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Geneva, 7–11 October 1996

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Introduction

The WHO Expert Committee on Biological Standardization met in Geneva from 7 to 11 October 1996. The meeting was opened on behalf of the Director-General by Dr F.S. Antezana, Assistant Director-General.

Dr Antezana explained that the shorter meeting that year followed the pattern established for other WHO Expert Committees. As recommended by the Committee in 1995, progress and development reports had been combined into a single agenda item so that attention could be directed to requirements and guidelines, reports of consultations and proposals for establishment and discontinuation of international reference materials.

He emphasized the importance of the work of the Committee to both developed and developing countries and the expansion of the field of biological products and its increasing diversity. Such diversification, together with the development of products derived by new biotechnologies, raised new and significant issues of safety, and he stressed that the challenge was to ensure public safety while not inhibiting the development of potentially important products through inappropriate or restrictive requirements.

Dr Antezana recalled the importance of obtaining international consensus regarding procedures for ensuring the safety and efficacy of new biological products. At the same time, it was necessary to ensure that requirements for existing products reflect scientific and technical advances. The role of well characterized reference materials, internationally established and supplied, was fundamental in contributing to the quality and safety of biological products.

Dr Antezana noted that a review of the scientific basis of the standardization and quality control of biological substances used in human medicine had been conducted recently for the National Biological Standards Board of the United Kingdom, which had responsibility for supervision of one of the WHO International Laboratories for Biological Standards. This review had been undertaken with the collaboration of WHO and the support of the European Medicines Evaluation Agency, the European Pharmacopoeia and the United States Food and Drug Administration. The report was to be presented during the meeting, and its conclusions had international implications that the Committee should consider.

He thanked the WHO International Laboratories for their important contribution to the programme of biological standardization, and

thanked the participants in WHO meetings and collaborative studies for the vital contribution they make to the international reputation and success of the programme.

The death of Dr D.I. Magrath, who served with distinction at WHO as Chief, Biologicals, from 1987 to 1994 was noted with deep regret.

General

Developments in biological standardization

The Committee was informed of the continued demand for international reference materials distributed by the International Laboratories for Biological Standards. As proposed by the Committee in 1995, work in progress in the various International Laboratories was summarized. Attention was drawn to a review of reference reagents for blood-typing sera; to a meeting planned for November 1996 to consider issues relating to immunoassays of cytokines; and to the need to review the requirements for antivenoms. In response to comments received concerning the measurement of antibodies, a WHO Informal Consultation would be held to prepare a report for consideration by the Committee in 1997.

The Committee discussed the importance of rapid dissemination of its decisions. National control authorities and manufacturers needed ready access to newly adopted guidelines and requirements, as well as information on the establishment of international reference materials. However, publication of the Committee's report could take longer than was ideal. Partly for these reasons, a summary of the major decisions of the 1995 meeting, including changes in the list of international reference materials, had appeared in the *Weekly epidemiological record*.[1] The Committee nevertheless urged WHO to seek other ways to speed up the dissemination of its decisions.

The Committee was informed of recent data concerning the presence of sequences of simian virus 40 (SV40) in certain human tumour tissues. It was also informed that simian virus 40 had not been present in poliomyelitis vaccine since the early 1960s and that tests to ensure its absence are performed routinely. The Committee requested the Secretariat to monitor developments.

The Committee was also informed that two WHO meetings had been held to discuss the public health implications of transmissible spongiform encephalopathies and the emergence of an apparent

[1] *Weekly epidemiological record*, 1996, **71**:105–109.

variant of Creutzfeldt-Jakob disease. A further meeting specifically to consider the safety of medicinal products in relation to human and animal transmissible spongiform encephalopathies (TSEs) was planned.[1] The Committee recognized the importance of these discussions for biological products and agreed to consider their implications at its next meeting.

Statens Seruminstitut

The Committee was informed that a review had been carried out by the Statens Seruminstitut (SSI) of its role in the field of biological standardization, in which it had been active for almost seventy-five years. The conclusion was that its efforts should in future concentrate on scientific contributions to the field. After discussions with the National Institute for Biological Standards and Control, Potters Bar (NIBSC), and with the cognizance of WHO, an agreement had been reached that existing stocks of international reference materials would be transferred from SSI to NIBSC during April 1997. The conditions of distribution would be the same as for international reference materials already held by NIBSC. Precautions were being taken to ensure safe and secure transfer from one site to the other.

On behalf of the Director-General, Dr Antezana here paid tribute to the long-standing and valuable contribution of SSI to the activities of WHO in the field of biological standardization and welcomed the assurance that there would be no discontinuity in the supply of reference materials. He also thanked NIBSC for its work in biological standardization and emphasized the gratitude of Member States towards those involved in this area.

On behalf of the Committee, the Chairman expressed thanks to the representatives from SSI, who had made such a significant and sustained contribution to the Committee's work.

Reviews of the field of biological standardization

During its recent meetings, the Committee had identified issues relating to WHO's biological standardization activities that it considered could benefit from in-depth examination. The Committee noted that WHO's role and functions in this area, as well as its own role and functions, had in large part been established nearly fifty years ago,

[1] A consultation on medicinal and other products in relation to human and animal transmissible spongiform encephalopathies was held in Geneva from 24 to 26 March 1997. The report of the consultation (WHO/BLG/97.2) is available on request from Biologicals, World Health Organization, 1211 Geneva 27, Switzerland.

when priorities and concerns in the field of biological standardization were very different. The Committee expressed concern about the limited resources available for WHO's biological standardization activities and once again emphasized the importance and consequences of this work to many WHO activities, especially the prevention of infectious and parasitic diseases. In light of the highly useful scientific review of biological standardization recently commissioned by the National Biological Standards Board of the United Kingdom, the Committee recommended that an independent high-level review of WHO's biological standardization activities, including its own activities, should likewise be carried out, and requested that ways of increasing the resources available for such activities should be considered.

The Committee endorsed a proposal from the National Biological Standards Board that the review it had commissioned should be disseminated under the auspices of WHO and urged that this should be undertaken as soon as possible.[1]

World Trade Organization

The attention of the Committee was drawn to the implications of recent agreements on the acceptance and circulation of goods in international trade. As it was not clear whether biological products are specifically included in such agreements, the Committee requested WHO to seek clarification concerning whether its requirements and guidelines might be regarded by the World Trade Organization (WTO) as the source of definitive information on specifications for biologicals, and thereby inadvertently lead to trade disputes. Pending resolution of this issue, the Committee emphasized that its responsibilities include:

- Establishing international reference materials for measuring the potency and other characteristics of biological products. These reference materials are recognized and used worldwide and play a crucial role in ensuring the comparability of products on a global basis, and thus facilitate international trade.
- Developing requirements and guidelines for biological products. This activity does not imply that WHO is assuming the role of a supranational regulatory authority. The requirements and guide-

[1] Issued as *Biological standardization and control: a scientific review commissioned by the UK National Biological Standards Board (NBSB)*. Geneva, World Health Organization, 1997 (unpublished document WHO/BLG/97.1; available on request from Biologicals, World Health Organization, 1211 Geneva 27, Switzerland).

lines are intended to be scientific and advisory in nature; they should not be construed as intending to set regulatory standards for the approval of products in individual countries or for the acceptability by individual countries of products moving in international commerce.

WHO Certification Scheme on the Quality of Pharmaceutical Products Moving in International Commerce

The Committee noted proposed guidelines for the certification of active pharmaceutical ingredients (WHO/PHARM/96.586 Rev.1 (draft)). The Committee considered that the draft text did not sufficiently take account of certain characteristics of biological substances. Although recognizing that perceptions of what constitutes a biological substance differed, the Committee nevertheless recommended that the proposed guidelines should make separate provisions for biological substances and considered that this might best be achieved by including a list of substances, such as vaccines, that would specifically be excluded from the scheme. The Committee requested its individual members to make detailed comments on the document.

Standardization of manufacturer-specific rDNA products or mutant forms of biological products

The Committee noted a report (BS/96.1842) discussing the need for a policy regarding preparation and establishment of reference materials for rDNA-derived products related to biological substances of natural origin but originating from a single manufacturer and having a different structure from the natural substances. The Committee recognized that the report raised a number of issues of importance, but considered that they should be addressed within the context of the review of biological standardization that it had recommended (see p. 3–4).

Hyaluronidase and pharmaceutical enzymes

The Committee took note of a report (BS/96.1835) reviewing the present status of the International Standard for Hyaluronidase. Apart from certain specialized enzymes that are blood products, this is the only enzyme used in medicine for which an international reference material established by the Committee is available. Reference materials for other enzymes are, however, available from the International Centre for Pharmaceutical Enzymes of the International Pharmaceutical Federation (FIP). The Committee requested the Secretariat to investigate whether the International Standard for Hyaluronidase should be maintained or whether some other arrangement should be made, and to make recommendations at the next meeting.

Reverse transcriptase activity in avian cells

The Committee was informed that, subsequent to the detection of very low levels of reverse transcriptase (RTase) activity in live viral vaccines prepared in chicken cells, extensive studies had been carried out to assess the implications of these findings. An international collaborative study had been coordinated by the National Institute for Biological Standards and Control, Potters Bar, and had demonstrated good agreement between eight laboratories in detecting RTase activity in a variety of vaccines, although results in a ninth laboratory showed some differences. Laboratory evidence had also been obtained that RTase activity is not transmissible. Thus, all data available to date present no cause for concern over the safety of vaccines derived from chicken cells, and the current requirements published by WHO for such vaccines remain appropriate. The Committee was informed that this conclusion was supported by studies that showed no evidence of antibody formation in vaccinees. Work was in progress to establish levels of RTase activity that would be indicative of the presence of an infective agent.

The amount of work performed in a short time was a good example of WHO's ability to take the lead on an important international health issue and demonstrated the value of good international cooperation and the importance of high levels of scientific competence in national control laboratories. The Committee recommended that WHO should convene a meeting to review the data and produce a report for dissemination.

Cytokines

The Committee noted the report of a WHO Informal Consultation on Cytokine Standards which had been held in June 1996 (BS/96/1847). This was the second meeting of the group constituted as endorsed by the Committee in its forty-fifth report (WHO Technical Report Series, No. 858, 1995, p. 5). The Committee endorsed a proposal that the report of the meeting should be published in a suitable scientific journal. The Committee was informed that the trend towards indicating the dose of therapeutic cytokine products by weight rather than biological potency had highlighted problems of differences in specific activity and noted that the availability of suitable reference materials is essential. The difficulties of developing suitable assays for some cytokines, such as glial-derived neurotropic factor (GDNF), also highlighted the critical role of reference materials.

The Committee agreed with a proposal from the Informal Consultation that adhesion molecules should be included within its terms of

reference, since they had therapeutic potential and no relevant standardization activity had yet been undertaken. In contrast, work on cytokine binding proteins and soluble receptors was only considered necessary where use of the cytokine itself would be inappropriate for measurement purposes. The Committee was pleased to learn that the Informal Consultation had welcomed the decision to establish a category of Reference Reagents to serve as interim reference materials until full collaborative studies justified the establishment of International Standards (WHO Technical Report Series, No. 872, 1998, p. 4).

The Committee noted that concerns had been expressed about the use of potency standards for immunoassays, for which they may not be suitable. A meeting was to be held in November 1996 to address this issue.[1]

Finally, the Committee noted that the future distribution in the USA of international reference materials for cytokines would be the responsibility of the Center for Biologics Evaluation and Research of the Food and Drug Administration.

Reference preparations for evaluating hepatitis B and C and HIV diagnostic kits

The Committee noted the report of a WHO Informal Consultation to assess the need for reference materials for evaluating hepatitis B, hepatitis C and human immunodeficiency virus (HIV) diagnostic kits, held at the National Institute for Biological Standards and Control, Potters Bar, during May 1996 (BS/96.1848). This consultation had been organized to consider priorities in this area and to make recommendations, which were discussed. The Committee recognized the importance of reference materials for ensuring the quality and safety of blood and blood products and recommended the continuing involvement of WHO in this work.

