-cell-line DNA per dose therefore does not apply to a product given orally. Acceptable limits should be set in consultation with the national control authority.

Growth-promoting proteins

Growth factors may be secreted by cells used to produce biologicals, but the risks from these substances are limited, since their growth-promoting effects are usually transient and reversible, they do not replicate, and many of them are rapidly inactivated *in vivo*. In exceptional circumstances, growth factors can contribute to oncogenesis, but even in these cases, the tumours apparently remain dependent upon continued administration of the growth factor. Therefore, the presence of known growth-factor contaminants at ordinary concentrations does not constitute a serious risk in the preparation of biological products from animal cells.

Proteins prepared using continuous-cell-line substrates need to be purified to permit their safe clinical use. Analytical methods to assure the purity of each batch should be proposed and validated by the manufacturer. The purification process should also be validated to demonstrate its capability to remove host-cell proteins to an acceptable level. In addition, batch-to-batch consistency should be shown for clinical trial batches and for three or more consecutive production batches. Subsequently, routine release testing for host cell proteins in the final purified batch may not be needed.

Requirements published by WHO

The first requirements published by WHO for cell cultures used for the production of biologicals were formulated in 1959 for the production of inactivated poliomyelitis vaccine in primary cell substrates (*22*). They were revised in 1965 (*23*). The successful use of primary cell cultures derived from the kidneys of clinically healthy monkeys for the production of both inactivated and oral poliomyelitis vaccine (*24*) led to confidence in the use of other cell cultures for the production of various viral vaccines. Many types of cell culture are now widely used for the production not only of viral vaccines, but also of other biologicals, such as monoclonal antibodies and a wide range of biologicals prepared using recombinant DNA technology.

Taking into account the latest available data relating to cell substrates and after extensive consultation, especially at a WHO/International Association of Biological Standardization/Mérieux Foundation International Symposium on the Safety of Biological Products prepared from Mammalian Cell Culture held in Annecy, France, in September 1996, the WHO Expert Committee on Biological Standardization adopted the text of this Annex as requirements appropriate for the quality control of animal cells used as *in vitro* substrates for the production of biologicals. They supersede previous requirements describing procedures for the growth and quality control of cell substrates for the production of biologicals (*5*, *6*) and should be read in conjunction with the requirements published by WHO for individual products.

The following requirements concern the characterization and testing of continuous-cell-line and diploid cell substrates for the production of both viral vaccines and other biologicals, such as monoclonal antibodies and products prepared using recombinant DNA technology. These requirements specifically exclude DNA vaccines manufactured in microbial cells. Some of the general manufacturing requirements given here (see sections A.2 and A.3) are also applicable to primary cell substrates. Specific requirements for primary cell cultures can be

found in the relevant requirements published by WHO (e.g. production of oral poliomyelitis vaccine in primary monkey kidney cells (25)).

Whenever practicable, manufacturers are encouraged to use cell substrates that can be generated from master cell banks that have been thoroughly characterized.

Requirements published by WHO are intended to be scientific and advisory in nature. The parts of each section printed in normal type have been written in the form of requirements so that, should a national control authority so desire, they may be adopted as they stand as the basis of national requirements. The parts of each section printed in small type are comments or recommendations for guidance.

Part A. General manufacturing requirements applicable to all types of cell culture production

A.1 Definitions

Cell bank: A cell bank is a collection of ampoules containing material of uniform composition stored under defined conditions, each ampoule containing an aliquot of a single pool of cells.

Cell seed: A quantity of well characterized cells of human, animal or other origin stored frozen at −100 °C or below in aliquots of uniform composition derived from a single tissue or cell, one or more of which would be used for the production of a master cell bank.

Master cell bank: A quantity of fully characterized cells of human, animal or other origin stored frozen at −100 °C or below in aliquots of uniform composition derived from the cell seed. The master cell bank is itself an aliquot of a single pool of cells generally prepared from a selected cell clone under defined conditions, dispensed into multiple containers and stored under defined conditions. The master cell bank is used to derive all working cell banks. The testing performed on a replacement master cell bank (derived from the same cell clone, or from an existing master or working cell bank) is the same as for the initial master cell bank, unless a justified exception is made.

Working cell bank: A quantity of cells of uniform composition derived from the master cell bank at a finite passage level, dispensed in aliquots into individual containers appropriately stored, usually frozen at −100 °C or below, one or more of which would be used for production purposes. All containers are treated identically and, once removed from storage, are not returned to the stock.

Production cell cultures: A collection of cell cultures used for biological production that have been prepared together from one or more containers from the working cell bank or, in the case of primary cell cultures, from the tissues of one or more animals.

Adventitious agents: Contaminating microorganisms of the cell culture or line including bacteria, fungi, mycoplasmas and viruses that have been unintentionally introduced.

In vitro culture age: Duration between the thawing of the master cell bank container(s) and the harvest of the production vessel's cell culture as measured by elapsed chronological time in culture, by the population doubling level of the cells, or by the passage level of the cells when subcultivated by a defined procedure for dilution of the culture.

A.2 Good manufacturing practices

The general manufacturing requirements contained in Good Manufacturing Practices for Pharmaceutical (*26*) and Biological (*27*) Products shall apply. Where open manipulations of cells are performed, simultaneous open manipulations of other cell lines shall be avoided to prevent cross-contamination.

Cell cultures shall be prepared by staff who have not, on the same working day, handled animals or infectious microorganisms. The personnel concerned shall be periodically examined medically and found to be healthy.

Particular attention shall be given to the recommendations in Good Manufacturing Practices for Biological Products (*27*) regarding the training and experience of the staff in charge of production and testing and of those assigned to various areas of responsibility in the manufacturing establishment, as well as to the registration of such personnel with the national control authority.

Penicillin or other β-lactam antibiotics shall not be present in production cell cultures.

> Minimal concentration of other antibiotics may be acceptable. However, the presence of any antibiotic in a biological process or product is discouraged.

A.2.1 *Selection of source materials*

For all types of cells, the donor shall be free of communicable diseases or diseases of uncertain etiology, such as Creutzfeldt-Jakob disease for humans and bovine spongiform encephalopathy (BSE) for cattle.

The national control authority may allow specific exceptions concerning donor health (e.g. myeloma and other tumour cells).

Cells of neurological origin may contain or be capable of amplifying the agent causing spongiform encephalopathies, and shall not be used in the manufacture of medicinal products, apart from cases for which a reasoned exception has been made (*28*).

The national control authority shall approve source(s) of animal-derived raw materials, such as serum and trypsin. These materials shall comply with the guidelines given in the *Report of a WHO Consultation on Medicinal and other Products in relation to Human and Animal Transmissible Spongiform Encephalopathies* (*29*). They shall be subjected to appropriate tests for quality and freedom from contamination by viruses, fungi, bacteria and mycoplasmas to evaluate their acceptability for use in production.

The reduction and elimination from the manufacturing process of raw materials derived from animals and humans is encouraged where feasible.

For some animal-derived raw materials used in the cell culture medium, such as insulin or transferrin, validation of the production process for the elimination of viruses can substitute for virus detection tests.

A.3 Tests applicable to all types of cell cultures

A.3.1 *Tests for viral agents*

Tests shall be undertaken to detect, and where possible identify, any endogenous or exogenous viral agents that may be present in the cells. Special attention shall be given to tests for agents known to be latent in the species from which the cells were derived (e.g. simian virus 40 in rhesus monkeys).

