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WHO Expert Committee on Biological Standardization

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WHO Expert Committee on Biological Standardization

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Introduction

The WHO Expert Committee on Biological Standardization met in Geneva from 17 to 21 November 2003. The meeting was opened by Dr Vladimir Lepakhin, Assistant Director-General, Health Technology and Pharmaceuticals, WHO, on behalf of the Director-General.

Dr Lepakhin emphasized the importance to Member States of the work of the Committee in preparing recommendations for biological products and for the development, establishment and distribution of reference materials for biologicals. During the meeting, a number of proposals for reference materials and draft recommendations would be considered. Dr Lepakhin informed the Committee that the World Health Assembly had endorsed many resolutions on the subjects of quality, safety and efficacy of medicines, blood products, vaccines and other biologicals. Despite considerable progress made both by WHO and its Member States in the implementation of such directives, urgent action was needed to sustain and expand basic normative regulatory functions underpinning public health efforts to assure access to quality biological medicines. Furthermore, as a result of rapidly changing global environments, increased international trade and the opening of borders, many biological medicines, including blood products, were circulating more freely than ever. Unless they were subject to control, biological medicines such as those derived from blood and plasma could be vehicles for the transmission of infectious diseases and/or other emerging agents. Dr Lepakhin reminded the Committee that the establishment and functioning of national regulatory systems with reference to WHO recommendations, norms and standards was essential to protect patients and public health from fraudulent practices and economic waste. WHO was therefore seeking political commitment and support from Member States to sustain and increase capacity building and training in all aspects of regulatory functions, including the efficient implementation of good regulatory practices. Finally, Dr Lepakhin reminded the Committee that its decisions should be based on sound science and common sense and not on partisan considerations.

General

Developments in biological standardization

The Committee was informed of the organizational changes that had taken place at WHO headquarters. The two teams concerned with

biological standardization work on an integrated programme although based in different clusters. The outcomes of the standardization programme include recommendations for quality standards, provision of reference materials, development of consensus on quality-related issues for biologicals and building technical capacity for biologicals in the different WHO Regions. The role of the International Laboratories in the development of this programme was acknowledged. The plans for further development of laboratory support for biological standardization by broadening geographical representation and improving working relationships with existing laboratories through regular meetings and better definition of priorities were outlined. A review of the Expert Advisory Panel on Biological Standardization had shown the need for more even geographical and gender representations and for the identification of more experts currently working the field. The need to enhance support for the Expert Committee had also been recognized and this could usefully be addressed through the greater use of Working Groups to prepare and review proposals for reference materials and recommendations. Consideration was also being given to how the work and operation of the Expert Committee might evolve to meet future needs in a more timely and efficient manner.

The place of standardization of vaccines within the Immunization, Vaccines and Biologicals department was outlined. Establishment of norms and standards is a critical early stage in the development of new vaccines. There is increasing appreciation by users of the norms and standards of the impact of standardization on global public health and trade, leading to increased support, both in terms of financial and human resources, and also to increased demand for standardization activities. These demands are likely to require innovative ways of providing support. As an example, the generous support of the Government of the Republic of Korea, through staff secondment from the Korean Food and Drug Administration, was acknowledged. The priorities for vaccine standardization for the next 2 years had been identified as: the provision of written guidelines and reference materials for new vaccines; improving the scientific basis for establishing vaccine quality; new and updated WHO recommendations on production and quality control; selected international reference materials; improved global coordination of standardization and control for vaccines; continued contributions to the safety of vaccines through guidelines on production and quality control; and polio vaccine standards for use after WHO has certified the global eradication of polio. These priorities are being addressed through the establishment of a group to discuss quality working through a controlled-access web site

and teleconferencing; better prioritization of recommendations being considered by the Expert Committee; production of updated guidelines for production of international biological reference materials and development of regional reference materials; and an initiative to establish a WHO repository for vaccine seeds of global importance to public health.

Attention was drawn once again to the unacceptable delays in publication of the formal reports of Expert Committee meetings. The Committee noted that such delays had persisted despite their previous recommendations to address the issue. The Committee was seriously concerned about the consequences of such delays particularly for regulatory authorities who are unable to implement unpublished recommendations. Pre-publication of the draft report and its annexes does not provide an adequate substitute for the published recommendations. The Committee recommended that WHO gives urgent attention to seeking ways to overcome the delays and so ensure that the value of work on biological standardization is not lost.