Standardization of gene-amplification methods for the viral safety testing of blood and blood products

The Committee was informed of meetings of a WHO Working Group concerned with the standardization of gene-amplification methods for the viral safety testing of blood and blood products. The objective of the Working Group's activities was to advance the scientific basis of

[1] An international meeting cosponsored by WHO was held at the National Institute for Biological Standards and Control, Potters Bar, from 14 to 15 November 1996. The report of the consultation was considered at the forty-eighth meeting of the Expert Committee in 1997.

standardization in this area, to exchange information and to organize collaborative studies leading to the establishment of international reference materials. Priority had been given to the development of materials for the detection of hepatitis C virus and HIV. The Committee was also informed that questions concerning the expression of the content of standards would have to be resolved. Another key issue to be investigated was the relationship between the detection of nucleic acid and infectivity. Therefore, the sensitivity and specificity of reference materials and test procedures are critical. The Committee considered this to be an important field of standardization and asked to be kept informed of developments.

Requirements and guidelines for biological substances

Requirements for cell substrates used for production of biologicals

The Committee noted a draft of proposed requirements for the use of animal cells as *in vitro* substrates for the production of biologicals (BS/95.1792 Rev.5) that had been revised following extensive discussion and comment on earlier drafts by a wide group of experts, in particular at a meeting held during October 1996. The Committee was informed that the proposed requirements represented a major departure from previous requirements published by WHO in that residual cellular DNA was no longer regarded as a significant risk factor requiring removal to extremely low levels. The Committee was further informed that certain requirements for well established diploid cell lines, such as MRC-5 and WI-38, had been relaxed. After making some additional modifications, the Committee adopted the text as Requirements for the Use of Animal Cells as *in vitro* Substrates for the Production of Biologicals, agreed that it should be annexed to its report (Annex 1) and noted that these requirements replaced the Requirements for Continuous Cell Lines Used for Biologicals Production (Requirements for Biological Substances, No. 37).

Requirements for acellular pertussis vaccines

The Committee noted a revised draft of proposed requirements for acellular pertussis vaccines (BS/96.1832 Rev) that had been prepared by the Secretariat in the light of comments and advice received at a recent international meeting. In view of the rapid changes and advances in the field, the introduction of vaccines containing acellular pertussis components, and the urgent need for guidance, the Committee agreed that the text should be published as guidelines instead of

requirements, since this would allow greater flexibility with respect to future developments in the field. After making some modifications, the Committee adopted the revised text as Guidelines for the Production and Control of the Acellular Pertussis Component of Monovalent or Combined Vaccines and agreed that it should be annexed to its report (Annex 2). However, the Committee recognized the need for further discussion on many aspects of requirements for, and use of, vaccines containing acellular pertussis components. It therefore recommended that WHO should arrange a working group to discuss issues such as collaborative studies to facilitate developments in the field (for example in assay methods), and to consider how the Guidelines could be extended.

Guidelines for assuring the quality of DNA vaccines

The Committee was informed that a preliminary discussion of issues regarding the quality assurance of DNA vaccines had been held at WHO in 1994 and that draft guidelines had been prepared, which had subsequently been examined at several meetings. The Committee had received a revised draft of the guidelines (BS/96. 1831 Rev.1), prepared following further comments. As the Committee recognized the importance of providing guidance to regulatory authorities in this area, after making some additional modifications, it adopted the text as Guidelines for Assuring the Quality of DNA Vaccines and agreed that it should be annexed to its report (Annex 3).

Guidelines for peptide vaccines

The Committee noted a revision of the draft guidelines for peptide vaccines (BS/96.1844), prepared after comments had been received from a wide group of experts. The Committee considered that issues relating to synthetic peptides and to the final vaccines made from them should be addressed in separate sections of the document. In view of this extensive revision, the Committee requested the Secretariat to revise the document in consultation with appropriate experts, circulate it for further comment and resubmit it at the next meeting.

International reference materials

Antibiotics

Review of reference materials

The Committee took note of a review (BS/96.1934) of the extent of use and changed international requirements for assay of several antibiotics. Certain standards had been established during the rapid growth phase in the development of antibiotics for substances that

had since disappeared from the market or have at most very limited use. This was reflected by negligible demand for the respective international reference materials. In many countries in recent years, technical and scientific advances in analytical methods had resulted in the discontinuation of the microbiological assay of certain other antibiotics. In view of the information provided, the Committee discontinued the international reference materials for oleandomycin, triacetyloleandomycin (INN = troleandomycin) and viomycin, on the grounds of lack of need. The Committee also discontinued the International Reference Preparation of Spectinomycin, as this substance has generally been assayed by non-microbiological methods for a number of years. The Committee requested the Secretariat to determine whether there continues to be a need for the international reference materials for capreomycin, lymecycline and novobiocin and whether any such needs could be met by the provision of standards at the national level. Finally the Committee requested the Secretariat to determine the extent of need for reference materials for five members of the tetracycline group: tetracycline, oxytetracycline, minocycline, doxycycline and demeclocycline, since these are now assayed by non-microbiological methods in a significant number of countries.

Where appropriate, the stocks of reference materials discontinued on the grounds of replacement of microbiological assay would be made available to the WHO Collaborating Centre for Chemical Reference Substances, Stockholm, Sweden.

Antibodies

Anti-rubella immunoglobulin and anti-rubella serum

The Committee noted that a preparation of normal immunoglobulin had been submitted to a collaborative study carried out by eleven laboratories in seven countries in comparison with the second International Reference Preparation of Anti-Rubella Serum, which had been prepared from normal human immunoglobulin (BS/96.1833). The candidate preparation exhibited satisfactory stability. The Committee noted the confusion in nomenclature that had arisen when the first International Reference Preparation of Anti-Rubella Serum (a serum preparation) was replaced by the second International Reference Preparation of Anti-Rubella Serum (an immunoglobulin preparation) and decided to take the necessary corrective action on replacement of the latter. In view of the results of the collaborative study, the Committee established the preparation coded RUBI-1-94 as the first International Standard for Anti-Rubella Immunoglobulin and assigned a potency of 1600 International Units to the contents of each vial.

The Committee discontinued the second International Reference Preparation of Anti-Rubella Serum.

It had previously been noted (WHO Technical Report Series, No. 858, 1995, p. 9) that reference materials were required for the calibration of diagnostic kits for measuring anti-rubella antibodies in serum. The Committee requested the Secretariat to determine whether a need still exists for two such standards (IgM and IgG) of anti-rubella serum.

Anti-thyroid microsome serum

The Committee noted a proposal to establish a preparation of high-titre human serum, coded 66/387, as an international reference material (BS/96.1837). The Committee recalled that it had previously considered a similar request (WHO Technical Report Series, No. 814, 1991, p. 11) and had concluded that more information was necessary. Although the proposal now provided some information about the stability of the preparation, no collaborative study had been performed. The Committee decided not to establish the preparation as an international reference material and requested the National Institute for Biological Standards and Control, Potters Bar, to ensure that the information accompanying distribution of the preparation makes clear its unofficial status.

Blood products and related substances

Blood coagulation factors II, VII, IX and X in plasma

The Committee noted a report on collaborative studies on a single plasma preparation, performed by eleven laboratories with respect to blood coagulation factors II, IX and X, and by ten laboratories with respect to factor VII (BS/96.1840). The studies compared the preparation with the first International Standard for Blood Coagulation Factors II, VII, IX and X in Human Plasma. The preparation showed good stability. The Committee established the preparation, in ampoules coded 94/746, as the second International Standard for Blood Coagulation Factors II, VII, IX and X, Plasma, Human and assigned potencies of 0.93 International Units of Factor II Activity, 1.25 International Units of Factor VII Activity, 0.90 International Units of Factor IX Activity and 0.95 International Units of Factor X Activity to the contents of each ampoule. The Committee was informed that the level of Factor VII Activity was rather high, which possibly reflected the age of the donors contributing to the plasma pool.

Blood coagulation factor IX concentrate

The Committee noted the results of a collaborative study performed in 38 laboratories in 17 countries on a highly purified preparation in

vials of factor IX concentrate, which had been compared with the second International Standard for Blood Coagulation Factors II, IX and X Concentrate, Human (BS/96/1845). A second preparation of purified factor IX concentrate in ampoules had also been included in the study. The Committee was informed that the preparation in vials had been adopted as a working standard by the Food and Drug Administration (USA) and the European Pharmacopoeia Commission. After considering the report and the properties of the two preparations, the Committee established the preparation in vials, coded 96/854, as the third International Standard for Factor IX Concentrate, Human and assigned a potency of 10.7 International Units of Factor IX Activity to the contents of each vial. However, the Committee requested the National Institute for Biological Standards and Control, Potters Bar, to continue to monitor the stability of this preparation for an extended period of time.

Blood coagulation factors II, IX and X concentrate

The Committee recognized that the third International Standard for Factor IX Concentrate, Human, would replace the second International Standard for Blood Coagulation Factors II, IX and X Concentrate, Human for the measurement of blood coagulation factor IX in concentrates. The Committee therefore discontinued the second International Standard for Blood Coagulation Factors II, IX and X Concentrate, Human, coded 84/633, with respect to factor IX but agreed to the continuation of the same preparation for the calibration of blood coagulation factors II and X in concentrates, with assigned potencies of 9.4 International Units of Factor II Activity and 11.1 International Units of Factor X Activity for the contents of each ampoule.

Recombinant ferritin

The Committee noted the results of a collaborative study on a preparation of recombinant ferritin in human plasma, which had been compared with the second International Standard for Ferritin, Human, in 18 laboratories in 9 countries (BS/96.1838). The Committee recalled that, because of the difficulty of obtaining suitable material of human origin, the candidate material was of recombinant origin (WHO Technical Report Series, No. 872, 1998, pp. 23–24). On the basis of the study and the stability of the preparation, the Committee established the preparation, coded 94/572, as the third International Standard for Ferritin, Human, Recombinant and assigned a value of 6.3 μg of ferritin to the contents of each ampoule. The Committee noted that although the preparation had not undergone secondary desiccation, its stability was satisfactory.

Whole blood folate

The Committee noted the results of a collaborative study on a preparation of blood for the measurement of blood folate, performed in 13 laboratories in 5 countries (BS/96.1806), and also noted stability data supplied in a supplementary report. The Committee further noted that the need for this reference material had been brought to its attention by the International Committee for Standardization in Haematology. On the basis of the study, the Committee therefore established the preparation, coded 95/528, as the first International Standard for Whole Blood Folate and assigned a value of 13 ng of folate to the contents of each ampoule. The Committee requested the National Institute for Biological Standards and Control, Potters Bar, to include a statement of the concentration of folate in molar terms in the information supplied with the preparation.

The Committee recognized that this standard could be considered a matrix standard (i.e. an analyte within the complex environment in which it is normally measured) and wished to emphasize that its establishment did not necessarily represent a decision to establish other such reference materials.

Thromboplastin, human, recombinant, plain

The Committee noted the report of a collaborative study carried out under the responsibility of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. The study, on a candidate replacement for the second International Reference Preparation of Thromboplastin, Human, Plain (BS/96.1846), had been carried out by 20 laboratories in 13 countries. The Committee was informed that a new source of material had become necessary because human brain was no longer appropriate as a source material. The Committee was also informed that the report had been approved by the above-mentioned Scientific and Standardization Committee.

On the basis of the results of the study, the Committee established the preparation in vials, coded rTF/95, as the third International Standard for Thromboplastin, Human, Recombinant, Plain[1] and assigned an International Sensitivity Index (ISI) of 0.940 to it. The Committee noted that the preparation is stable. The second International Reference Preparation of Thromboplastin, Human, Plain, coded BCT/253, was discontinued.

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[1] The new reference material is designated the "third" International Standard in accordance with the provisions agreed by the Committee at its thirty-seventh meeting (WHO Technical Report Series, No. 760, 1987, p. 16).