For primary cell cultures, the principles and procedures outlined in Part C, Requirements for Poliomyelitis Vaccine (Oral) (*25*), together with those in section A.4 of Requirements for Measles, Mumps and Rubella Vaccines and Combined Vaccine (Live) (*30*) may be followed. For continuous cell lines and diploid cell substrates see parts B and C below.

A.3.2 *Serum used in cell-culture media*

Serum used for the propagation of cells shall be tested to demonstrate freedom from cultivable bacteria, fungi and mycoplasmas, as specified in Part A, sections 5.2 (*31*) and 5.3 (*32*) of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances), and from infectious viruses.

Suitable tests for detecting viruses in bovine serum are given in Appendix 1 of the revised Requirements for Biological Substances No. 7 (Requirements for

Poliomyelitis Vaccine, Oral) (*25*). Where appropriate, more sensitive methods may be used.

In some countries, sera are also examined for freedom from certain phages.

In some countries, irradiation is used to inactivate potential contaminant viruses.

The acceptability of the source(s) of serum of bovine origin shall be approved by the national control authority (see A.2.1).

Human serum shall not be used. If human albumin is used, it shall meet the revised Requirements for Biological Substances No. 27 (Requirements for the Collection, Processing and Quality Control of Blood, Blood Components and Plasma Derivatives) (*33*), as well as the guidelines contained in *Report of a WHO Consultation on Medicinal and other Products in relation to Human and Animal Transmissible Spongiform Encephalopathies* (*29*).

A.3.3 *Trypsin used for preparing cell cultures*

Trypsin used for preparing cell cultures shall be tested and found free of cultivable bacteria, fungi, mycoplasmas and infectious viruses, especially bovine or porcine parvoviruses, as appropriate. The methods used to ensure this shall be approved by the national control authority.

The source(s) of trypsin of bovine origin shall be approved by the national control authority (see A.2.1).

In some countries, irradiation is used to inactivate potential contaminant viruses.

A.3.4 *Tests for bacteria, fungi and mycoplasmas at the end of production*

A volume of 20 ml of the pooled supernatant fluids from the production cell cultures shall be tested for bacteria, fungi and mycoplasmas as specified in Part A, sections 5.2 (*31*) and 5.3 (*32*) of the Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances), by a method approved by the national control authority.

A.3.5 *Tests for adventitious viruses at the end of production*

For virus-based products, control cell cultures are necessary when the product interferes with the test systems used to monitor the absence of adventitious agents. These control cell cultures shall be observed at the end of the production period for viral cytopathic effects and tested for haemadsorbing viruses. If multiple harvest pools are prepared at

different times, the cultures shall be observed and tested at the time of the collection of each pool.

In some countries, 25% of the control cell cultures are tested for haemadsorbing viruses using guinea-pig red cells. If the red cells have been stored, the duration of storage should not have exceeded 7 days, and the temperature of storage should have been in the range 2–8 °C. In tests for haemadsorbing viruses, calcium and magnesium ions should be absent from the medium.

In some countries, the national control authority also requires that other types of red cells, including cells from humans (blood group IV O), monkeys and chickens (or other avian species), should be used in addition to guinea-pig cells. In all tests, readings should be taken after incubation for 30 minutes at 0–4 °C, and again after a further incubation for 30 minutes at 20–25 °C. For the test with monkey red cells, readings should also be taken after a final incubation for 30 minutes at 34–37 °C.

For recombinant DNA proteins, monoclonal antibodies and other cell-based products, the unprocessed bulk harvest or a lysate of cells and their production culture medium shall be tested.

At the time of production of each unprocessed bulk pool, an appropriate volume of the pool shall be inoculated onto monolayer cultures of at least the following cell types:

- Cultures (primary or continuous cell line) of the same species and tissue type as that used for production. This may not be possible for some continuous cell lines (e.g. hybridomas).
- Cultures of a human diploid cell line.
- Cultures of another cell line from a different species.

The unprocessed bulk-pool sample to be tested shall be diluted as little as possible. Material from at least 10^7 cells and spent culture fluids shall be inoculated onto each of the three cell types. The resulting co-cultivated cell cultures shall be observed for evidence of adventitious viruses for at least 2 weeks. If the product is from a continuous cell line known to be capable of supporting the growth of human cytomegalovirus, human diploid cell cultures shall be observed for at least 4 weeks.

Extended cell culture for the purposes of identifying human cytomegalovirus can be replaced by the use of specific probes to detect cytomegalovirus nucleic acid.

At the end of the observation period, aliquots of each of the three co-cultivated cell culture systems shall be tested for haemadsorbing viruses.

Part B. Requirements for continuous-cell-line substrates

B.1 General considerations

Several types of continuous cell line have been employed as substrates in the production of biologicals, including Vero cells in the preparation of live and inactivated viral vaccines and the use of CHO cells in the production of a number of recombinant proteins. The advantage of such cell lines is that they grow relatively rapidly and provide high yields of monolayer or, in some cases, suspension cultures.

Continuous cell lines may have biochemical, biological and genetic characteristics that differ from primary or diploid cells. In particular, they may produce transforming proteins and may contain potentially oncogenic DNA. In some cases, continuous cell lines may cause tumours when inoculated into animals. The manufacturing process for the production of biologicals in continuous-cell-line substrates should take these factors into account in order to ensure the safety of the product. Generally, purification procedures will result in the extensive removal of cellular DNA, other cellular components and potential adventitious agents. Procedures that extensively degrade or denature DNA might be appropriate for some products (e.g. rabies vaccine). When continuous cell lines are being contemplated for use in the development of live viral vaccines, careful consideration must be given to the possible incorporation of oncogenic cellular DNA into the virions.

Production of biologicals from continuous-cell-line substrates should be based on well defined master and working cell banks. The master cell bank is generally derived from a selected cell clone. The working cell bank is derived by expansion of one or more containers of the master cell bank.

Evidence that the cell line is free from cultivable bacteria, mycoplasmas, fungi and infectious viruses, and where appropriate, potentially oncogenic adventitious agents should be provided. Special attention should be given to viruses that commonly contaminate the animal species from which the cell line is derived. Cell seed should preferably be free from all adventitious agents. However, certain cell lines express endogenous viruses, e.g. retroviruses. Tests capable of detecting such agents should be carried out on cells grown under production conditions, and the results should be reported. Specific contaminants identified as endogenous agents in the master and working cell banks should be shown to be inactivated and/or removed by the purification

procedure used in production. The validation of the purification procedure used is also considered essential (*34*) (see Appendix).

The data required for the characterization of any continuous cell line to be used for the production of biologicals include: a history of the cell line and a detailed description of the production of the cell banks, including methods and reagents used during culture, *in vitro* culture age, and storage conditions; the results of tests for infectious agents; distinguishing features of the cells, such as biochemical, immunological or cytogenetic patterns which allow them to be clearly distinguished from other cell lines; and the results of tests for tumorigenicity, including data from the scientific literature.

Special consideration should be given to products derived from cells that contain known viral genomes (e.g. Namalva cells). Cells modified by recombinant DNA technology have been increasingly used in the manufacture of novel medicinal products and specific considerations for those products are addressed elsewhere (*35*, *36*).