Finally, the Committee was updated on several topics not considered elsewhere during the meeting. The Committee was informed of an informal WHO consultation held recently on the development of a vaccine for severe acute respiratory syndrome (SARS). Several approaches are under investigation and it is expected that trials of candidate inactivated SARS *coronavirus* vaccines may start shortly. In this context it was noted that WHO had recommended that all work with live SARS *coronavirus*, including the large-scale production of virus during the initial stages of production of inactivated vaccine, should be performed under biosafety level 3 conditions. Work is under way to identify validated animal models for efficacy and safety. The safety model should address concerns about immune enhancement, a phenomenon seen with one inactivated animal *coronavirus* vaccine. WHO would facilitate the continuation of work on quality and safety issues. Studies should be initiated on establishing antibody standards for the calibration of assays and diagnostic tests, and for the calibration of immunotherapy products.

The Committee was informed of new initiatives within WHO that would have an impact on activities in the area of blood products and related biologicals during the next biennium, 2004–2005. This area, which is part of the overall biologicals programme, is placed within the new Department of Essential Health Technologies which, in turn, is part of the Health Technology and Pharmaceuticals cluster. The new initiatives are centred around a strong commitment to capacity building of national regulatory authorities, and aimed at

strengthening their technical capacity especially in the area of blood products. Basic operational frameworks have been developed, based on the four objectives: policy; quality and safety; access; and use. The basic operational framework is an assessment tool that will be used to identify priorities and activities at the level of WHO Regions and countries during the next biennium, and also to assist countries to identify gaps in their own health systems. Two countries would be selected from each WHO region to pilot-test the assessment tool. The area of biological standardization would be given special emphasis to highlight the need to develop regulatory mechanisms for plasma fractionation activities, either where these activities take place in the country already or where countries are expected to become involved in offering contracts for plasma fractionation in the near future. Stringent regulatory control is vital for assuring the quality and safety of products derived from human blood. Special efforts will be devoted to strengthening the technical capacity of national regulatory authorities to ensure the appropriate control of blood products and related in vitro diagnostics worldwide.

The priorities for 2004–2005 will include the updating of the WHO requirements for the collection, processing and quality control of blood, blood components and plasma derivatives. Training activities for good manufacturing practices at blood and plasma collection centres will be initiated in the Region of the Americas with the collaboration of the Regional Office. Efforts will be made to facilitate the availability of those international biological reference materials with the greatest importance to public health, such as the reference panel for hepatitis B surface antigen (HbsAg) (see p.27), in countries where resources are limited with the collaboration of WHO Regional Advisers. Activities relating to the standardization of in vitro biological diagnostic materials, particularly those applicable to testing for virological safety, will continue.

An overall need in the area of biological standardization is for coordination of the process of setting international standards. To achieve this WHO, and the Expert Committee on Biological Standardization, in particular should enhance their work with regulators in the international sphere, for example through the International Conference of Drug Regulatory Authorities as well as with other standard-setting bodies such as the International Standards Organisation and the International Office for Weights and Measures.

The Committee welcomed the comprehensive review of the work on biological standardization but expressed concern that despite the importance of the extensive programme proposed, and its likely impact

on public health, the relevant teams did not have sufficient personnel and resources to effectively perform the proposed work. The Committee recommended that these concerns be addressed.

Progress reports and work programmes

The Committee was informed of recent developments at the various WHO International Laboratories for biological standardization.