Reference preparations for blood coagulation factor VIII concentrate

The Committee was informed of a pilot collaborative study on two lots of purified factor VIII, one of which would be selected as a candidate reference material and offered to WHO. The Committee welcomed this proposal and asked to be kept informed of developments. The Committee asked the organizers to take account in planning the full international collaborative study of the different assay methods used in different countries and to ensure the continuity of the International Unit.

The Committee was also informed of apparent discrepancies in the measurement of factor VIII in plasma and concentrates. Taking account of the regulatory issues involved, the Committee recommended that the Secretariat should seek advice on this matter from the International Society on Thrombosis and Haemostasis, and that the subject should be discussed at the next meeting of the Committee.

Cytokines

The Committee recalled its decision in 1995 (WHO Technical Report Series, No. 872, 1998, p. 4) to establish a new class of interim Reference Reagents on the basis of limited data in rapidly changing fields, in particular that of cytokines, in order to allow the use of such materials before the full programme for establishment of an International Standard could be completed. The following are the first results of this decision.

Interleukin-5

The Committee noted the report on a preparation of interleukin-5 (BS/96.1849), and on this basis established the preparation, coded 90/586, as the first Reference Reagent[1] for Interleukin-5, with an assigned potency of 5000 units per ampoule.

Interleukin-7

The Committee noted the report on a preparation of interleukin-7 (BS/96.1849), and on this basis established the preparation, coded 90/530, as the first Reference Reagent[1] for Interleukin-7, with an assigned potency of 100 000 units per ampoule.

Interleukin-9

The Committee noted the report on a preparation of interleukin-9 (BS/96.1849), and on this basis established the preparation, coded 91/

[1] This interim Reference Reagent was established in accordance with the provisions agreed by the Committee at its forty-sixth meeting (WHO Technical Report Series, No. 872, 1998, p. 4).

678, as the first Reference Reagent[1] for Interleukin-9, with an assigned potency of 1000 units per ampoule.

Interleukin-11

The Committee noted the report on a preparation of interleukin-11 (BS/96.1849), and on this basis established the preparation, coded 92/788, as the first Reference Reagent[1] for Interleukin-11, with an assigned potency of 5000 units per ampoule.

Interleukin-12

The Committee noted the report on a preparation of interleukin-12 (BS/96.1849), and on this basis established the preparation, coded 95/544, as the first Reference Reagent[1] for Interleukin-12, with an assigned potency of 10000 units per ampoule.

Interleukin-13

The Committee noted the report on a preparation of interleukin-13 (BS/96.1849), and on this basis established the preparation, coded 94/622, as the first Reference Reagent[1] for Interleukin-13, with an assigned potency of 1000 units per ampoule.

Interleukin-15

The Committee noted the report on a preparation of interleukin-15 (BS/96.1849), and on this basis established the preparation, coded 95/554, as the first Reference Reagent[1] for Interleukin-15, with an assigned potency of 10000 units per ampoule.

Leukaemia inhibitory factor

The Committee noted the report on a preparation of leukaemia inhibitory factor (BS/96.1850), and on this basis established the preparation, coded 93/562, as the first Reference Reagent[1] for Leukaemia Inhibitory Factor, with an assigned potency of 10000 units per ampoule.

Oncostatin M

The Committee noted the report on a preparation of oncostatin M (BS/96.1851), and on this basis established the preparation, coded 93/564, as the first Reference Reagent[1] for Oncostatin M, with an assigned potency of 25000 units per ampoule.

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[1] This interim Reference Reagent was established in accordance with the provisions agreed by the Committee at its forty-sixth meeting (WHO Technical Report Series, No. 872, 1998, p. 4).

Tumour necrosis factor, beta

The Committee noted the report on a preparation of tumour necrosis factor, beta (BS/96.1852), and on this basis established the preparation, coded 87/640, as the first Reference Reagent[1] for Tumour Necrosis Factor, beta with an assigned potency of 150 000 units per ampoule. The Committee requested the National Institute for Biological Standards and Control, Potters Bar, to supply details of stability studies to the Secretariat.

Nerve growth factor

The Committee noted the report on a preparation of nerve growth factor (BS/96.1836), and on this basis established the preparation, coded 93/556, as the first Reference Reagent[1] for Nerve Growth Factor, with an assigned potency of 10 000 units per ampoule. The Committee considered that the report on this preparation was more detailed than necessary for a preparation intended to be a Reference Reagent of this type and recommended that future reports should follow more closely the format of the other reports discussed above.

Endocrinological and related substances

Thyroid-stimulating hormone, human, recombinant

The Committee noted the report on the collaborative study of a preparation of recombinant thyroid-stimulating hormone (TSH), which had been compared with the second International Reference Preparation of Thyroid Stimulating Hormone, Human, for Immunoassay, a preparation of pituitary origin. The study had been carried out by 33 laboratories in 11 countries (BS/96.1843). The Committee also noted that the aims of the collaborative study were to compare the two preparations in a variety of immunoassay systems, to assess the suitability of the recombinant preparation for calibration of diagnostic immunoassays, to assess its stability and to confirm its bioactivity. Of the laboratories, 28 had employed immunoassays. In view of the results of the study, the Committee established the preparation, coded 94/674, as the first Reference Reagent[1] for Thyroid-Stimulating Hormone (TSH), Human, Recombinant and assigned a potency of 0.0067 units to the contents of each ampoule. The Committee noted that the data obtained in the collaborative study had been insufficient to permit reliable calibration of the new standard by bioassay and

[1] This interim Reference Reagent was established in accordance with the provisions agreed by the Committee at its forty-sixth meeting (WHO Technical Report Series, No. 872, 1998, p. 4).

therefore requested that the intended use of the standard be made clear in the memorandum supplied with it.

Miscellaneous

MAPREC analysis of poliovirus type 3 (Sabin)

The Committee noted a proposal to establish a reference material for a method of molecular analysis (mutant analysis by polymerase chain reaction and restriction enzyme cleavage, or MAPREC) for poliovirus type 3 (Sabin) (BS/96.1841). The candidate material, a novel preparation of a synthetic nucleotide sequence, plays a central role in this *in vitro* method of indicating the presence in poliomyelitis vaccines of an RNA sequence critical for producing neurovirulence in monkeys. A collaborative study had been performed, and the status of the test had been discussed at a WHO Informal Consultation in 1995. Although the synthetic nucleotide preparation did not carry an assigned unitage, the Committee considered that its importance justified its establishment as an International Standard. In view of the results of the study, the Committee established the preparation, coded 95/542, as the first International Standard for MAPREC Analysis of Poliovirus Type 3 (Sabin) and assigned a value of 0.9% 472-C nucleotide to the contents of each ampoule.

The Committee recognized the importance of the research leading to this development, which was a good example of the type of work essential for advances in the regulatory field. The Committee again congratulated those involved in this work and requested WHO to continue to monitor developments. The Committee was informed that data were being collected on the application and validation of the MAPREC procedure, with the aim of reviewing the data in 1997, and it awaited the outcome of these efforts with interest. It also strongly supported the work in progress on poliovirus types 1 and 2 (Sabin) vaccine.

Further research should be encouraged to establish the value of the MAPREC method in assessing the overall quality and consistency of other live viral vaccines.

Endotoxin

The Committee noted the report on the collaborative study of a preparation of endotoxin carried out by 26 laboratories in 13 countries (BS/96.1830 Rev.1). This preparation was part of a batch (in vials) produced on behalf of the United States Pharmacopeia (USP) that was proposed as a USP reference material and which had also been offered to the European Pharmacopoeia Commission. The study included the first International Standard for Endotoxin for

Limulus Gelation Tests, as well as EC-5, the original preparation against which that standard had been calibrated. On the basis of the results of the study, the Committee established the preparation in vials, coded 94/580, as the second International Standard for Endotoxin and assigned a potency of 10 000 International Units per vial. The Committee noted that an International Unit of the preparation is equivalent to 1 endotoxin unit (EU), and that parity was thus maintained between units. The Committee further noted that the new International Standard had been examined in and is suitable for different assay procedures, unlike the previous standard which was established only for use in gelation tests. The stability of the preparation should continue to be monitored.

The Committee also noted that the results of the extensive recent collaborative study indicated that the potency of the first International Standard for Endotoxin might have been underestimated because the previous collaborative study had been more limited. Nevertheless, the Committee noted that an underestimate of potency of the standard would result in an overestimate of the endotoxin content of pharmaceutical products and so add a margin of safety.

The Committee discontinued the first International Standard for Endotoxin for *Limulus* Gelation Tests on the grounds that it was no longer needed and that its potency appeared to have been underestimated.

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.

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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.

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Annex 2
Guidelines for the production and control of the acellular pertussis component of monovalent or combined vaccines

1. General considerations

Coordinated efforts on the part of manufacturers, research institutions and national control authorities have led to the development of a variety of acellular pertussis vaccines that appear to be effective in clinical studies. These positive developments have led to the need for international guidelines to assure the quality of this new generation of pertussis vaccines.

These guidelines are concerned with the acellular pertussis component of monovalent and combined vaccines. They are intended to cover the production and control of pertussis vaccines composed of one or more individually purified or co-purified antigens. The vaccines tested in studies of clinical efficacy have been mainly products where the acellular pertussis component has been formulated with diphtheria and tetanus antigens.

There is as yet no consensus about the antigenic composition of an ideal acellular pertussis vaccine. Acellular pertussis vaccines currently available from different manufacturers should be considered as different and unique products because of the presence of one or more different components (chemically or genetically detoxified pertussis toxin, filamentous haemagglutinin (FHA), 69 kDa outer-membrane protein (also known as pertactin), fimbrial-2 and fimbrial-3 antigens) in different concentrations, and with different degrees of adsorption to different adjuvants. In addition, these individual antigens may be derived from different strains of *Bordetella pertussis* and have been purified by different methods. For these reasons the protective efficacy in humans of various manufacturers' products may be based on different mechanisms, which complicates the direct comparison of the protective activity of various products and new formulations by means of simple laboratory tests. Indeed, no unequivocal immunological correlates of protection against pertussis have yet been demonstrated, nor has a generally accepted animal model to predict clinical efficacy been validated.

In the light of this difficulty, any change in the manufacture or formulation of an acellular pertussis vaccine that has been shown to be safe and effective in clinical studies should be treated with the utmost caution. While, ideally, the safety and efficacy of a new formulation should be demonstrated in the target population, it is becoming increasingly difficult to undertake efficacy trials. Consequently, other criteria may be accepted by a national control authority as predictors of clinical performance. One possibility is to demonstrate the induction of immune responses equivalent to those induced by an approved homologous acellular pertussis vaccine of proven safety and efficacy. However, additional information about the physicochemical and immunological characteristics of a new vaccine formulation will also be necessary to demonstrate its equivalence with a homologous approved pertussis vaccine. Such criteria should be discussed with the national control authority.

In view of these considerations, it is essential that research to identify immunological markers of protection against pertussis be actively supported and pursued, and that there be rigorous post-licensing monitoring of vaccines for safety and effectiveness.

Consistency of manufacture

In the absence of internationally accepted indicators of protective efficacy, manufacturers should demonstrate consistency in manufacturing and formulation and should adhere strictly to the production

process used for the manufacture of the vaccine lots used to prove efficacy and safety in clinical trials. In addition, laboratory tests should show equivalence in safety, potency, physicochemical and immunological characteristics of new vaccine lots compared with a homologous reference vaccine. Such a reference vaccine should in turn show equivalence to lots of known clinical efficacy. Only by these means can maximum assurance be given of the safety and efficacy of vaccine production lots. Manufacturers should ensure that sufficient quantities of homologous reference vaccine of adequate stability are available for routine in-house testing and for confirmatory tests undertaken by the national control authority. This approach is only meaningful when the immunological and physicochemical characteristics of the reference vaccine are the same as they were when clinical trials demonstrating efficacy were performed.

Characterization of antigens

The immunological, biological and biochemical characterization of the individual antigens claimed to contribute to vaccine efficacy is critical for the demonstration of the structural and/or functional integrity of these components in vaccine production lots. The relevant tests must be done before any procedure, such as detoxification or chemical treatment, known to modify the immunological or biological characteristics of the components in carried out. Other tests, for example the test for residual activity of pertussis toxin, are more appropriately undertaken after detoxification or chemical treatment of antigen lots. If antigen lots are not detoxified or chemically modified, then all tests indicated in section 2.2.3 should be performed on the purified antigen lots.