Continuous cell lines should be characterized so that appropriate controls for the purity and safety of the final product can be included. For example, if a continuous cell line contains an endogenous virus, tests to ensure the absence of any detectable biological activity of that virus could be incorporated as one of the requirements for products derived from that cell line. Alternatively, process validation may replace testing at the end of production for endogenous viruses when a high degree of assurance of consistency of virus clearance can be provided.

There has been considerable discussion internationally on general criteria for the acceptability of products (e.g. hormones, blood components, viral vaccines) prepared from continuous cell lines. A consensus has emerged on the general desirability of achieving a high degree of purification of the product, involving significant removal or destruction of DNA of cell substrate origin. Manufacturers considering the use of continuous cell lines should be aware of the need to develop and evaluate efficient methods for purification as an essential element of any product development programme.

While all continuous cell lines, by definition, have an infinite life span, they may express no tumorigenic properties below a certain passage (or population-doubling) level, but subsequently display increasing evidence of the tumorigenic phenotype with increasing passage. It is therefore important to establish an age limit for *in vitro* cultures beyond which they cannot be used for production. The limit should be based on data derived from production cells expanded under pilot

plant-scale or full-scale conditions to the proposed *in vitro* culture age limit or beyond. Generally, the production cells are obtained by expansion of the working cell bank; however, the master cell bank could be used to prepare the production cells, given appropriate justification. Increases in the established *in vitro* culture age limit for production should be supported by data from cells that have been expanded to an *in vitro* culture age that is equal to or greater than the proposed new limit.

The following Requirements concern the characterization and testing of continuous cell lines used for the production of biologicals. They should be read in conjunction with the general manufacturing requirements applicable to all cell cultures contained in part A of these Requirements. Specific requirements for purity as well as other quality control procedures will be incorporated in requirements published by WHO for individual biological products.

B.2 Manufacturing requirements

B.2.1 *Certification of continuous cell lines for use in the production of biologicals*

A continuous cell line used for biologicals production shall be approved by the national control authority and shall be identified by historical records that include information on the origin of the cell line, its method of development and the *in vitro* culture age limit for production.

A continuous cell line used for biologicals production shall also be characterized with respect to genealogy, genetic markers (e.g. histocompatibility leukocyte antigen (HLA), DNA fingerprinting), viability during storage, and growth characteristics at passage levels (or population doublings or time-in-culture, as appropriate) equivalent to, or beyond, those of the master and working cell banks and the cell cultures used for production.

B.2.2 *Cell banks*

The use of continuous cell lines for the manufacture of biological products shall be based on the cell bank system, which shall include a well defined master cell bank and working cell bank.

The cell bank used for the production of biologicals shall be that approved by and registered with the national control authority. The continuous cell line from which the master cell bank has been derived shall be characterized as described in section B.1. The working cell bank shall be shown to yield cell cultures capable of producing biologicals that are both safe and efficacious in humans.

In section B.2.3, extensive testing directed at identifying exogenous and endogenous agents that may be present in the cell line is described; special attention is given to agents known to be present in a latent state in the species from which the cells were derived. Such extensive testing need only be performed once, on either the master cell bank or a working cell bank. Once a continuous cell line has been characterized in this respect, further testing of working cell banks or production cell cultures is restricted to tests directed at detecting common adventitious agents that could have contaminated the cultures during their preparation.

The tumorigenicity testing described in section B.2.3.7 shall be performed only once on cells of either the master cell bank or a working cell bank propagated to an *in vitro* culture age at or beyond the limit for production. If the cell line has already been documented to be tumorigenic or if the class of cells to which it belongs (e.g. hybridomas) is tumorigenic, the cell line may be presumed to be tumorigenic and tumorigenicity tests need not be undertaken.

Both the master and working cell banks shall be stored at –100 °C or below (i.e. in either the liquid or vapour phase of liquid nitrogen). The location, identity and inventory of individual ampoules of cells shall be thoroughly documented.

It is recommended that the master and working cell banks should each be stored in at least two widely separated areas within the production facility in order to avoid accidental loss of the cell line.

B.2.3 ***Identification and characteristics of continuous cell lines***

The characterization of a continuous cell line intended for use in the manufacture of biologicals shall include information on: the history and general characteristics of the cell line; the cell bank system; and quality control testing. These data shall be made available to the national control authority.

B.2.3.1 *Identity test*

The cell banks shall be identified by a method approved by the national control authority.

Methods for identity testing include, but are not limited to, biochemical (e.g. isoenzyme analyses), immunological (e.g. HLA assays), cytogenetic tests (e.g. for chromosomal markers), and tests for genetic markers (DNA fingerprinting).

B.2.3.2 *Sterility tests*

A volume of 20 ml of supernatant fluids from cell cultures derived from at least one ampoule of the master and working cell banks shall

be tested for bacteria, fungi and mycoplasmas. Tests shall be performed as specified in Part A, sections 5.2 (*31*) and 5.3 (*32*) of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances, by a method approved by the national control authority.

B.2.3.3 *Tests for viral agents using cell cultures*

Live or disrupted cells and spent culture fluids of the master or working cell bank shall be inoculated onto monolayer cultures or cocultivated with monolayer cultures, as appropriate, of the following cell types:

- Cultures (primary or continuous cell line) of the same species and tissue type as the continuous cell line. This may not be possible for some continuous cell lines, e.g. hybridomas.
- Cultures of a human diploid cell line.
- Cultures of another cell line from a different species.

The sample to be tested shall be diluted as little as possible. Material from at least 10^7 cells and spent culture fluids shall be inoculated onto each of the three cell types. The resulting cultures shall be observed for at least 2 weeks for evidence of adventitious viruses. If the continuous cell line being tested is known to be capable of supporting the growth of human cytomegalovirus, human diploid cell cultures shall be observed for at least 4 weeks.

> Extended cell culture for the purposes of identifying human cytomegalovirus can be replaced by the use of specific probes to detect cytomegalovirus nucleic acid.

At the end of the observation period, aliquots of each of the three cell culture systems shall be tested for haemadsorbing viruses.

B.2.3.4 *Tests for viral agents using animals and eggs*

The cells of the master and working cell banks are suitable for production if none of the animals or eggs shows evidence of the presence of any viral agent attributable to the cell banks.

Tests in animals. Tests in animals for pathogenic viruses shall include the inoculation by the intramuscular route of each of the following groups of animals with cells from the master or working cell banks, propagated to or beyond the maximum *in vitro* culture age (or population doubling, as appropriate) used for production, where at least 10^7 viable cells are divided equally among the animals in each group:

- two litters of suckling mice, comprising a total of at least ten animals, less than 24 h old; and
- ten adult mice weighing 15–20 g.

In some circumstances, tests in five guinea-pigs weighing 350–450 g and five rabbits weighing 1.5–2.5 kg may be considered.

The test in rabbits for the presence of B virus in cell lines of simian origin may be replaced by a test in rabbit kidney-cell cultures.

The animals shall be observed for at least 4 weeks. Any animals that are sick or show any abnormality shall be investigated to establish the cause. The test is not valid if more than 20% of the animals in the test group become sick for non-specific reasons and do not survive the observation period.

In some countries, the suckling and adult mice are also inoculated by the intracerebral route.

If the cell line is of rodent origin, at least 10^6 viable cells shall be injected intracerebally into each of ten susceptible adult mice to test for the presence of lymphocytic choriomeningitis virus.