National Institute for Biological Standards and Control, Potters Bar, England

The Committee was provided with an updated programme of work proposed by the National Institute for Biological Standards and Control (NIBSC) for the period 2003–2008 covering work in progress, reference materials expected to require replacement and new projects. The Committee was pleased to note progress made with the work programme since its previous review, represented by a number of items considered elsewhere on the Agenda. The new Centre for Biological Reference Materials at the Institute had now been opened. This facility provides increased capacity for small- and large-scale fills of ampoules and vials, manufactured under negative pressure conditions to high quality standards. One filling suite can process up to 20 000 ampoules in a single batch and the other is capable of processing up to 23 000 vials in a batch. The previous maximum batch size was of the order of 3000 ampoules or vials. The complex and technically advanced building is currently undergoing validation. The facility represents a very significant investment by the United Kingdom Department of Health that will be of benefit to international biological standardization. The Committee was also informed of some organizational changes planned within the Institute to take best advantage of the new facility. Another new biological resource at NIBSC is the establishment of the UK stem-cell bank intended to hold and distribute cells for research purposes and for "clinical grade" cells under a regime that complies fully with regulatory requirements for quality assurance. An independent committee will be formed to oversee decisions on distribution of cells. There will be no involvement in basic research on stem-cell biology or in any commercial product development.

Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, (Sanquin), Amsterdam, the Netherlands

The Central Laboratory of the Netherlands Red Cross Blood Transfusion Service makes available reference materials for use in the blood products field. The Committee was informed about reference

materials distributed during the past year. The Committee was also informed of the need to replace two standards because of depletion of stocks (thromboplastin, rabbit, plain and anti-double stranded DNA, serum, human) and of the development of new human serum standards each monospecific for a specific hepatitis C antigen. Candidate materials would be obtained for evaluation. Sanquin works with NIBSC in the blood products field, particularly on reference materials for quality control in the context of blood virology. A number of such materials had been identified and work on them is in progress. Another area in which there is a need for reference materials is quantitative measurement using nucleic acid amplification techniques (NAT). The Committee noted that further information on the need for the anti-double stranded DNA serum would be helpful in determining the priority assigned to this work and endorsed the work programme under way at Sanquin.

c) Center for Biologics Evaluation and Research, Bethesda, MD, United States

The Committee was informed of recent developments in the vaccine field in the United States. Two new vaccines, a nasally-administered live attenuated influenza vaccine and a diphtheria and tetanus vaccine for use in adults, had been licensed during 2003. Current safety concerns were the implications of contamination of some vaccines more than 40 years ago with Simian virus 40 (SV40) virus and the adequacy of past and current testing for SV40. There is strong evidence to suggest that SV40 is a transforming virus and moderate evidence to suggest that exposure to SV40 could lead to human cancer under natural conditions. However, the evidence for accepting or rejecting a causal relationship between SV40-containing vaccines administered in the 1950s and cancer is inadequate. The current concerns about SV40 emphasize the potential for very long-term implications of issues of vaccine quality. The Committee was also informed that one consequence of the case of bovine spongiform encephalopathy (BSE) identified in Canada in 2003 was that the current US regulations concerning ruminants and ruminant products from countries with mimimal risk of BSE may be revised.

The Committee was further informed of current developments in the regulation of blood products including the management of emerging infectious diseases. There had been a rapid development of investigational NAT techniques for West Nile virus in the United States following the emergence of this virus in 2002. As a result a number of contaminated donations had been identified in 2003 and withdrawn from the blood supply. A control panel was under development. The

implications of other infections, including monkeypox, smallpox and anthrax, on selection of blood donors were reviewed. Current policy calls for donor deferral (i.e. a delay between vaccination and clearance to donate blood) subsequent to smallpox vaccination because of the uncertainty over viraemia, and there are concerns over the impact of a mass vaccination campaign against smallpox on the blood supply. Progress in the development of diagnostic tests for screening the blood supply was described, and information given about recent guidance from the US Food and Drug Administration in the blood products field.

The Committee noted the information supplied, endorsed the work programmes proposed by the laboratories and expressed gratitude for their contributions. The Committee further noted those activities intended to anticipate developments in standardization and considered that these activities would allow time to ensure adequate resources for this work.