The quantification of individual antigens in the final bulk and assessment of their adsorption to the adjuvant are important for assuring consistency of formulation. However, the effect of the detoxification process and the potential interference of adjuvant with certain quantitative tests, such as enzyme-linked immunosorbent assays (ELISAs) for the individual antigens, may complicate standardization of the final bulk. For this reason manufacturers are encouraged to develop reliable methods for monitoring individual antigens in the final bulk.

There is as yet no internationally accepted direct method of measuring the potency of acellular pertussis vaccine that can guarantee protective immunity will be elicited in the target population. However, the characteristics of the various antigens claimed to contribute to vaccine efficacy, together with data on vaccine composition and

dosage, consistency of production, and conformity with the specifications of the vaccine used in clinical trials give some indication, though not definitive proof, of the ability to elicit protective immunity.

At the present time, immunogenicity assays in mice that compare test vaccine with homologous reference vaccine are widely used for the lot release of the vaccine final bulk. The adoption of this approach should not be interpreted to imply that such assays possess all the desirable characteristics of a potency assay. However, in the absence of laboratory correlates of human protection, the immunogenicity test in mice is a simple means of complementing other tests for consistency of manufacture. To standardize the antibody tests used to evaluate the immunogenicity of individual antigen components in mice, manufacturers are encouraged to include appropriate mouse reference sera.

Research to identify assays that better predict protective efficacy in humans is strongly encouraged.

Recent information suggests that evidence of induction of cell-mediated immunity, cytokine induction, and protection against aerosol or intranasal challenge with *B. pertussis* may give useful information for further characterization of the vaccine. In addition, a modified intracerebral protection test in mice, used in some countries for lot release, may merit further investigation.

Toxicity testing

Despite advances in the immunochemical knowledge of the toxins and other potentially reactogenic components produced by *B. pertussis*, uncertainty remains regarding the exact role played by these substances in the pathogenesis of pertussis and in vaccine reactions. This has hampered the establishment of scientifically sound limits for the residual activity of these components in vaccines containing pertussis antigens.

Chemically or genetically inactivated pertussis toxin is a component of all acellular pertussis vaccines currently produced. For that reason, toxicity tests to monitor residual pertussis toxin activity and, where appropriate, the possible reversion of pertussis toxoid to pertussis toxin during storage are critical for ensuring the safety of the product.

Acceptable limits should be based on consistency of manufacture, i.e. the amount of active pertussis toxin in a new production lot should not exceed that present in lots shown to be safe in clinical studies. The histamine-sensitizing test appears to be suitable for lot release.

The histamine-sensitizing test is based on the fact that exposure of certain strains of mice to active pertussis toxin increases their sensitivity to the lethal effect of histamine by a factor of approximately 100. Although the physiological basis of this phenomenon is not well understood, an assay based on histamine sensitization has been shown to be specific and adequately sensitive for the detection of active pertussis toxin in acellular-pertussis-vaccine preparations. Many factors influence the histamine-sensitizing activity of pertussis toxin, including the strain, age and sex of mice used for the assay, the route of administration of the preparations, the amount of histamine used for the challenge and a number of less-well-characterized environmental factors.

The manufacturing process should be designed to reduce the level of lipooligo-saccharide (LOS) endotoxin from *B. pertussis* associated with the antigens constituting the vaccine. The LOS content of the final bulk pertussis vaccine is usually measured for lot release by means of the *Limulus* amoebocyte lysate (LAL) test. LOS content should not exceed the amount present in lots shown to be safe in clinical trials.

In addition to these lot-release tests, manufacturers are required to submit evidence of the absence of residual activity of heat-labile toxin, tracheal cytotoxin, adenylate cyclase toxin, as well as the lack of reversion to toxicity in the final product, as part of the validation of the manufacturing process. Where appropriate, detailed information about the kinetics of the detoxification of the pertussis toxin must also be submitted. The Chinese-hamster ovary-cell (CHO) toxicity test is a convenient and sensitive test for this purpose, and can be used instead of the histamine-sensitizing test.

It should be noted that the mouse weight-gain test and leukocytosis-promotion test, which are currently in use to monitor the toxicity of whole-cell pertussis vaccine, are considered to be of insufficient sensitivity to demonstrate residual pertussis-toxin activity in acellular pertussis vaccines.

Purity

Tests for the presence of residual levels of reagents used in bacterial culture or antigen purification (e.g. cyclodextrin or fetuin) should be included, and limits should be set. In addition, the purity of preparations of individually purified antigens should be assessed either for every lot or as an important part of process development and validation.

2. Scope

The guidelines apply to the production of acellular pertussis vaccines. Where the acellular pertussis component is to be combined with other antigens (e.g. diphtheria, tetanus toxoids), tests recommended for the final bulk of acellular pertussis vaccine must be performed on the final bulk of the combination vaccine.

The guidelines cover control of the following three areas:

- the starting materials
- the manufacturing process
- the final product.

The general manufacturing requirements contained in Good Manufacturing Practices for Pharmaceutical (*1*) and Biological (*2*) Products apply to the production of the acellular pertussis component of monovalent or combined vaccines.

Written descriptions of the standard operating procedures used for the preparation and testing of the acellular pertussis component of monovalent or combined vaccines, together with evidence of appropriate validation of each production step, should be submitted for approval to the national control authority as part of the licensing application. Proposals for any modifications of the manufacturing and/or control methods should be submitted for approval to the national control authority before they are implemented.

2.1 Control of starting materials

2.1.1 *Strains of B. pertussis*

Strains of *B. pertussis* used in preparing vaccine should be identified by a full record of their history, including origin and characteristics. If genetically modified *B. pertussis* is used, all relevant modified DNA sequences should be clearly delineated and fully characterized. The strains of *B. pertussis* used should be approved by the national control authority.

2.1.2 *Seed-bank system*

The production of the acellular pertussis component of monovalent or combined vaccines should be based on a well characterized seed-bank system. Cultures from the working seed bank should have the same characteristics as cultures from the master seed bank. If genetically modified *B. pertussis* is used, the relevant modified DNA sequences should be reconfirmed at the working-seed-bank level.

The strains should be maintained by a method approved by the national control authority. The method should preserve the ability of the seed to yield potent vaccine in terms of the quality of the antigens produced.

If validated, freeze-drying or storage in liquid nitrogen is a satisfactory method of maintaining strains.

2.1.3 *Culture media for production of bacteria*

The media used should enable *B. pertussis* to grow and produce the antigens of interest in good yields. Human blood or blood products should not be used in culture media, neither for seed banks nor for vaccine production. Where possible, animal blood or blood products should likewise not be used, but, if they are, they should be derived from animals in good general health, and the final product should be tested for the presence of contaminating antigens and allergenic substances.

Media constituents or other materials of bovine origin should comply with the guidelines given in the *Report of a WHO Consultation on Medical and other Products in relation to Human and Animal Transmissible Spongiform Encephalopathies* (*3*) and should be approved by the national control authority.

2.2 Control of the manufacturing process

2.2.1 *Control of production cultures*

Production cultures should be shown to be consistent with respect to growth rate, pH and the rate of production of the desired antigen or antigens. Acceptance specifications should be established.

2.2.2 *Control of bacterial purity*

Samples of individual cultures should be tested for microbial purity by microscopic examination of stained smears, by inoculation of appropriate culture media, or by any other suitable procedure. For microscopic examination, several fields should be examined at high magnification. If a contaminant is found, the culture and any product derived from it should be discarded.

2.2.3 *Control of purified antigens*

Tests undertaken prior to detoxification/chemical treatment

Characterization of antigens. Rigorous characterization of the antigens by physicochemical, immunological or functional (biological) assays, as appropriate, is essential before any step is undertaken that

is capable of modifying these characteristics. Particular attention should be given to employing a range of analytical techniques based on different principles. Suitable assays include sodium-dodecyl-sulphate–polyacrylamide-gel electrophoresis (SDS-PAGE), single radial immunodiffusion, immunoblotting, the CHO-cell toxicity test, haemagglutination and high-performance liquid chromatography (HPLC).

In cases where two or more antigens are co-purified, the proportion of each antigen claimed to contribute to vaccine efficacy should be measured by a suitable method, e.g. SDS-PAGE, HPLC, electrophoresis on non-denaturing gels, or densitometry, and shown to be within the range of values found for vaccine lots shown to be efficacious in clinical trials.

Antigenic purity of the vaccine component(s). The purity of the individual or co-purified antigens should be determined by SDS-PAGE, HPLC or other appropriate analyses before detoxification. It is important for the techniques used to be based on as wide a range of properties of the vaccine components as possible. Limits should be specified for all impurities detected.

The purity of the individual or co-purified antigens should be within the range of values found for vaccine lots shown to be efficacious in clinical trials.

Residual levels of endotoxin. The antigens should be tested for residual endotoxin content by means of the LAL test or other appropriate assay. Endotoxin content should be consistent with levels found to be acceptable in vaccine lots used in clinical trials and approved by the national control authority.

Tests undertaken after detoxification/chemical treatment

Residual activity of pertussis toxin. The amount of residual biologically active pertussis toxin in the individually or co-purified antigens should be estimated after detoxification by means of a sufficiently sensitive test, for example the CHO-cell test. When diluted to vaccine strength, the total amount of residual pertussis toxin from all pertussis antigens should not exceed that found in vaccine lots shown to be safe in clinical trials and approved by the national control authority.

Antigen content. The amount of individually or co-purified antigens that have been characterized, purified and detoxified, as appropriate, and are ready for formulation of the final bulk, should be estimated by means of a validated quantitative assay of sufficient sensitivity, such as an assay for protein content and, where available, a suitable quantitative immunoassay.

Sterility test. Each purified antigen lot should be tested for bacterial and mycotic sterility in accordance with the requirements of Part A, section 5, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (*4*), or by a method approved by the national control authority.

If a preservative is added, appropriate measures should be taken to prevent interference with the sterility test.

2.3 Control of final bulk

2.3.1 *Preparation*

The final bulk is prepared by mixing the adjuvant (as appropriate) with suitable quantities of individually or co-purified antigens so as to meet the specifications of vaccine lots shown to be safe and efficacious in clinical trials. A preservative may be added.

2.3.2 *Detoxifying/stabilizing agents*

The content of free residual detoxifying or stabilizing agents in the final bulk should be determined by methods approved by the national control authority. Limits should be specified. If formaldehyde has been used, the residual content should not exceed 0.2 g/l.

2.3.3 *Preservative*

If preservative is added, the content should be determined by a method approved by the national control authority.

The amount of preservative in the final bulk should be shown not to have any deleterious effect on the antigens for which a claim of protective efficacy is made, and should be shown not to cause any unexpected adverse reaction in humans. The preservative and its concentration should be approved by the national control authority.

2.3.4 *Adjuvant*

When adjuvant is added, its content in the final bulk (or final lot) should be determined by a method approved by the national control authority.

When aluminium or calcium compounds are used as adjuvants, the concentration of aluminium should not exceed 1.25 mg per single human dose, and that of calcium 1.3 mg. Adsorption of antigens to the adjuvant should be investigated, where possible, by tests designed to determine which, and how much of each, are adsorbed. Consistency

of adsorption is important, and the adsorption of production lots should be demonstrated to be within the range of values found for vaccine lots shown to be clinically effective.

2.3.5 *Sterility*

Each final bulk should be tested for bacterial and mycotic sterility as indicated in section 2.2.3, *Sterility test.*

2.3.6 *Residual activity of pertussis toxin*

Each final bulk of vaccine should be tested for the presence of active pertussis toxin using a sufficiently sensitive histamine sensitization test (see Appendix). The acceptable amount of active residual pertussis toxin in the final bulk diluted to vaccine strength should meet the specification approved by the national control authority on the basis of vaccine lots shown to be safe in clinical studies.