Tests in eggs. At least 10^6 viable cells from the master or working cell banks, propagated to or beyond the maximum *in vitro* culture age (or population doubling, as appropriate) shall be injected into the allantoic cavity of each of ten embryonated chicken eggs, and the yolk sac of each of another ten embryonated chicken eggs. The eggs shall be examined after not less than 5 days of incubation. The allantoic fluids of the eggs shall be tested with red cells from guinea-pig and chickens (or other avian species) for the presence of haemagglutinins. The test is not valid if more than 20% of the embryonated chicken eggs in the test group are discarded for non-specific reasons.

Usually, the eggs used for the yolk sac test should be 5–6 days old. The eggs used for the allantoic cavity test should be 9–11 days old.

Alternative ages for the embryonated chicken eggs and alternative incubation periods are acceptable if they have been determined to be capable of detecting the presence of routine adventitious agents in the test samples.

B.2.3.5 *Tests for retroviruses and other endogenous viruses or viral nucleic acid*

Test samples from the master or working cell banks, propagated to or beyond the maximum *in vitro* culture age (or population doubling, as appropriate) shall be examined for the presence of retroviruses using the following techniques:

- infectivity assays (if the infectivity assay is positive, tests for reverse transcriptase are not necessary);

- transmission electron microscopy (TEM); and
- reverse transcriptase (RTase) assays (performed in the presence of magnesium and manganese) on pellets obtained from fluids by high speed centrifugation (e.g. 125000*g* for 1 h) at 4 °C.

Recently developed highly sensitive RTase assays may be considered, but the results need to be interpreted with caution because RTase activity is not unique to retroviruses and may derive from other sources, such as retrovirus-like elements which do not encode a complete genome or cellular DNA polymerase.

It is often possible to increase the sensitivity of cell-culture infectivity assays by first inoculating the test material onto human cell lines that can support retroviral growth in order to amplify any retrovirus contaminant that may be present at low concentrations. For non-murine retroviruses, test cell lines should be selected for their capacity to support the growth of a broad range of retroviruses, including viruses of human and non-human primate origin (*37*, *38*).

For murine retroviruses, amplification of low-level contaminants may be achieved by co-cultivation of cells with a highly susceptible cell line, e.g. *Mus dunni* cells (*39*). The latter are susceptible to infection by all tested murine leukaemia viruses except Moloney murine leukaemia virus. For that reason, another susceptible cell, for example SC-1 (*40*), should also be used. Fluid from the resulting co-cultures should be further passaged on *Mus dunni* or other susceptible cells and subsequently assayed for murine leukaemia virus.

A variety of other assays may be useful, depending on the circumstances. Some examples of such assays include viable cell immunofluorescence (IFA) on *Mus dunni* cells co-cultivated with the test cells using a broadly reactive monoclonal antibody (e.g. HY95) for the detection of ecotropic, xenotropic, mink-cell focus-forming and amphotropic viruses; feline S + L assays using PG4 cells (*41*) for detection of amphotropic viruses; mink S + L assays for detection of xenotropic viruses (*12*) and mouse S + L assays using D56 (*42*) cells for detection of ecotropic viruses.

Murine and other rodent cell lines or hybrid cell lines containing a rodent component should be assumed to be inherently capable of producing infectious retroviruses. For murine cell lines used for monoclonal antibody production, the extent of testing for specific retroviruses may be reduced. However, the manufacturing process should be evaluated for removal and/or inactivation of retroviruses. For murine–human hybrid cell lines, additional concerns arise. Any proposed testing should be discussed with the national control authority on a case-by-case basis.

Probe hybridization/polymerase-chain-reaction amplification and virus-specific monoclonal antibody detection may provide additional information on the presence or absence of specific contaminants.

B.2.3.6 *Tests for selected viruses*

The following tests shall be undertaken on a selected basis on samples from the working cell bank propagated to or beyond the maximum *in vitro* culture age (or population doubling, as appropriate).

Murine cell lines shall be tested for species-specific viruses using mouse, rat and hamster antibody production tests. *In vivo* testing for lymphocytic choriomeningitis virus, including a challenge for non-lethal strains, is required for such cell lines as specified in B.2.3.4.

Human cell lines shall be screened for human viral pathogens such as Epstein-Barr virus, cytomegalovirus, human retroviruses, and hepatitis B and C viruses with appropriate *in vitro* techniques. Selection of the viruses to be screened for shall take into account the tissue source and medical history of the person from whom the cell line was derived. Tests for retroviruses are specified in section B.2.3.5.

The use of other cell cultures also may be appropriate for the characterization of cell banks, depending on the cell type and source of the cell line being characterized (*17*). Under certain circumstances, specific testing for the presence of other transforming viruses, such as papillomavirus, adenovirus and herpesvirus 6 and 7, may also be indicated.

B.2.3.7 *Tests for tumorigenicity*

If the continuous cell line has already been demonstrated to be tumorigenic (e.g. BHK21, CHO, C127), or if the class of cells to which it belongs, for example hybridoma, is tumorigenic, it is not necessary to require additional tumorigenicity tests. A new cell line shall be presumed to be tumorigenic unless data demonstrate that it is not. If a manufacturer proposes to characterize the cell line as non-tumorigenic, the following tests shall be undertaken.

Tests in vivo. Cells from the master or working cell bank propagated to or beyond the *in vitro* culture age limit for production shall be examined for tumorigenicity in a test approved by the national control authority. The test shall involve a comparison between the continuous cell line and a suitable positive reference preparation (e.g. HeLa, Hep 2 or FL cells).

A negative control is not essential but desirable. For that purpose non-tumorigenic diploid cell lines such as WI-38 or MRC-5 may be used.

Animal systems that have been shown to be suitable for this test include:

(a) athymic mice (*Nu/Nu* genotype);
(b) newborn mice, rats or hamsters that have been treated with antithymocyte serum or globulin; and
(c) thymectomized and irradiated mice that have been reconstituted (T–, B+) with bone marrow from healthy mice.

Whichever animal system is selected, the cell line and the reference cells are injected into separate groups of ten animals each. In both cases, the inoculum for each animal is 10^7 cells suspended in a volume

of 0.2 ml, and the injection is by either the intramuscular or the subcutaneous route. In the case of newborn animals (b), the animals are treated with 0.1 ml of antithymocyte serum or globulin on days 0, 2, 7 and 14 after birth. A potent serum or globulin is one that suppresses the immune mechanisms of the growing animals to the extent that the subsequent inoculum of 10^7 positive reference cells regularly produces tumours and metastases.

At the end of the observation period all animals, including the reference group(s), shall be killed and examined for gross and microscopic evidence of the proliferation of inoculated cells at the site of injection and in other organs (e.g. lymph nodes, lungs, kidneys and liver).

In all test systems, the animals shall be observed and palpated at regular intervals for the formation of nodules at the sites of injection. Any nodules formed should be measured in two perpendicular dimensions, the measurements being recorded regularly to determine whether there is progressive growth of the nodule. Animals showing nodules which begin to regress during the period of observation shall be killed before the nodules are no longer palpable, and processed for histological examination. Animals with progressively growing nodules shall be observed for 1–2 weeks. Among those without nodule formation, half shall be observed for 3 weeks and half for 12 weeks before they are killed and processed for histological examination. A necropsy shall be performed on each animal and shall include examination for gross evidence of tumour formation at the site of inoculation and in other organs such as lymph nodes, lungs, brain, spleen, kidneys and liver. All tumour-like lesions and the site of inoculation shall be examined histologically. In addition, since some cell lines may give rise to metastases without evidence of local tumour growth, any detectable regional lymph nodes and the lungs of all animals shall be examined histologically.