Contamination of oral poliomyelitis vaccine with wild poliovirus

The Committee was informed that, in the period from November 2002 to February 2003, several cases of poliomyelitis had been reported in one country after vaccination, and the MEF-1 reference wild-type poliovirus type 2 strain was isolated in each case. Initial investigations excluded cross-contamination within the WHO polio laboratory network as an explanation for the findings. Further examination demonstrated the presence of MEF-1 in vials of one vaccine batch that had been filled locally from imported bulk material. Samples of the batch that had been collected from the field, from retained samples and from other bulks were tested. Only samples from the field tested positive, which indicated that contamination had taken place downstream from manufacture although it could not be established whether this was during storage or distribution. Enhanced security measures have now been put in place by the manufacturer concerned. The Committee was asked to consider the wider implications of this episode, whether current WHO guidance on good manufacturing practice (GMP) is adequate and whether additional control measures on the final product should be specified. The Committee considered that the prevention of deliberate interference with a product requires safeguards different to those normally covered by GMP and that no changes to existing GMP guidance for this reason were necessary. However, the Committee drew attention to measures that ensure that vials are tamper-proof and to procedures that are available to detect counterfeiting. In this context WHO was advised to

obtain additional specialist advice, and to make this available to all vaccine manufacturers, their distributors and national regulatory authorities.

Feedback from the field on WHO recommendations and guidelines and capacity building in quality assurance

The Committee was informed of feedback on the recommendations and guidelines that it had produced on GMP. This had been obtained in part from the field through the WHO Global Training Network courses on GMP organized for Indian and Chinese GMP inspectors and quality assurance managers of vaccine manufacturers, in July 2002, August 2003 and November 2003. The problems that had been identified included some ambiguities and some inconsistencies in wording; these were outlined for future revision. It was pointed out that GMP inspectors need to understand the background of the requirements and that proper interpretation of WHO guidelines is critical when national guidelines are being prepared.

The Committee was reminded that the recommendations for GMP for biologicals published in 1992 (WHO Technical Report Series No. 822, 1992) apply to all biologicals. However, the special considerations applicable to blood products are not adequately addressed. Some of these issues are covered by the Guidelines on collection, processing and quality control of blood, blood components and plasma derivatives (WHO Technical Report Series No. 1994, 840). Some are also covered in a Pharmaceutical Inspection Convention (PIC)/Blood circle document. Application of GMP to blood collection establishments is seen as a critical area for the improvement of blood safety. Training for GMP should have high priority and steps are being taken to improve this through courses and the establishment of regional networks and regulatory forums. Feedback from these courses should be provided to the Committee.

The Committee noted the issues that had been raised and agreed that priority should be given to a review of GMP for blood products. Moreover, this review should be given a higher priority than the revision of the Guidelines on collection, processing and quality control of blood, blood components and plasma derivatives (WHO Technical Report Series No. 840, 1994).

International guidelines, recommendations and other matters related to the manufacture and quality control of biologicals

Guidelines on nonclinical evaluation of vaccines

Because of scientific and technical developments, improvements are being made to existing vaccines and a broad range of novel vaccines are under development. There is a need for guidance on the type and extent of nonclinical evaluation needed for such products, based on best scientific knowledge, since this forms an essential part of the development of the vaccines. The Committee noted the draft WHO Guidelines on Nonclinical Evaluation of Vaccines (WHO/BS/03.1969), which had been prepared following the preliminary review of an earlier draft at the fifty-third meeting of the Committee. The Guidelines are intended to set out principles for nonclinical evaluation of vaccines and to provide information and guidance to vaccine manufacturers and recommendations for national regulatory authorities. The document outlines regulatory expectations and is intended to complement, and therefore should be read in conjunction with, the Guidelines for clinical evaluation of vaccines: regulatory expectations (WHO Technical Report Series, No. 924, 2004). After making a number of changes to the text, the Committee adopted the revised text as the Guidelines on nonclinical evaluation of vaccines and agreed that it should be annexed to its report (Annex 1).

Recommendations for the production and control of pneumococcal conjugate vaccines

The Committee reviewed the draft WHO Recommendations for the production and control of pneumococcal conjugate vaccines (WHO/BS/03.1968). The Recommendations are intended to be scientific and advisory and provide information and guidance to national regulatory authorities and vaccine manufacturers. Infections caused by *Streptococcus pneumoniae*, the pneumococcus, are responsible for substantial morbidity and mortality, particularly in the very young and in the elderly. Several pneumococcal vaccines containing polysaccharide conjugated to protein carriers are available and others are at an advanced stage of development. Controlled clinical trials of these vaccines have demonstrated that such conjugates are both safe and highly immunogenic. Differences in the incidence of the serotypes causing disease from one continent to another have led to the development of pneumococcal vaccine formulations consisting of increasing numbers of conjugated components. The experience

gained with identification of reference levels of antibodies that supported the successful licensure of one product in a number of countries will guide the review of clinical trial data from other countries and with other products.