2.3.7 *Reversion to toxicity*

Manufacturers should demonstrate to the satisfaction of the national control authority that chemically inactivated pertussis toxin present in the final bulk does not revert to its toxic form before the vaccine expiry date. The national control authority may base approval on tests performed on the product (in the final containers and after storage at the recommended temperature for a period at least as long as the validity period) as described in section 2.3.6. In addition, accelerated reversion testing, such as by subjecting the final bulk to the above test after storage for at least 4 weeks at 37 °C, may be useful, and the national control authority may require this test as part of the initial validation of the inactivation process rather than as a test required for the release of individual lots.

2.3.8 *Immunogenicity*

The immunogenicity of each vaccine component claimed to contribute to efficacy should be tested by comparison with a homologous reference vaccine. At present, this test is usually performed in mice (see Appendix). The strain or strains of mouse used should allow a sufficient immune response to be detected for each antigen.

The reference vaccine should preferably be appropriately stabilized to allow for a meaningful comparison between the immunogenic activity of the test vaccine and that of the original lots of vaccine shown to be effective in clinical trials. Careful attention must be given to the demonstration of any effects the stabilizing procedure may have on the immunogenic activity of the reference vaccine. The reference

vaccine should either be drawn from a vaccine lot with proven clinical efficacy, or from a subsequent lot manufactured by the same process used to manufacture the clinical trial lots, and with adequate potency as demonstrated in the immunogenicity assay described in the Appendix.

The immunogenic activity of each antigen claimed to contribute to vaccine efficacy should be within the specification approved by the national control authority on the basis of the immunogenic activity of the corresponding antigen in the reference vaccine.

An alternative method to the immunogenicity test described in the Appendix is a modified intracerebral protection test in mice, which is used for lot release in some countries (see General considerations).

2.4 Control of final lot

The following tests should be performed on each final lot of vaccine (i.e. in the final containers).

2.4.1 *Identity*

An identity test should be performed on at least one labelled container from each final lot by means of a validated method approved by the national control authority.

2.4.2 *Sterility*

Each final lot should be tested for sterility as specified in section 2.2.3, *Sterility test.*

2.4.3 *Adjuvant content*

Unless determined for the final bulk, each final lot should be assayed for adjuvant content (where appropriate). The method used and the adjuvant concentration permitted should be approved by the national control authority. The latter should be within the range of values found for vaccine lots shown to be clinically safe and effective.

2.4.4 *Preservative content*

Where appropriate, each final lot should be assayed for preservative content, if this has not been done for the final bulk. The method used and content permitted should be approved by the national control authority.

2.4.5 ***pH***

The pH of each final lot should be within the range of values found for vaccine lots shown to be clinically safe and effective.

2.5 Stability, storage and expiry date

Adequate stability studies form an essential part of vaccine development. The stability of the vaccine in its final containers, maintained at the recommended storage temperature, should be demonstrated to the satisfaction of the national control authority. Containers from at least three consecutive final lots, and derived from different antigen production lots, should be tested.

For each of the antigens claimed to contribute to protective efficacy, real-time stability studies should support immunogenicity and lack of specific toxicity of the product up to the expiry date.

The product must be manufactured in such a way that reversion to toxicity of the inactivated pertussis toxin in the vaccine does not occur during the validity period, provided the product is stored under the conditions stated on the label.

The desorption of antigens from the adjuvant, which may occur with time, should be investigated, and where possible, limits agreed to with the national control authority.

Accelerated stability studies may provide additional evidence of product stability but cannot replace real-time studies.

When any changes that may affect the stability of the product are made in the production procedure, the stability of the vaccine produced by the new method should be demonstrated.

2.6 Reference materials

Manufacturers should set aside, as reference material, a vaccine lot equivalent to the vaccine lots tested in the clinical trials on the basis of which the licence was granted. Manufacturers should also maintain adequate stocks of reference sera.

No formally established international reference materials that would allow standardization of the acellular pertussis component of vaccines are currently available, but their development is under consideration. In the absence of such materials, the following reagents are offered through the courtesy of different manufacturers and national control authorities.

(1) Pertussis toxin for confirmation of the sensitivity of histamine sensitization tests (J-NIH 5).

(2) Serotyping agents for *B. pertussis* strain characterization.
(3) Mouse reference serum for standardization of the immunogenicity test in mice (US Standard Pertussis Antiserum (mouse), Lot 1 (SPAM-1)). SPAM-1 has been calibrated by ELISA against J-NIH 11 (mouse anti-pertussis-FHA serum) and J-NIH 12 (mouse anti-pertussis-toxin serum) to derive anti-FHA and anti-pertussis-toxin unitage, respectively. It has also been assigned anti-pertactin unitage. As supply is limited, a new mouse reference serum is under development.
(4) Human reference sera for standardization of clinical serology (US Reference Pertussis Antiserum (human), Lots 3 and 4).
(5) FHA for quantification of FHA content of vaccine (J-NIH 4).
(6) Pertactin, for quantification of pertactin content of vaccine.

Reagents (1), (2), (5) and (6) are available in limited quantities from: Division of Bacteriology, WHO International Laboratory for Biological Standards, National Institute for Biological Standards and Control, South Mimms, Potters Bar, Herts., England EN6 3QG.

Reagents (3) and (4) are available in limited quantities from: Center for Biologics Evaluation and Research, Food and Drug Administration, 1401 Rockville Pike, Rockville, MD 20852-1448, USA. Additional reagents are under development; details are available upon request.

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4. 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 (WHO Technical Report Series, No. 530).

Appendix

Methods currently used in some countries for quality control of acellular pertussis vaccines

In this Appendix, immunogenicity and histamine-sensitization tests in mice are described. These methods are currently widely used for the lot release of final bulk pertussis vaccine. However, this should not be interpreted to imply that the immunogenicity test in mice possesses all the desirable characteristics of a potency assay. Further research will be necessary to develop improved assays for predicting the protective efficacy of pertussis vaccine in humans.

Histamine-sensitization test for active pertussis toxin

A histamine sensitization test for the detection of active pertussis toxin is as follows. Groups of between 10 and 20 mice each, defined with respect to strain,[1] sex and age, should be randomly allocated to the different treatment protocols. One such group (the positive control group) should be injected with three or more serial dilutions of a preparation of pure pertussis toxin. Another group should be injected with one or more dilutions of the vaccine final bulk. Dilution factors should be chosen so as to obtain a graded response; however, dilution factors should be no greater than five. The dilution scheme should be optimized for each preparation. An additional group of mice (the negative control group) should be injected with diluent. The positions of the cages on the storage shelves during the testing period should be allocated at random. All mice should be challenged by injection with a defined dose of histamine between 4 and 5 days after sensitization or injection with diluent. Histamine challenge should follow the place-order of the cages on the shelves. Deaths within 24 hours of histamine injection should be recorded. For the assay to be considered valid, mice injected with diluent must not show substantial sensitization to the lethal effect of histamine. In addition, the susceptibility to sensitization of the mice used in each test should be validated and the median histamine-sensitizing dose (HSD_{50}) of pertussis toxin should be shown to be acceptable. The HSD_{50} should be calculated by using a suitable statistical method, such as probit analysis. Departure from linearity of the log dose–response relation for the positive control

[1] Several strains of mouse (all with Swiss-Webster ancestry) are highly responsive to histamine sensitization, but a number of Swiss-Webster strains, both inbred and outbred, are weakly responsive. A limit for the strain chosen should be established on a statistical basis. Strains considered acceptable show a median histamine-sensitizing dose (HSD_{50}) (point estimate) for pure pertussis toxin below 50 ng. When validating mice used in the assay, variability of the estimate of HSD_{50} should be taken into account.

group should not be demonstrable (with 99% confidence). Once linearity has been established by repeated experiments, the assay may be simplified so as to include in each test only a single positive control group.

An estimate of the amount of residual active pertussis toxin in the final bulk can be expressed in terms of an HSD_{50} dose. Alternatively, the amount of residual active pertussis toxin can be expressed as the proportion of animals that die upon sensitization with a single dose of pertussis vaccine (usually a single human dose) and subsequent histamine challenge. The residual pertussis toxin activity in the final bulk should not exceed that of vaccine lots shown to be safe in clinical trials. If a vaccine lot fails in a single test, it should pass two additional consecutive and independent assays to be considered suitable for release.

Immunogenicity test in mice

The immunogenicity test for acellular pertussis vaccine is a standardized assay designed to demonstrate consistent immunogenicity in mice from lot to lot for each antigen in the vaccine. Immunogenicity can be measured as either the geometric-mean amount of antibody produced in mice injected with a test dose of vaccine, or as the minimal dose of each antigen inducing a measurable antibody response in a certain proportion of mice (e.g. the median effective immunizing dose (ED_{50})).

In the first method, a group of mice is injected with a pre-selected dose of vaccine that is within the linear-response region of the dose–response curve (vaccine dose versus antibody production) for a given antigen. After an appropriate length of time, another test dose of vaccine may be required for preparations containing multiple antigens, because of the differential immunogenicity of the antigens in mice. In the second method, groups of mice are injected with serial dilutions of vaccine. After consistency in manufacturing and testing has been demonstrated to the satisfaction of the national control authority, the serial-dose method may be simplified to a single-dose (e.g. ED_{50} for the antigen) assay.

Regardless of test design, the antibody content of test sera is calculated relative to a stabilized reference serum by means of a validated and standardized ELISA.

For all antigens, reproducibility of the antibody response in the chosen strain of mice should be verified in every test by the inclusion of a group of mice injected with homologous reference vaccine. The

reference vaccine ensures that the test mice respond in a way consistent with previous testing. It is therefore essential that the reference vaccine be appropriately stabilized, preferably by lyophilization.

The results of the mouse immunogenicity test for new lots of acellular pertussis vaccine are compared with those from lots for which efficacy was directly demonstrated in human clinical trials, or with lots shown in human immunogenicity studies to be equivalent to such lots. Immunogenicity of vaccine as measured in this test is not an index of clinical efficacy, but rather a means of showing that newly manufactured lots have an equivalent immunogenicity to clinically efficacious vaccine lots when tested with a standardized assay in mice.

Two components of the test require careful attention:

Mouse. Strains of mouse (if necessary more than one) should be selected so that a sufficient antibody response is obtained for each antigen; the optimal age for mouse immunization (e.g. more than 5 weeks of age), the optimal time for bleeding (e.g. 4 to 6 weeks after immunization), and the isotype of the antibody response should be thoroughly studied.

Antibody detection system. The ELISA used for detection of antibodies should be subjected to thorough validation and standardization studies. The studies should include determination of the biochemical integrity and immunological purity of antigens used for coating assay plates and determination of the optimal antigen-coating concentration. For this purpose, the production and standardization of a working-reference mouse-serum is of utmost importance. Studies for reference serum standardization should include an evaluation of the parallelism of the dose–response curves of reference and test sera.

Another component of the antibody detection system requiring careful study is the anti-mouse-immunoglobulin–enzyme conjugate. This reagent should be characterized in terms of isotype specificity and subclass reactivity, and a suitable working dilution should be determined.

The reproducibility (intra- and inter-assay) of the assay for sera containing different amounts of antibody should be studied. Two parameters of the assay system that define its performance are the minimal amount of antibody that can be accurately distinguished from background (the limit of detection, LOD), and the minimal amount of antibody that can be measured with a conventional precision (the limit of quantification, LOQ). These limits are necessary for evaluating the capacity of the assay to discriminate between a mouse that has responded to a given antigen and one that has not.

The development of criteria for acceptance of a vaccine lot subjected to the immunogenicity test should take into account the following ELISA validity criteria:

- The average absorbance value for normal mouse serum should be below a historically defined upper limit. Normal mouse serum should be obtained from mice injected with diluent and housed with vaccinated mice for the duration of the immunization period. The absorbance of normal mouse serum should be measured in the same ELISA as the sera of immunized mice.
- The parameters of the curve relating absorbance to dilution for the reference serum should be within historically defined upper and lower limits.
- A control serum with characteristics similar to the test sera and stored in a separate location from the reference serum should be included in every ELISA plate. The ratio of the ELISA units calculated for the control serum to those for the reference serum should be within historically defined upper and lower limits.