For the test to be considered valid, progressively growing tumours must be produced in at least nine of ten animals injected with the positive reference cells.

In vitro tests may be considered sufficient by some national control authorities.

Two *in vitro* tests that have been found to provide useful additional information on tumorigenicity are: colony formation in soft agar gels, and production of invasive cell growth following inoculation onto organ cultures. They may be used to characterize more fully the cell lines that show no evidence of tumorigenicity in animal tests (see above), or when the results are equivocal.

As the cells used in the production of biologicals may contain activated oncogenes, assays of cell transformation with DNA derived from a continuous cell line at the limit for *in vitro* culture age for production should be considered in order to determine whether or not activated oncogenes can be detected. The 3T3 assay system has been found useful for *ras* assays. Additional tests may also be considered as new techniques are developed for the detection of a broader range of oncogenes.

B.2.3.8 *Tests on cells carrying a recombinant-DNA expression system*

Data shall be obtained demonstrating that a continuous cell line can be used for its intended purpose. If a continuous cell line contains an expression construct to produce a recombinant DNA-derived protein, data shall be obtained to demonstrate the consistent quality and quantity of the protein it produces throughout the proposed *in vitro* culture age range for production (*14*, *15*). Studies shall be performed to determine whether manipulation of the cell line in order to produce a product by transfection changes its biological characteristics significantly, for instance conversion to the tumorigenic phenotype. Any such change must be taken into account in product development and in assessing approaches taken to assure an acceptable product.

The International Conference on Harmonisation has issued additional useful information (*43*).

B.2.4 *Production cell cultures*

Characterization of the product and routine monitoring for adventitious agents during the production process are part of the quality control of biological products.

The choice of method for quality control of the production cell substrate depends on the nature of the propagation system used. Cell substrates are propagated as monolayer cultures, in suspension cultures or in bioreactors, and can be maintained on a short-term, a long-term or even on a potentially indefinite basis. The product is obtained either from a single harvest of cell culture fluid or from multiple harvests. In some cases, quality control testing may need to be performed on each harvest before pooling into a bulk lot. The management of cell substrates for the purposes of quality control testing should be designed to optimize sensitivity of the testing.

B.2.4.1 *Serum used in cell-culture media*

Serum used in cell-culture media shall be tested as specified in section A.3.2.

B.2.4.2 *Trypsin used for preparing cell cultures*

Trypsin used for preparing cell cultures shall be tested as specified in section A.3.3.

B.2.4.3 *Identity test*

For viral vaccines, an identity test shall be performed on the control cell culture as described in section B.2.3.1. For recombinant DNA proteins and monoclonal antibodies, the presence of the protein at consistent levels in the harvest is an adequate confirmation of identity and purity.

B.2.4.4 *Tests for bacteria, fungi and mycoplasmas at the end of production*

Tests for bacteria, fungi and mycoplasmas shall be conducted on the production culture supernatant or lysate as specified in section A.3.4.

B.2.4.5 *Tests for adventitious viruses at the end of production*

Tests for adventitious viruses shall be conducted on the production culture supernatant or lysate as specified in section A.3.5.

Part C. Requirements for diploid cell substrates

C.1 General considerations

Two human diploid cell lines, WI-38 and MRC-5, derived from embryo lung tissue, have been in widespread use for many years for the production of live virus vaccines, including oral poliomyelitis, measles, mumps, rubella and varicella vaccines, and inactivated vaccines, for example, rabies and hepatitis A vaccines. In addition, a rhesus diploid cell line, FRhL-2, has been in limited use for rabies vaccine production. These substrates have been found to be safe and to produce vaccines that stimulate effective immunity without untoward reactions attributable to the cell substrate.

The following requirements concern the characterization and testing of diploid cell lines used for the production of biologicals. They should be read in conjunction with the general manufacturing requirements applicable to all cell cultures contained in Part A of these Requirements.

C.2 Manufacturing requirements

C.2.1 *Certification of diploid cell lines for use in the production of biologicals*

A diploid cell line used for biologicals production shall be approved by the national control authority and shall be identified by historical

records that include information on the origin of the cell line, its method of development and the range of passage levels at which it can be used in biologicals production.

A new diploid cell line (e.g. other than WI-38, MRC-5 and FRhL-2) used for biologicals production shall be characterized with respect to genealogy, genetic markers (e.g. HLA, DNA fingerprinting), or other markers of identity acceptable to the national control authority, as well as for viability during storage. In addition, data must be obtained to establish the cell line's diploid character and growth characteristics at *in vitro* culture ages equivalent to, or beyond, those of the master and working cell banks, and of the cell cultures used for production.

Accumulated experience suggests that WI-38 and MRC-5 can be used for production until 10 generations before senescence.

C.2.2 ***Cell banks***

C.2.2.1 *Master cell bank and working cell bank*

Tests shall be performed on the master and working cell banks as described in section C.2.3, where appropriate and approved by the national control authority. In addition, for a new diploid cell line (e.g. other than WI-38, MRC-5, and FRhL-2) the cells of the working cell bank shall be shown to be diploid and stable with respect to karyology by the tests outlined in section C.2.3.5.

C.2.3 ***Identification and characteristics of diploid cell lines***

The characterization of a diploid cell intended for use in the manufacture of biologicals shall include information on: the history and general characteristics of the cell line; the cell bank system; and quality control testing. These data shall be made available to the national control authority.

C.2.3.1 *Identity tests*

An identity test shall be performed on the master cell bank by a method approved by the national control authority.

Methods for identity testing include, but are not limited to, biochemical tests (e.g. isoenzyme analyses), immunological tests (e.g. HLA assays), cytogenetic tests (e.g. for chromosomal markers), and tests for genetic markers (DNA fingerprinting).

Tests to ensure that the master cell bank is not contaminated with a continuous cell line shall be performed.

Tests of identity such as DNA fingerprinting of appropriate sensitivity, karyology at different levels of passage or studies of lifespan in culture may be used for this purpose if approved by the national control authority.

C.2.3.2 *Sterility tests*

Tests for bacteria, fungi and mycoplasmas shall be conducted in cell cultures as specified in section B.2.3.2.

C.2.3.3 *Tests for viral agents using cell cultures*

Tests for viral agents shall be conducted in cell cultures as specified in section B.2.3.3.

C.2.3.4 *Tests for viral agents using animals and eggs*

Tests for viral agents shall be conducted in animals and eggs as specified in section B.2.3.4.

C.2.3.5 *Chromosomal characterization of a diploid cell line*

The usefulness of chromosomal characterization depends on the nature of the product and the manufacturing process. In general, products that might contain live cells or which have little "downstream" purification will require chromosomal characterization of the cell line. Such products manufactured in cells identified to be WI-38, MRC-5 or FRhL-2 cells do not require recharacterization of the cell substrate by karyology, unless the cells have been genetically modified.

The utility of chromosomal monitoring of the cell substrate for unpurified products manufactured in other cell lines shall be evaluated on a case-by-case basis. However, products that contain no cells and are highly purified will not require this test.