The draft was based on the outcome of an informal WHO Consultation held in 2003. The Committee was reminded of the large number of serotypes of *S. pneumoniae* and of the need to allow flexibility in the recommendations to cover different conjugation chemistries and carrier proteins. After making several changes to the text, the Committee adopted the revised text as the Recommendations for the production and control of pneumococcal conjugated vaccines and agreed that it should be annexed to its report (Annex 2).

Recommendations for the production and control of influenza vaccine (inactivated)

The Committee reviewed the draft WHO recommendations for the production and control of influenza vaccine (inactivated) (WHO/BS/03.1967). The recommendations are intended to provide information and guidance for manufacturers of vaccines and recommendations for national regulatory authorities. The draft was based on the outcome of an informal WHO Consultation held in Ferney-Voltaire, France, in July 2003, during which a previous draft had been revised. The Committee was reminded of the significant developments in influenza vaccines during the past years. Subunit and split vaccines are now widely used, and the effective dose of haemagglutinin has been established. In addition vaccines containing adjuvants had been developed and approved. The danger of pandemics caused by the appearance of novel and highly pathogenic strains of virus presents a number of challenges for the production and administration of suitable vaccines. The existing recommendations therefore require revision to reflect these and other developments. After making several changes to the text, the Committee adopted the revised text as the Recommendations for the production and control of influenza vaccine (inactivated) and agreed that it should be annexed to its report (Annex 3). The Committee noted that the recommendations from the informal Consultation included: work to standardize the virus neutralization test; to establish its correlates of immunity and to evaluate its use for virus strain characterization; and to prepare vaccines through reverse genetic techniques and evaluate their use for vaccine production subject to resolution of intellectual property issues relating to the technique.

Requirements for the use of animal cells as *in vitro* substrates for the production of biologicals

The WHO requirements for the use of animal cells as *in vitro* substrates for the production of biologicals (WHO Technical Report Series No. 878, 1998, Annex 1) provide, inter alia, information about a WHO cell bank of Vero cells. These cells were developed in 1987 and designated as a Master Cell Bank in 1998. Cultures of the cells are available to manufacturers and national control authorities. As at its fifty-third meeting (WHO Technical Report Series, No. xxx, in press), the Committee had been informed of possible deficiencies in the records relating to the cell bank that might have regulatory implications for the establishment of master cell banks, and a revision of the Requirements was therefore proposed. A draft amendment to the section "General considerations — continuous-cell-line substrates" in the Requirements had been prepared (WHO/BS/03.1970). The Committee adopted the draft text as the Addendum 2003 to the requirements for the use of animal cells as in vitro substrates for the production of biologicals and agreed that it should be annexed to its report (Annex 4).

Requirements for diphtheria, tetanus and pertussis and combined vaccines

The Committee was informed of the developments that had taken place in methods of assay of diphtheria and tetanus vaccines since the Requirements for diphtheria, tetanus and pertussis and combined vaccines (WHO Technical Report Series, No. 800, 1990, Annex 2) had been published. These developments had been directed to overcoming difficulties in potency testing and several in vitro assays had been developed and validated. A series of reviews and meetings held during 1999–2000 had resulted in proposals for amendment of the WHO Requirements, but the technical details could not be finalized at that time. Further discussion resulted in a recommendation to move to a harmonized and simplified batch release assay using guinea-pigs. The Committee noted draft amendments to the WHO requirements for diphtheria, tetanus and pertussis and combined vaccines (WHO/BS/03.1984). The draft was based on the outcome of an informal meeting of a WHO Expert Group held in Geneva from 30 June to 2 July 2003. The main changes to the present Requirements constitute an updating of the sections on reference materials, and splitting sections on potency into two addressing licensing and batch release, respectively. After making a number of changes to the draft, the Committee adopted the modified text as the Amendments 2003 to the Requirements for diphtheria, tetanus and pertussis and combined vaccines

(WHO Technical Report Series, No. 800, 1990, Annex 2) and agreed that it should be annexed to its report (Annex 5).