If the ELISA meets these validity criteria, the antibody values for mice immunized with the reference vaccine and the test vaccine should be calculated. Sera with ELISA-unit values below the LOD or LOQ should be qualified as belonging to non-responder mice. For the purpose of calculating geometric mean antibody level, an arbitrary value (e.g. 1/2 LOQ) may be assigned to such sera. The number of mice responding to each antigen should be used to calculate ED_{50}. If the ELISA validity criteria are not met, the ELISA should be repeated.

After either a geometric mean or ED_{50} has been calculated for the reference vaccine, the value should be compared with the criteria for sufficient antibody response established when the strain of mouse used in the assay was validated. If the criteria are met, the test vaccine should be examined. If the criteria are not met, the ELISA should be repeated on all sera (from mice inoculated with both reference and test vaccine). If the criteria are not met after a second ELISA, immunization should be repeated.

To pass the immunogenicity test, the geometric mean antibody levels, or ED_{50}, for mice immunized with test vaccine should meet the criteria established when the assay was validated. Such limits should be determined by performing several tests on all available lots shown to be clinically efficacious. If geometric mean antibody levels are below the established limit or the ED_{50} is above the established limit, as determined in valid ELISAs, immunizations and ELISAs should be repeated for those antigens that fail the test. After a second test (if

valid), the geometric mean antibody levels or ED_{50} should be calculated and results of the two tests should be combined by appropriate statistical methods. The limit values to consider when two tests are performed should be statistically adjusted. If the results of single or double tests for all antigens in the vaccine satisfy their corresponding limits, the vaccine passes the potency test. If any antigen does not satisfy its adjusted limit after two assays, the vaccine fails the potency test.

The method used to calculate antibody response, as well as the treatment of non-responder mice in the calculation of vaccine potency, should be approved by the national control authority.

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Annex 3
Guidelines for assuring the quality of DNA vaccines

1. Introduction

Vaccination consists of stimulating the immune system with an infectious agent, or components of an infectious agent, modified in such a manner that no harm or disease is caused, but ensuring that when the host is confronted with that infectious agent, the immune system can adequately neutralize it before it causes any ill effect. For over a hundred years vaccination has been effected by one of two approaches: either introducing specific antigens against which the immune system reacts directly; or introducing live attenuated infectious agents that replicate within the host without causing disease and synthesize the antigens that subsequently prime the immune system.

Recently, a radically new approach to vaccination has been developed. It involves the direct introduction into appropriate tissues of a plasmid containing the DNA sequence encoding the antigen(s) against which an immune response is sought, and relies on the *in situ* production of the target antigen. This approach offers a number of potential advantages over traditional approaches, including the stimulation of both B- and T-cell responses, improved vaccine stability, the absence of any infectious agent and the relative ease of large-scale manufacture. As proof of the principle of DNA vaccination, immune responses in animals have been obtained using genes from a variety of infectious agents, including influenza virus, hepatitis B virus, human immunodeficiency virus, rabies virus, lymphocytic choriomeningitis virus, malarial parasites and mycoplasmas. In some cases, protection from disease in animals has also been obtained. However, the value and advantages of DNA vaccines must be assessed on a case-by-case basis and their applicability will depend on the nature of the agent being immunized against, the nature of the antigen and the type of immune response required for protection.

The field of DNA vaccination is developing rapidly. Vaccines currently being developed use not only DNA, but also include adjuncts that assist DNA to enter cells, target it towards specific cells, or that may act as adjuvants in stimulating or directing the immune response. Ultimately, the distinction between a sophisticated DNA vaccine and a simple viral vector may not be clear. Many aspects of the immune response generated by DNA vaccines are not understood. However, this has not impeded significant progress towards the use of this type of vaccine in humans, and clinical trials have begun.

The first such vaccines licensed for marketing are likely to use plasmid DNA derived from bacterial cells. In future, others may use RNA or may use complexes of nucleic acid molecules and other entities. These

guidelines address the production and control of vaccines based on plasmid DNA intended for use in humans. The purpose of these guidelines is to indicate:

- appropriate methods for the production and control of plasmid DNA vaccines; and
- specific information that should be included in submissions by manufacturers to national control authorities in support of applications for the authorization of clinical trials and marketing.

It is recognized that the development and application of nucleic acid vaccines are evolving rapidly. Thus, their control should be approached in a flexible manner so that it can be modified as experience is gained in production and use. The intention of these guidelines is to provide a scientifically sound basis for the production and control of DNA vaccines intended for use in humans, and to assure their consistent safety and efficacy. Individual countries may wish to use these guidelines to develop their own national guidelines for DNA vaccines.

2. Special safety considerations

There are several safety issues associated with administering plasmid DNA to humans: (i) the injected DNA taken up by cells of the host may be integrated into the host's chromosomes and cause an insertional mutagenic event; (ii) the long-term expression of a foreign antigen may result in an undesired immunopathological reaction; (iii) the use of genes encoding cytokines or co-stimulatory molecules may pose additional risks; (iv) antibodies against injected DNA may form and may contribute towards undesired autoimmune reactions; and (v) the expressed antigen may have biological activity.

(i) It is known that DNA taken up by mammalian cells in culture can be integrated into the cellular genetic material and be faithfully maintained during replication. This is the basis of the production of some recombinant therapeutic proteins. Theoretically, the introduction of extraneous DNA into a susceptible cell type *in vivo* could cause a transformational event leading to the formation of tumour cells by the insertion of an active oncogene, by the insertional activation of a host-cell proto-oncogene or by insertional deactivation of a suppressor gene. DNA insertion can occur in one of three ways: by random integration, by homologous recombination or by retroviral insertion. The most likely means in the present context would be random integration.

In the early 1980s, a major cause of concern regarding the use of continuous (transformed) cell lines (CCLs) for the production of biologicals was the possible transmission of an oncogene through DNA contamination of the biological product (*1*). The perceived problem was not DNA itself but its coding sequence, and there has been little concern regarding the residual bacterial DNA in recombinant protein products produced by genetically engineered bacteria. However, recently, a group of experts at an International Symposium on the Safety of Biological Products prepared from Mammalian Cell Culture held in Annecy, France, and co-sponsored by WHO, the International Association of Biological Standardization and the Mérieux Foundation, concluded that residual CCL DNA should be considered a cellular contaminant rather than a significant risk factor requiring removal to extremely low levels.[1]

After injection of DNA into an animal, only a small proportion of the DNA molecules enters cells, and of those that do only a fraction is likely to enter the nucleus. The probability of an extraneous DNA molecule being integrated into a chromosome is very low. When consideration is given to the probability of insertional mutation occurring at a growth-regulatory gene, and to the multi-step process of oncogenesis, the risk of insertional mutagenesis is seen to be exceedingly low. This argument is based upon the known low frequency of DNA insertions *in vitro* in replicating cells specifically treated to enhance DNA uptake. There is relatively little data on the frequency of DNA insertion in tissue cells *in vivo*, and none to suggest that it may be higher than that observed *in vitro*. Nevertheless, an important aspect of the preclinical safety testing of a DNA vaccine is investigation of the potential of *in vivo* integration of plasmid DNA into the vaccinated subject's chromosomes, especially since such vaccines are likely to contain strong eukaryotic- or viral-transcription promoters.

(ii) The mechanism of the immune response to an antigen expressed from injected DNA is poorly understood. There is also insufficient knowledge concerning the duration of expression of an antigen by injected DNA, although some reports suggest it could continue for many months, raising concerns regarding the

[1] Requirements for the use of animal cells as *in vitro* substrates for the production of biologicals (Requirements for Biological Substances, No. 50). In: *WHO Expert Committee on Biological Standardization. Forty-seventh Report.* Geneva, World Health Organization, 1998, Annex 1 (WHO Technical Report Series, No. 878).

possibility of tolerance. Other immunopathological reactions are theoretically possible and could be difficult to reverse.

(iii) The co-administration of genes encoding regulatory cytokines to improve responses may have adverse consequences, such as the possibility of stimulating one arm of the immune response at the expense of the other, and could lead to immunopathological reactions, including reactions directed against the cytokines themselves.

(iv) In view of the presence of specific high-affinity anti-DNA antibodies in autoimmune disorders like systemic lupus erythematosus, there is some concern that the administration of plasmid DNA may result in the production of high levels of anti-DNA antibodies and thereby contribute towards an autoimmune disorder. Although experimental attempts to generate anti-DNA antibodies that may contribute to disease have generally been unsuccessful, it is not known whether unintended antibody induction would have adverse consequences such as autoimmune disorders or tolerance. Furthermore, specific bacterial DNA sequences known to be mitogenic in animals may be incorporated into plasmid-DNA vaccines in order to enhance the immune response.

(v) Consideration must be given to the possibility that an antigen synthesized *in vivo* may exhibit unwanted biological activity. If necessary, appropriate steps must be taken, e.g. deletion mutagenesis, to eliminate this activity while retaining the desired immune response.

While these issues must be addressed experimentally, especially during the early stages of the development of DNA vaccines, it is possible that further evidence for the safety of DNA vaccines will accrue with time.

3. Scope

These guidelines cover control of the following three areas:

- the starting materials, including background data concerning the production in bacterial cells of a plasmid containing the gene(s) of interest, the sequence of the gene(s) and details of the bacterial host cell;
- the manufacturing process;
- the final product.

Plasmid DNA vaccines are similar to bacterial and viral vaccines produced by traditional methods in that adequate control of the

starting materials and manufacturing process is as important as control of the finished product. These guidelines therefore place considerable emphasis on "in-process" controls for ensuring safety and effectiveness, in addition to comprehensive characterization of the vaccine itself.

The general manufacturing requirements contained in Good Manufacturing Practices for Pharmaceutical (*2*) and Biological (*3*) products apply to plasmid DNA vaccines. Appropriate attention therefore needs to be given to the quality of all reagents used in production, including components of culture media. Many of the general issues related to the quality control of biological products, such as tests for potency, endotoxin, stability and sterility, also apply to DNA vaccines. Nucleic-acid vaccines made entirely by chemical means will require separate consideration. Guidelines for good clinical practice (GCP) for trials on pharmaceutical products (*4*) should also be applied during all stages of the development of DNA vaccines.

While the guidelines set out below should be considered generally applicable, individual vaccines may present particular problems of quality control. The production and quality control of each vaccine must therefore be given careful consideration so that any special features can be taken into account. Furthermore, the application of these guidelines to a particular vaccine should reflect its intended clinical use. Thus, different criteria would apply to a vaccine intended for prophylactic use in healthy children than to one for therapeutic use for a life-threatening condition.

4. Overview of development

A complete description of the plasmid DNA vaccine, including its nucleotide sequence, should be provided. The description should include the identification, source, means of isolation and sequence of the gene encoding the antigen(s); information on the construction of the entire plasmid; a detailed functional map of the plasmid with special attention given to regions derived from eukaryotic sources; information on the source and function of component parts of the plasmid, such as the origins of replication, viral or eukaryotic promoters, and genes encoding selection markers. The gene encoding the antigen should be shown to possess the appropriate codons for mammalian-cell expression. Certain sequences, such as retroviral-like long terminal repeats (LTRs) and oncogenes, should be avoided, as should sequences with homology to the human genome, which may promote homologous recombination. Also, a check with international databases of DNA-sequence homology should be performed to ensure

that the plasmid does not unintentionally include sequences of biological significance, such as those encoding cellular-growth functions, or present the possibility of alternative and unanticipated reading frames. A clear rationale should be provided for the use of specific regions of DNA, such as a promoter or a gene encoding a selection marker, and the nature of a selection marker should be given special attention. Representative restriction-enzyme maps should be presented. A description of the bacterial host cell used for plasmid production, including its source, phenotype and genotype, should be provided.