For the determination of the general character of a new diploid cell line (i.e. other than WI-38, MRC-5 and FRhL-2), samples from the master cell bank shall be examined at approximately four equally spaced intervals over the life span of the cell line during serial cultivation through to senescence. Each sample shall consist of a minimum of 200 cells in metaphase and shall be examined for exact counts of chromosomes and for frequency of hyperdiploidy, hypoploidy, polyploidy, breaks and structural abnormalities. The acceptablity of any new diploid cell line shall be determined by the national control authority.

It is recommended that photographic reconstruction should be employed to prepare chromosome-banded karyotypes of an additional ten metaphase cells.

Stained slide preparations of the chromosomal characterization of the diploid cell line, or photographs of these, shall be maintained permanently as part of the cell line record.

C.2.3.7 *Tests for tumorigenicity*

The tumorigenic potential of a new diploid cell line (i.e. other than WI-38, MRC-5 and FRhL-2) shall be tested as specified in section B.2.3.7 as part of the characterization of the cell line, but is not required on a routine basis.

If satisfactory data from at least two independent laboratories are available, further tumorigenicity testing may not be required. The adequacy of tumorigenicity testing of a new diploid cell line should be discussed with the national control authority. Positive results should be discussed with the authority, taking into consideration the purity of the product, including residual cellular DNA.

C.2.3.8 *Tests on cells carrying a recombinant-DNA expression construct*

Data shall be obtained demonstrating that a diploid cell line can be used for its intended purpose. If a cell line contains an expression construct to produce a recombinant-DNA-derived protein, data shall be obtained to demonstrate the consistent quality and quantity of the protein produced throughout the proposed *in vitro* culture age range for production (*33*, *34*).

The International Conference on Harmonisation has issued additional useful information (*43*).

C.2.4 *Production cell cultures*

C.2.4.1 *Serum used in cell-culture media*

Serum used in cell-culture media shall be tested as specified in section A.3.2.

C.2.4.2 *Trypsin used for preparing cell cultures*

Trypsin used for preparing cell cultures shall be tested as specified in section A.3.3.

C.2.4.3 *Identity test*

An identity test shall be performed on the control cell culture as specified in section B.2.3.1.

C.2.4.4 *Tests for bacteria, fungi and mycoplasmas at the end of production*

Tests for bacteria, fungi and mycoplasmas shall be conducted on the production culture supernatant or lysate as specified in section A.3.4.

C.2.4.5 *Tests for adventitious viruses at the end of production*

Tests shall be conducted on the product at the end of production but before further processing as specified in section A.3.5. If the presence of the product interferes, tests shall be performed on the control cell culture as specified in section A.3.5.

Authors

The first draft of these Requirements was prepared by Dr V. Grachev, Scientist, Biologicals, Dr D. Magrath, Chief (1987–1994), Biologicals, and Dr E. Griffiths, Chief (from 1994), Biologicals, World Health Organization, Geneva, Switzerland.

A revised draft was formulated by Dr V. Grachev, Deputy Director, Institute of Poliomyelitis and Viral Encephalitides, Russian Academy of Medical Sciences, Moscow, Russian Federation, Dr E. Griffiths, Chief, Biologicals, WHO, Geneva, Switzerland and Dr J.C. Petricciani, Vice-President, Genetics Institute, Cambridge, MA, USA.

After extensive consultation, including discussion of the main scientific issues at a WHO/IABS meeting held at the Mérieux Foundation, Annecy, France, from 29 September to 1 October 1996, further amendments to the text were proposed by the following group of experts:

Dr Y.Y. Chiu, Director, Division of New Drug Chemistry, Food and Drug Administration, Rockville, MD, USA

Dr R. Dobbelaer, Acting Chief, Biological Standardization, Institute for Hygiene and Epidemiology, Brussels, Belgium

Dr V. Grachev, Institute of Poliomyelitis and Viral Encephalitides, Russian Academy of Medical Sciences, Moscow, Russian Federation

Dr E. Griffiths, Chief, Biologicals, World Health Organization, Geneva, Switzerland

Dr I. Gust, CSL Limited, Parkville, Victoria, Australia

Dr M.C. Hardegree, Director, Office of Vaccine Research and Review, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD, USA

Dr T. Hayakawa, National Institute of Health Science, Tokyo, Japan

Professor F. Horaud, Pasteur Institute, Paris, France

Dr A.S. Lubiniecki, SmithKline Beecham, King of Prussia, PA, USA

Dr P. Minor, Head, Division of Virology, National Institute for Biological Standards and Control, Potters Bar, England

Dr B. Montagnon, Pasteur Mérieux Sera and Vaccines, Marcy l'Etoile, France

Dr J. Peetermans, SmithKline Beecham Biologicals, Rixensart, Belgium

Dr J. Petricciani, Vice-President, Genetics Institute, Cambridge, MA, USA

Dr A. Ridgeway, Head, Biotechnology Section, Health and Welfare Canada, Ottawa, Ontario, Canada

Dr J. Robertson, National Institute for Biological Standards and Control, Potters Bar, England

Dr G. Schild, Director, National Institute for Biological Standards and Control, Potters Bar, England

Dr K.B. Seamon, Immunex Corporation, Seattle, WA, USA

Acknowledgements

Acknowledgements are due to the following experts for their comments and advice: Dr S.C. Arya, Centre for Logistical Research and Innovation, New Delhi, India; Dr T. Bektimirov, Deputy Director, Tarasevic State Institute for the Standardization and Control of Medical Biological Preparations, Moscow, Russian Federation; Dr M. Duchêne, Director, Quality Control, SmithKline Beecham Biologicals, Rixensart, Belgium; Dr B.D. Garfinkle, Vice-President, Vaccine Quality Operations, Merck & Co., West Point, PA, USA; Dr R.K. Gupta, Assistant Director, Massachusetts Public Health Biologics Laboratory, Boston, MA, USA; Dr K. Healy, Head, Quality Assurance Department, CSL Limited, Parkville, Victoria, Australia; Dr A. Homma, Regional Adviser in Biologics, WHO Regional Office for the Americas, Washington, DC, USA; Dr J.G. Kreeftenberg, Head, Quality and Regulatory Affairs, National Institute of Public Health and Environmental Protection, Bilthoven, Netherlands; Dr L. Lavese, Head of International Affairs Department, Farmindustria, Rome, Italy; Dr R. Netter, Rue Vaugirard, Paris, France; Dr A.S. Outschoorn, Scientist Emeritus, United States Pharmacopeia, Rockville, MD, USA; Dr E. Walker, Head, Molecular Biology Section, Therapeutic Goods Administration, Woden, Australia.

References

1. Van Wezel AL. Microcarrier technology — present status and prospects. *Developments in biological standardization*, 1984, **55**:3.

2. Jacobs JP et al. Guidelines for the acceptability, management and testing of serially propagated human diploid cells for the production of live virus vaccines for use in man. *Journal of biological standardization*, 1981, **9**:331–342.

3. Hayflick L, Plotkin S, Stevenson R. History of the acceptance of human diploid cell strains as substrates for human virus vaccine production. *Developments in biological standardization*, 1987, **68**:9–17.

4. Grachev, V. World Health Organization attitude concerning the use of continuous cell lines as substrate for production of human virus vaccines. In: Mizrahi A, ed. *Advances in biotechnological processes. Vol. 14. Viral vaccines*. Wiley-Liss, 1990:37–67.

5. *Acceptability of cell substrates for production of biologicals. Report of a WHO Study Group*. Geneva, World Health Organization, 1987 (WHO Technical Report Series, No. 747).

6. Requirements for continuous cell lines used for biologicals production. In: *WHO Expert Committee on Biological Standardization. Thirty-sixth Report*. Geneva, World Health Organization, 1987, Annex 3 (WHO Technical Report Series, No. 745).

7. WHO bank of Vero cells for the production of biologicals. In: *WHO Expert Committee on Biological Standardization. Fortieth Report*. Geneva, World Health Organization, 1990, p. 11 (WHO Technical Report Series, No. 800).

8. Temin HM. Overview of biological effects of addition of DNA molecules to cells. *Journal of medical virology*, 1990, **31**:13–17.

9. **Kurth R.** Risk potential of the chromosomal insertion of foreign DNA. *Annals of the New York Academy of Sciences*, 1995, **772**:140–150.

10. **Petricciani JC, Horaud FN.** DNA, dragons, and sanity. *Biologicals*, 1995, **23**:233–238.

11. **Nichols WW et al.** Potential DNA vaccine integration into host cell genome. *Annals of the New York Academy of Science*, 1995, **772**:30–38.

12. **Coffin JM.** Molecular mechanisms of nucleic acid integration. *Journal of medical virology*, 1990, **31**:43–49.

13. **Petricciani JC, Regan PJ.** Risk of neoplastic transformation from cellular DNA: calculations using the oncogene model. *Developments in biological standardization*, 1986, **68**:43–49.

14. **Strain AJ.** The uptake and fate of DNA transfected into mammalian cells *in vitro*. *Developments in biological standardization*, 1986, **68**:27–32.

15. **Doehmer J.** Residual cellular DNA as a potential transforming factor. *Developments in biological standardization*, 1986, **68**:33–41.

16. **Wierenga DE, Cogan J, Petriccaini JC.** Administration of tumor cell chromatin to immunosuppressed and non-immunosuppressed primates. *Biologicals*, 1995, **23**:221–224.

17. **Greenwald P et al.** Morbidity and mortality among recipients of blood from pre-leukemic and pre-lymphomatous donors. *Cancer*, 1976, **38**: 324–328.

18. **Fleckenstein B, Daniel MD, Hunt RD.** Tumor inductions with DNA of oncogenic primate herpesviruses. *Nature*, 1978, **274**: 57–59.

19. **Duxbury M et al.** DNA in plasma of human blood for transfusion. *Biologicals*, 1995, **23**:229.

20. **Nawroz H et al.** Microsatellite alterations in serum DNA of head and neck cancer patients. *Nature medicine*, 1996, **2**:1035–1037.

21. **Perrin P, Morgeaux S.** Inactivation of DNA by beta-propiolactone. *Biologicals*, 1995, **23**:207.

22. Requirements for Poliomyelitis Vaccine (Inactivated). In: *Requirements for Biological Substances: 1. General Requirements for Manufacturing Establishments and Control Laboratories; 2. Requirements for Poliomyelitis Vaccine (Inactivated). Report of a Study Group*. Geneva, World Health Organization, 1959 (WHO Technical Report Series, No. 178).

23. Requirements for Poliomyelitis Vaccine (Inactivated). In: *Requirements for Biological Substances. Manufacturing and Control Laboratories. Report of a WHO Expert Group*. Geneva, World Health Organization, 1966 (WHO Technical Report Series, No. 323).

24. **Hilleman M.** *Cells, vaccines and pursuit of precedent*. Bethesda, MD, National Cancer Institute, 1968 (National Cancer Institute Monograph, No. 29).

25. Requirements for Poliomyelitis Vaccine (Oral) (Requirements for Biological Substances No. 7, revised 1989). In: *WHO Expert Committee on Biological*

Standardization. Fortieth Report. Geneva, World Health Organization, 1990, Annex 1 (WHO Technical Report Series, No. 800).

26. Good manufacturing practices for pharmaceutical products. In: *WHO Expert Committee on Specifications for Pharmaceutical Preparations. Thirty-second Report.* Geneva, World Health Organization, 1992, Annex 1 (WHO Technical Report Series, No. 823).

27. Good manufacturing practices for biological products. In: *WHO Expert Committee on Biological Standardization. Forty-second Report.* Geneva, World Health Organization, 1992. Annex 1 (WHO Technical Report Series, No. 822).

28. Questions de santé publique liées aux encéphalopathies spongiformes chez l'animal et chez l'homme: Mémorandum d'une réunion de l'OMS. *Bulletin of the World Health Organization*, 1992, **70**:573–582.

29. *Report of a WHO Consultation on Medicinal and Other Products in Relation to Human and Animal Transmissible Spongiform Encephalopathies.* Geneva, World Health Organization, 1997 (unpublished document WHO/BLG/97.2; available on request from Biologicals, World Health Organization, 1211 Geneva 27, Switzerland).

30. Requirements for Measles, Mumps and Rubella Vaccines and Combined Vaccine (Live) (Requirements for Biological Substances No. 47, 1992). In: *WHO Expert Committee on Biological Standardization. Forty-fourth Report.* Geneva, World Health Organization, 1994, Annex 3 (WHO Technical Report Series, No. 840).

31. General Requirements for the Sterility of Biological Substances (Requirements for Biological Substances No. 6, revised 1973). In: *WHO Expert Committee on Biological Standardization. Twenty-fifth Report.* Geneva, World Health Organization, 1973, Annex 4 (Technical Report Series, No. 530).

32. General Requirements for the Sterility of Biological Substances (Requirements for Biological Substances No. 6, revised 1973, amendment 1995). In: *WHO Expert Committee on Biological Standardization. Forty-sixth Report.* Geneva, World Health Organization, 1998, Annex 3 (WHO Technical Report Series, No. 872).

33. Requirements for the Collection, Processing and Quality Control of Blood, Blood Components and Plasma Derivatives (Requirements for Biological Substances No. 27, revised 1992). In: *WHO Expert Committee on Biological Standardization. Forty-third Report.* Geneva, World Health Organization, 1994, Annex 2 (WHO Technical Report Series No. 840).

34. *Viral safety evaluation of biotechnology products.* Geneva, International Conference on Harmonisation, 1997 (unpublished document available on request from International Conference on Harmonisation Secretariat, International Federation of Pharmaceutical Manufacturers Associations, 30 rue de St-Jean, 1211 Geneva 18, Switzerland).

35. Guidelines for assuring the quality of pharmaceutical and biological products prepared by recombinant DNA technology. In: *WHO Expert Committee on Biological Standardization. Forty-first Report.* Geneva, World

Health Organization, 1991, Annex 3 (WHO Technical Report Series, No. 814).

36. Requirements for Hepatitis B Vaccines made by Recombinant DNA Techniques. In: *WHO Expert Committee on Biological Standardization. Thirty-ninth Report.* Geneva, World Health Organization, 1989, Annex 2 (WHO Technical Report Series, No. 786).

37. **Peebles PT.** An *in vitro* focus-induction assay for xenotropic murine leukemia virus, feline leukemia virus C, and the feline-primate viruses RD-114/CC/M-7. *Virology*, 1975, **67**:288–291.

38. **Sammerfelt MA, Weiss RA.** Receptor interference groups of 20 retroviruses plating on human cells. *Virology*, 1990, **176**:58–69.

39. **Lander MR, Chattopadhyay SK.** A *Mus dunni* cell line that lacks sequences closely related to endogenous murine leukemia viruses and can be infected by ecotropic, amphotropic, xenotropic, and mink cell focus-forming viruses. *Journal of virology*, 1984, **52**:695–698.