The identity of the plasmid after transfection into the bacterial host cell used for production should be confirmed, as should the phenotype of the bacterial cell. Plasmid rearrangements within the bacterial host cell leading to, for example, a plasmid in which an antibiotic resistance gene is under the control of a mammalian promoter, are undesirable, and the stability of the plasmid within the bacterial cell should therefore be demonstrated. The nucleotide sequence of the gene(s) to be expressed and of flanking control regions should be indicated. The precise sequence intended to be expressed in the vaccinated subject's tissues should also be clearly delineated.

5. Control of production

5.1 Master cell bank and working cell bank

The production of a plasmid DNA vaccine should be based on a cell bank system in which the working cell bank (WCB) is derived from a master cell bank (MCB). A well characterized bacterial cell containing the plasmid should be cloned and used to establish the MCB. During establishment of the MCB and WCB, no other microorganisms should be handled in the laboratory or by the same personnel.

Full information should be provided concerning the origin, form, storage conditions and life expectancy of the MCB at its anticipated rate of use. Evidence for viability of the bacterial-cell–plasmid system under storage and recovery conditions in the MCB and WCB should also be provided. New cell banks should be fully characterized and meet established acceptance criteria. Specific phenotypic features that can identify the bacterial cell containing the plasmid should be described.

The DNA sequence of the entire plasmid should normally be confirmed after establishment of the MCB. Evidence that the MCB and WCB are free from extraneous microbial agents should be provided.

5.2 Production culture

Procedures and materials used for bacterial cell growth should be described in detail. For each production run, immediately before harvesting, data on the extent and nature of any extraneous microbial contamination of the culture vessels should be provided. Acceptable limits for contamination should be set, and the sensitivity of the methods used to detect it indicated.

Data on the consistency of culture conditions, bacterial growth and plasmid yield should be presented. Criteria for the rejection of culture lots should be established. The maximum level of bacterial cell growth permitted during production should be specified, on the basis of information regarding stability of the bacterial-cell–plasmid system up to and beyond the level of growth used in production.

Bacterial cell and plasmid characteristics should be investigated at the end of production cycles. The plasmid copy number, the degree of retention of the plasmid in the cell and restriction-enzyme mapping of the plasmid are some characteristics of interest.

5.3 Purification

The methods used for harvesting the bacteria, as well as extraction and purification of the plasmid, should be described in detail. Special attention should be given to the elimination of bacterial RNA and chromosomal DNA and other materials such as linear plasmid DNA or substances added during culture. Special attention should also be given to the removal of endotoxin.

The ability of the purification procedure to remove unwanted nucleic acid, as well as irreversibly denatured plasmids, bacterial proteins and carbohydrates or other impurities, including residual media components and undesirable chemicals introduced in the course of purification, should be investigated thoroughly, as should the reproducibility of the process. Data from validation studies of purification procedures may be required to demonstrate the overall clearance of contaminants, as well as at each purification step. These studies will indicate the extent to which contaminants can theoretically be removed during purification.

6. Characterization of the purified plasmid bulk and formulated vaccine

The plasmid vaccine should be characterized with respect to identity, purity, potency and stability. The type of testing necessary and the

degree of purity expected will depend on several factors, including the intended use of the vaccine, the method of production and purification, including the possible use of adjuvants and facilitators, and experience with the production of several batches of the vaccine.

6.1 Characterization of the purified plasmid bulk

Rigorous characterization of the purified plasmid bulk by chemical, physical and biological methods is essential. A wide range of analytical techniques based on different principles should be used. Sequence verification is of paramount importance. The degree of verification required may depend on the scope of other characterization tests. For some purposes, partial sequence determination and mapping of the plasmid may suffice; for others, full sequence determination may be necessary. Attention should be paid to possible modification of the DNA (e.g. methylation, the formation of irreversibly denatured molecules or partial degradation by nucleases), since such modifications may influence the biological and immunological properties of the plasmid vaccine.

Whenever possible, the antigen should be expressed *in vitro* by transfection of a suitable cell line, and the expressed protein should be characterized, for example by immunofluorescence or Western blot, with respect to relevant features (e.g. glycosylation). The plasmid should be shown to possess the desired immunogenic activity in animals.

An appropriate potency assay should be established for the plasmid vaccine. Potency could be determined by quantitative *in vitro* expression but may require titration in an animal model to determine the minimum quantity of vaccine inducing an appropriate immune response. An approved in-house reference preparation should be established for assay standardization. Wherever possible, an appropriate national or international reference material calibrated in appropriate units of biological activity should be used for calibration of the in-house reference preparation.

6.2 Purity

Data on contaminants present in the purified plasmid bulk should be provided, including estimates of maximum levels. Some data suggest that supercoiled plasmid DNA molecules are more efficiently expressed than relaxed circular or linear molecules. The proportion of supercoiled plasmid should be determined, and specifications should be set. The degree of acceptable contamination and criteria for the rejection of a production batch should be specified.

It is important to demonstrate purity on the basis of as wide a range of physicochemical properties as possible. Attention should be given to tests for contamination by bacterial chromosomal DNA and other substances of bacterial origin, as well as substances added during the production or purification process. Limits should be specified for all impurities detected, and impurities should be identified and characterized as appropriate.

Plasmids to be administered more than once, or in large doses, should be assayed for traces of antigenic constituents and product-related impurities, such as irreversibly denatured or degraded DNA, and strict upper limits should be specified. Techniques such as immunoblotting, radio-immunoassays or enzyme-linked immunosorbent assays (ELISAs) using high-affinity antibodies raised against bacterial-cell lysates, appropriate subcellular fractions and culture medium constituents can be used to detect contaminating antigens. As the detection of antigens is limited by the specificity and sensitivity of the antisera used, other techniques, such as sodium-dodecyl-sulphate–polyacrylamide-gel electrophoresis (SDS-PAGE) may be useful.

6.3 Multi-plasmid vaccines

Additional considerations should be taken into account when more than one plasmid is contained in the formulated vaccine. Plasmids in multi-component vaccines may encode additional antigens, cytokines or other biologically active molecules that enhance the efficacy or alter the safety of the vaccine. For each plasmid, an overview of development should be described and control of production and characterization of the purified plasmid bulk should be carried out as above. Careful consideration should be given to control of the final dosage form, since potency can depend on the combination, rather than on any single plasmid component vaccine.

6.4 Adjuvants and facilitators

Details of adjuvants and other non-plasmid components, such as facilitators, should be provided, including their source, specification, method of conjugation if appropriate, and their final concentration in the formulated vaccine. Their effect on vaccine efficacy should be demonstrated, and any adverse effect noted. The potency of the formulated vaccine should be established unless otherwise justified.

6.5 Stability

Adequate stability studies are an essential part of vaccine development. The stability of the formulated vaccine should therefore be determined, and the results should be used to set a maximum shelf-life under appropriate storage conditions. Real-time stability studies should be undertaken, but accelerated studies at elevated temperatures may provide useful supporting evidence.

6.6 Consistency

A number of successive batches of purified plasmid bulk, as well as the formulated vaccine, should be characterized as fully as possible to determine consistency. Any differences between batches should be noted. The data obtained from such studies should be used as the basis for the vaccine specification.

7. Routine control of purified plasmid bulk and formulated vaccine

Not all the tests described above need to be carried out on each production batch of vaccine. Some are required to establish the validity or acceptability of a procedure, while others might be performed on a limited series of batches in order to establish consistency of production. Thus, a comprehensive analysis of initial production batches should establish consistency of identity, purity, potency and stability, but thereafter a more limited series of tests may be appropriate. The appropriateness of performing tests on the purified plasmid bulk versus formulated vaccine should be considered on a case-by-case basis.

7.1 Identity

Each batch of vaccine should be appropriately tested to confirm the identity and characteristics of the purified plasmid. The specific tests that will adequately characterize any particular plasmid on a lot-to-lot basis, however, may depend on the nature of the plasmid and its method of production and purification. Depending on the scope of the tests, confirmation of the DNA sequence or restriction-enzyme mapping and verification of antigen expression following transient transfection will be necessary.

7.2 Purity

The purity of each batch of vaccine should be determined to be within specified limits. The analysis should include sensitive and reliable

assays for contaminants of bacterial origin, and strict upper limits should be specified for contaminants in the purified plasmid bulk.

7.3 Potency

The potency of each batch of vaccine should be determined by comparison with an in-house reference preparation in a suitable assay.

7.4 Stability

The need for lot-to-lot stability testing should be determined, with approval from the national control authority, on the basis of stability studies carried out on a number of successive batches as indicated in section 6.5.

8. Reference materials

Data from the studies described in sections 6 and 7 should be considered in determining a definitive specification for the product.

A suitable batch of the formulated vaccine, preferably one that has been clinically evaluated, should be fully characterized with respect to its chemical composition, purity and biological activity, including, where possible, full determination of its sequence, and retained for use as a chemical and biological reference material. The characteristics of this reference material should be the basis for determining specifications for production batches.

9. Preclinical safety evaluation

The general aim of preclinical safety evaluation is to screen medicinal products for the potential to cause unexpected and undesirable effects. However, classical safety or toxicological testing, in particular toxicity testing in animals, as recommended for chemical drugs, may be of limited relevance for plasmid DNA products, and their safety evaluation must take into account a large number of factors. For these and other reasons, it is likely that a flexible approach will be necessary for the preclinical safety evaluation of DNA vaccines. As outlined in section 2, there are special considerations regarding the safety of DNA vaccines. Assays to assess the distribution, duration and potential integration of the plasmid DNA, including an assessment of the germ line, should be performed in experimental animals.

Preclinical studies should evaluate the possibility of immunopathological reactions arising from use of the plasmid vaccine. Consideration should be given to assays for anti-DNA antibodies and to the possibility that antigens synthesized *in vivo* may exhibit unwanted biological activity. Although safety testing will certainly be required, the range of tests that need to be carried out should be decided on a case-by-case basis in consultation with the national control authority. A wide range of techniques — biological, molecular, biochemical, immunological, toxicological and histopathological — should be used, where appropriate, to assess the plasmid vaccine's effect over a range of doses, and following both acute and chronic exposure.

10. Additional considerations

The mode of delivery of the vaccine should be indicated, and information should be provided on the efficacy of delivery.

Individuals given abnormally large or repeated doses of a plasmid vaccine during clinical trials should be monitored for the presence of antibodies to contaminating antigens.

Authors

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Annex 4
Biological substances: International Standards and Reference Reagents

A list of International Biological Standards, International Biological Reference Preparations and International Biological Reference Reagents is issued as a separate publication.[1] Copies may be obtained from appointed sales agents for WHO publications or from: Distribution and Sales, World Health Organization, 1211 Geneva 27, Switzerland.

The Expert Committee made the following changes to the previous list.

Additions

Antibodies

Anti-rubella immunoglobulin	1600 IU/vial	First International Standard 1996

This substance is held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, Potters Bar, Herts., EN6 3QG, England.

Blood products and related substances

Blood coagulation factor IX concentrate, human	10.7 IU/ampoule	Third International Standard 1996
Blood coagulation factors II, VII, IX, X, plasma, human	0.93 IU/ampoule of factor II	Second International Standard 1996
	1.25 IU/ampoule of factor VII	Second International Standard 1996
	0.90 IU/ampoule of factor IX	Second International Standard 1996
	0.95 IU/ampoule of factor X	Second International Standard 1996

[1] *Biological substances: International Standards and Reference Reagents, 1990.* Geneva, World Health Organization, 1991.

Ferritin, human, recombinant	6.3 µg/ampoule	Third International Standard 1996
Whole blood folate	13 ng/ampoule	First International Standard 1996

These substances are held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, Potters Bar, Herts., EN6 3QG, England.

Thromboplastin, human, recombinant, plain	International Sensitivity Index: 0.940	Third International Standard 1996

This substance is held and distributed by the International Laboratory for Biological Standards, Central Laboratories, Netherlands Red Cross Blood Transfusion Centre, Amsterdam, Netherlands.