40. **Hartley RH, Rowe WP.** Clonal cell lines from a feral mouse embryo which lack host-range restrictions for murine leukemia viruses. *Virology*, 1975, **65**:128–134.

41. **Bassin RH et al.** Normal DBA/2 mouse cells synthesize a glycoprotein which interferes with MCF virus infection. *Virology*, 1982, **123**:139–151.

42. **Bassin RH, Tuttle N, Fischinger PJ.** Rapid cell culture assay technic for murine leukeamia viruses. *Nature*, 1971, **229**:564–566.

43. *Analysis of the expression construct in cells used for production of a DNA-derived protein product.* Geneva, International Conference on Harmonisation, 1995 (unpublished document available on request from International Conference on Harmonisation Secretariat, International Federation of Pharmaceutical Manufacturers Associations, 30 rue de St-Jean, 1211 Geneva 18, Switzerland).

Appendix

Validation of viral elimination from monoclonal antibodies and biologicals prepared using recombinant DNA technology (except viral vaccines)

Traditionally, cell lines used as cell substrates for the production of biologicals have been tested to ensure the absence of contamination with adventitious viruses. As continuous cell lines have been introduced, it has become necessary to approve for production cell lines that produce virus-like particles and even infectious viruses. These efforts have resulted in an enhanced understanding of the significance of virus-like particles in cell lines and have demonstrated that certain findings, such as the presence of intracisternal A particles, are only of remote theoretical concern. As experience has been gained with monoclonal antibodies produced in cell lines that produce murine retroviruses, evidence has accumulated that such products can be safe, and methods have been developed to minimize both the potential for contamination of the products with retroviruses and the theoretical risk associated with such contamination. In particular, manufacturers have used manufacturing procedures that include steps that inactivate and/or remove viruses from the product, and have performed studies to validate the effectiveness of these procedures. When the manufacturing process is known to eliminate significantly more virus than is present in the unprocessed bulk, and when the purified product is tested for the presence of virus, there is reasonable assurance of freedom from contamination.

Validation studies assist in the quantification of risk, but do not of themselves prove absence of risk. They are relevant for the evaluation of production using cell lines potentially carrying any type of virus (e.g. Epstein-Barr virus, papillomavirus), but risk assessment also includes consideration of the type of virus and the potential use of the product. Validation studies are not a means of demonstrating that introduction of an adventitious virus during manufacture is acceptable. Validation is accomplished by evaluating the ability of downstream processing steps to remove and/or inactivate virus from the bulk harvest: virus is added to test the efficacy of selected steps in a scaled-down model of the manufacturing process.

Design

The design of procedures to validate the elimination of virus during processing should take into account the following variables.

Selection of appropriate virus or viruses

The virus or viruses to be used may be the virus which is known or suspected to contaminate the cell line, or it may be a model virus (or viruses) selected because of its similarity to the virus of concern and because of practical considerations, such as availability of material of high titre and the ease of assay. The viral contaminant may be added in a labelled (i.e. radioactive) or non-labelled form. It may be necessary to use more than one virus when, for example, the use of a single virus does not provide an adequate basis for the evaluation of the purification process.

Scaled-down manufacturing system

If a scaled-down model of the purification process is used for validation, it should accurately reflect the actual manufacturing process. Bed height, flow rate, flow-rate to bed-height ratio, types of buffer, pH, and the concentration of protein, buffer and product should all be evaluated, and equivalence to the full-scale manufacturing system demonstrated.

Analysis of step-wise elimination of virus

In many cases it is desirable to evaluate the individual contribution to virus elimination of different manufacturing steps. Sufficient virus should be present before each critical step so that an adequate evaluation of the effectiveness of each step is obtained. In some cases, the addition of high-titre virus to the unpurified bulk and the testing of its concentration between steps will be sufficient. In other cases, the addition of virus to material during the manufacturing process will also be necessary. The virus titre should be determined before and after each tested step.

Determining physical removal or inactivation

The type of contribution (removal or inactivation) of each step should be identified by determining, when feasible, what portion of the reduction in titre is due to virus inactivation and what portion is due to physical removal of the virus from the product.

Kinetics of inactivation

In some cases, the kinetics of virus inactivation at the critical inactivation step should be determined. This is particularly important where the virus is known to be a human pathogen and it is necessary to design a completely effective inactivation process.

Estimation of combined effects

The combined effect of each tested step on the reduction of virus titre should be calculated in order to establish the total virus inactivation/removal capacity of the purification procedure. Where a process involves several steps that achieve a reduction in titre by the same mechanism, unless otherwise justified the results of only one such step should be considered in calculating the overall titre reduction.

Regeneration of columns

When chromatographic procedures are used for virus elimination, it is critical that validation studies should employ columns that are representative of those actually used in manufacturing. Routine procedures for the regeneration of columns should be such that the design of the validation study is relevant to the manufacturing process.

Specific precautions

- Validation usually takes place outside the manufacturing facility in order to prevent possible viral contamination of the facility.
- Care should be taken in preparing virus preparations to avoid the aggregation of viral particles. This may facilitate physical removal and hinder inactivation, thereby reducing comparability with the actual manufacturing process.
- The virus preparation to be added to the product should constitute a small volume so as not to dilute or change the characteristics of the product.
- Care should be taken to avoid even small differences in, for example, buffers, media or reagents as these can substantially affect virus clearance and comparability with the manufacturing process.
- As virus removal/inactivation is time dependent, the amount of time the product remains in a buffer solution or on a chromatography column should reflect the conditions of the full-scale manufacturing process.
- Buffers and product should be evaluated independently for interference with the assays used to determine the virus titre, since these components may adversely affect the indicator cells. If the buffer solutions are toxic for the indicator cells, dilution, adjustment of pH or dialysis of the virus-containing buffer may be necessary. If the biological product itself has an antiviral activity, it may be possible to perform the validation study without the product, although omission of product or substitution of a similar protein without antiviral activity could affect behaviour of the virus in some manufacturing steps.
- Many purification schemes repeatedly use the same or similar buffers or chromatography columns. The effects of this approach

should be taken into account when analysing the data. The effectiveness of virus removal/inactivation by a particular process may vary according to the stage in manufacture at which it is used.

Interpretation

The purpose of a validation study is to show that a process, when conducted according to standard operating procedures, will reliably produce a certain result. For viral contaminants, it is important to show that not only is the virus removed and/or inactivated, but also that there is excess capacity for this built into the purification process that will assure an appropriate level of safety for the final product. It is recommended that a purification process should include at least one viral inactivation step when infectious virus is known to be routinely present in the unpurified bulk product.

The following potential limitations of validation studies for virus removal or inactivation should be addressed when interpreting results.

- A model virus may not behave identically to relevant potential viral contaminants.
- The full-scale manufacturing process may differ from the scaled-down process used for validation purposes.
- Unrecognized differences in the materials or procedures used for validation as compared with those used for manufacturing may overestimate virus removal or inactivation.
- The effects of repeated steps, particularly of those with little individual effect, may not be additive, and summation of the effects of such steps may result in overestimation.
- The efficacy of chromatography columns and other devices used in the purification scheme may change on repeated use.

Statistics

Validation studies should analyse data statistically. Validation studies should be duplicated, and the statistical variation within and between them evaluated.

The design of the validation study should be statistically valid, i.e. it should be capable of supporting the conclusions reached.