Cytokines[1]

Interleukin-5	5000 units/ampoule	First Reference Reagent 1996
Interleukin-7	100000 units/ ampoule	First Reference Reagent 1996
Interleukin-9	1000 units/ampoule	First Reference Reagent 1996
Interleukin-11	5000 units/ampoule	First Reference Reagent 1996
Interleukin-12	10000 units/ ampoule	First Reference Reagent 1996
Interleukin-13	1000 units/ampoule	First Reference Reagent 1996
Interleukin-15	10000 units/ ampoule	First Reference Reagent 1996
Leukaemia inhibitory factor	10000 units/ ampoule	First Reference Reagent 1996
Oncostatin M	25000 units/ ampoule	First Reference Reagent 1996
Tumour necrosis factor, beta	150000 units/ ampoule	First Reference Reagent 1996

[1] These reference materials are interim Reference Reagents, as distinct from International Reference Reagents, and are established on the basis of limited data for certain rapidly developing fields where there is a need for standardization in measurement before an International Standard can be established.

Nerve growth factor	10000 units/ ampoule	First Reference Reagent 1996

These substances are held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, Potters Bar, Herts., EN6 3QG, England.

Endocrinological and related substances[1]

Thyroid-stimulating hormone (TSH), human, recombinant	0.0067 units/ ampoule	First Reference Reagent 1996

This substance is held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, Potters Bar, Herts., EN6 3QG, England.

Miscellaneous

Endotoxin	10000IU/vial	Second International Standard 1996
MAPREC analysis of poliovirus type 3 (Sabin)	0.9% 472-C nucleotide	First International Standard 1996

These substances are held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, Potters Bar, Herts.. EN6 3QG, England.

Discontinuations

Anti-rubella serum	Second International Reference Preparation 1970
Thromboplastin, human, plain	Second International Reference Preparation 1983
Factor IX component of blood coagulation factors II, IX and X concentrate, human	Second International Standard 1994
Oleandomycin	First International Standard 1964

[1] These reference materials are interim Reference Reagents, as distinct from International Reference Reagents, and are established on the basis of limited data for certain rapidly developing fields where there is a need for standardization in measurement before an International Standard can be established.

Spectinomycin	First International Reference Preparation 1975
Triacetyloleandomycin	First International Reference Preparation 1962
Viomycin	Second International Reference Preparation 1968
Endotoxin for *Limulus* gelation tests	First International Standard 1986

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Annex 5
Requirements for Biological Substances and other sets of recommendations

The specification of requirements to be fulfilled by preparations of biological substances is necessary in order to ensure that these products are safe, reliable and potent prophylactic or therapeutic agents. International recommendations on requirements are intended to facilitate the exchange of biological substances between different countries and to provide guidance to workers responsible for the production of these substances as well as to others who may have to decide upon appropriate methods of assay and control.

Recommended requirements and sets of recommendations concerned with biological substances are formulated by international groups of experts and are published in the Technical Report Series of the World Health Organization,[1] as listed here.

Requirements

1. General Requirements for Manufacturing Establishments and Control Laboratories
   Revised 1965, TRS **323** (1966)
   Replaced by "Good manufacturing practices for biological products", TRS **822** (1992) and "Guidelines for national authorities on quality assurance for biological products", TRS **822** (1992)
2. Requirements for Poliomyelitis Vaccine (Inactivated)
   Revised 1981, TRS **673** (1987)
   Addendum 1985, TRS **745** (1987)
3. Requirements for Yellow Fever Vaccine
   Revised 1975, TRS **594** (1976)
   Addendum 1987, TRS **771** (1988)
   Revised 1995, TRS **872** (1998)
4. Requirements for Cholera Vaccine
   Revised 1968, TRS **413** (1969)
   Addendum 1973, TRS **530** (1973)

[1] Abbreviated here as TRS.

5. Requirements for Smallpox Vaccine
   Adopted 1966, TRS **323** (1966)

6. General Requirements for the Sterility of Biological Substances
   Revised 1973, TRS **530** (1973)
   Amendment 1995, TRS **872** (1998)

7. Requirements for Poliomyelitis Vaccine, Oral
   Revised 1989, TRS **800** (1990)

8.& 10. Requirements for Diphtheria, Tetanus, Pertussis and Combined Vaccines
   Revised 1989, TRS **800** (1990)

9. Requirements for Procaine Benzylpenicillin in Oil with Aluminium Monostearate
   Revised 1966, TRS **361** (1967)
   Discontinued 1989, TRS **800** (1990)

11. Requirements for Dried BCG Vaccine
   Revised 1985, TRS **745** (1987)
   Amendment 1987, TRS **771** (1988)

12. Requirements for Measles Vaccine (Live)
   Revised 1987, TRS **771** (1988)
   Replaced by Requirements No. 47

13. Requirements for Anthrax Spore Vaccine (Live, for Veterinary Use)
   Adopted 1966, TRS **361** (1967)

14. Requirements for Human Immunoglobulin
   Adopted 1966, TRS **361** (1967)
   Replaced by Requirements No. 27

15. Requirements for Typhoid Vaccine
   Adopted 1966, TRS **361** (1967)

16. Requirements for Tuberculins
   Revised 1985, TRS **745** (1987)

17. Requirements for Influenza Vaccine (Inactivated)
   Revised 1990, TRS **814** (1991)

18. Requirements for Immune Sera of Animal Origin
   Adopted 1968, TRS **413** (1969)

19. Requirements for Rinderpest Cell Culture Vaccine (Live) and Rinderpest Vaccine (Live)
    Adopted 1969, TRS **444** (1970)

20. Requirements for *Brucella abortus* Strain 19 Vaccine (Live, for Veterinary Use)
    Adopted 1969, TRS **444** (1970)
    Addendum 1975, TRS **594** (1976)

21. Requirements for Snake Antivenins
    Adopted 1970, TRS **463** (1971)

22. Requirements for Rabies Vaccine for Human Use
    Revised 1980, TRS **658** (1981)
    Amendment 1992, TRS **840** (1994)

23. Requirements for Meningococcal Polysaccharide Vaccine
    Adopted 1975, TRS **594** (1976)
    Addendum 1980, TRS **658** (1981)

24. Requirements for Rubella Vaccine (Live)
    Adopted 1976, TRS **610** (1977)
    Addendum 1980, TRS **658** (1981)
    Replaced by Requirements No. 47

25. Requirements for *Brucella melitensis* Strain Rev. 1 Vaccine (Live, for Veterinary Use)
    Adopted 1976, TRS **610** (1977)

26. Requirements for Antimicrobic Susceptibility Tests
    I. Agar Diffusion Tests Using Antimicrobic Susceptibility Discs
    Revised 1981, TRS **673** (1982)
    Addendum 1982, TRS **687** (1983)
    Addendum 1985, TRS **745** (1987)
    Addendum 1987, TRS **771** (1988)
    Addendum 1989, TRS **800** (1990)
    Addendum 1990, TRS **814** (1991)
    Addendum 1991, TRS **822** (1992)
    Discontinued 1991, TRS **822** (1992)

27. Requirements for the Collection, Processing and Quality Control of Blood, Blood Components and Plasma Derivatives
    Revised 1992, TRS **840** (1994)

28. Requirements for Influenza Vaccine (Live)
    Adopted 1978, TRS **638** (1979)

29. Requirements for Rabies Vaccine for Veterinary Use
    Adopted 1980, TRS **658** (1981)
    Amendment 1992, TRS **840** (1994)

30. Requirements for Thromboplastins and Plasma Used to Control Oral Anticoagulant Therapy
    Revised 1982, TRS **687** (1983)

31. Requirements for Hepatitis B Vaccine Prepared from Plasma
    Revised 1987, TRS **771** (1988)

32. Requirements for Rift Valley Fever Vaccine
    Adopted 1981, TRS **673** (1982)

33. Requirements for Louse-Borne Human Typhus Vaccine (Live)
    Adopted 1982, TRS **687** (1983)

34. Requirements for Typhoid Vaccine (Live, Attenuated, Ty 21a, Oral)
    Adopted 1983, TRS **700** (1984)

35. Requirements for Rift Valley Fever Vaccine (Live, Attenuated) for Veterinary Use
    Adopted 1983, TRS **700** (1984)

36. Requirements for Varicella Vaccine (Live)
    Revised 1993, TRS **848** (1994)

37. Requirements for Continuous Cell Lines Used for Biologicals Production
    Adopted 1985, TRS **745** (1987)
    Replaced by Requirements No. 50

38. Requirements for Mumps Vaccine (Live)
    Adopted 1986, TRS **760** (1987)
    Replaced by Requirements No. 47

39. Requirements for Hepatitis B Vaccines Made by Recombinant DNA Techniques in Yeast
    Adopted 1986, TRS **760** (1987)
    Replaced by Requirements No. 45

40. Requirements for Rabies Vaccine (Inactivated) for Human Use Produced in Continuous Cell Lines
    Adopted 1986, TRS **760** (1987)
    Amendment 1992, TRS **840** (1994)

41. Requirements for Human Interferons Made by Recombinant DNA Techniques
    Adopted 1987, TRS **771** (1988)

42. Requirements for Human Interferons Prepared from Lymphoblastoid Cells
    Adopted 1988, TRS **786** (1989)

43. Requirements for Japanese Encephalitis Vaccine (Inactivated) for Human Use
    Adopted 1987, TRS **771** (1988)

44. Requirements for Haemorrhagic Fever with Renal Syndrome (HFRS) Vaccine (Inactivated)
    Adopted 1993, TRS **848** (1994)

45. Requirements for Hepatitis B Vaccines Made by Recombinant DNA Techniques
    Adopted 1988, TRS **786** (1989)

46. Requirements for *Haemophilus* Type b Conjugate Vaccines
    Adopted 1990, TRS **814** (1991)

47. Requirements for Measles, Mumps and Rubella Vaccines and Combined Vaccine (Live)
    Adopted 1992, TRS **840** (1994)
    Note, TRS **848** (1994)

48. Requirements for Vi Polysaccharide Typhoid Vaccine
    Adopted 1992, TRS **840** (1994)

49. Requirements for Hepatitis A Vaccine (Inactivated)
    Adopted 1994, TRS **858** (1995)

50. Requirements for the Use of Animal Cells as *in vitro* Substrates for the Production of Biologicals
    Adopted 1996, TRS **878** (1998)

Requirements for Immunoassay Kits [unnumbered]
    Adopted 1980, TRS **658** (1981)

Other documents

Recommendations for the assessment of binding-assay systems (including immunoassay and receptor assay systems) for human hormones and their binding proteins (A guide to the formulation of requirements for reagents and assay kits for the above assays and notes on cytochemical bioassay systems)
    TRS **565** (1975)

Development of national assay services for hormones and other substances in community health care
    TRS **565** (1975)

Report of a WHO Working Group on the Standardization of Human Blood Products and Related Substances
TRS **610** (1977)

Guidelines for quality assessment of antitumour antibiotics
TRS **658** (1981)

The national control of vaccines and sera
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TRS **673** (1982)

Standardization of interferons (reports of WHO informal consultations)
TRS **687** (1983)
TRS **725** (1985)
TRS **771** (1988)

Production and testing of the WHO yellow fever virus primary seed lot 213–77 and reference batch 168–73
TRS **745** (1987)

Report of a WHO Meeting on Hepatitis B Vaccines Produced by Recombinant DNA Techniques
TRS **760** (1987)

Procedure for evaluating the acceptability in principle of vaccines proposed to United Nations agencies for use in immunization programmes, revised 1988
TRS **786** (1989)

Guidelines for the preparation, characterization and establishment of international and other standards and reference reagents for biological substances, revised 1989
TRS **800** (1990)

Guidelines for assuring the quality of pharmaceutical and biological products prepared by recombinant DNA technology
TRS **814** (1991)

Good manufacturing practices for biological products
TRS **822** (1992)

Guidelines for national authorities on quality assurance for biological products

TRS **822** (1992)

Guidelines for assuring the quality of monoclonal antibodies for use in humans

TRS **822** (1992)

Regulation and licensing of biological products in countries with newly developing regulatory authorities

TRS **858** (1995)

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Summary protocol for the batch release of virus vaccines

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