Potency assays for acellular pertussis vaccines

The Committee was informed of the meeting of a WHO Working Group on standardization and control of pertussis vaccines held in Ferney-Voltaire, France, in May 2003. The purpose of the meeting was to advise WHO on updating the guidelines for the acellular pertussis component of monovalent or combined vaccines. Previous meetings had resulted in the initiation of collaborative studies to assess the value of intranasal challenge assays and modified intracerebral challenge assays. Data from the studies were reviewed at the meeting. One study showed that the intranasal assay was transferable between laboratories and differentiated between the collaborative study samples, but needed to be optimized to allow estimates of relative potencies of products. A reference preparation included in the study had performed satisfactorily, but may not be an ideal reference for all types of acellular pertussis vaccines. A second study evaluated a modified intracerebral assay. This was found to be effective for assigning relative potencies to acellular pertussis vaccines although it was noted that active pertussis toxin increased the apparent potency of preparations in the assay. A reference material, JNIH-3, included in the study had proved satisfactory.

The modified intracerebral challenge test is used in some parts of the world whereas an immunogenicity assay is used in other countries for lot release. Although it was acknowledged that it would be difficult to introduce changes for products and countries that had effectively monitored acellular pertussis vaccines for more than 20 years, it was agreed that additional methods for the assessment of functional activity for new products and formulations, and for technology transfer, were needed. The Committee noted that the Working Group had recommended that position papers about several assays and tests should be prepared by small subgroups for eventual submission to the Committee. The Working Group also recommended that the current WHO Recommendations for whole cell pertussis vaccines should be updated; that steps should be taken to make available an international reference antiserum for pertussis antigens before the currently widely used Center for Biologics Evaluation and Research (CBER) material runs out; and that NIBSC should be asked to confirm that supplies of monoclonal antibodies to Fim 2 and Fim 3 for use as serotyping agents are adequate. The Committee endorsed these recommendations.

Requirements for the collection, processing and quality control of material for transplantation

The Committee was informed of the growing numbers of transplants involving organs, tissues and cells worldwide. Limitations of supply determine the number of transplants that can be performed and prevent demand being met. There is a wide variety of tissue banks, and cross-boundary circulation of material takes place within and between all WHO Regions. Complex processes may be required for handling tissues and cells for transplantation. Ethical and public health concerns arise with respect to use of human tissue, the risk of transmission of pathogens and misuse of resources. These concerns were addressed at a WHO meeting in Madrid, Spain, in October 2003 which recommended that WHO develop quality, technical and ethical standards for transplantation. These should address safety and efficacy, GMP and quality management systems, and be consistent with WHO recommendations for other therapeutic materials. The Committee was invited to participate in this work. Although this represents a new sphere of activity, the Committee agreed that it had a role to play and asked for detailed and prioritized proposals to be submitted to it.

Human and animal spongiform encephalopathies and safety of biologicals

The Committee was reminded that an informal WHO Consultation on transmissible spongiform encephalopathies (TSEs) in relation to biological and pharmaceutical products had been held in Geneva in February 2003. The objectives of the Consultation were to provide evidence-based information to regulatory authorities, especially in countries where BSE had not yet been reported, regarding the risk assessment and precautionary and control measures for biological and pharmaceutical products. An additional goal was to promote worldwide harmonization of the regulations concerning TSE. Updated scientific and geographical information was presented at the Consultation and a new tissue classification was agreed. This tissue classification was now being used as the basis of worldwide regulations. At its previous meeting, the Committee had requested to see the report so that the implications for biological standardization could be considered. The Committee noted the report (WHO/BCT/QSD/03.01) and requested that this document be referenced where appropriate in WHO Recommendations and Guidelines for the production and quality control of biological medicines.

Gene transfer medicinal products

The Committee was informed that the WHO Gene Transfer Monitoring Group had met in Geneva in June 2003. During the meeting the lessons learned from the adverse events that had been reported in gene therapy trials were considered together with experience gained in preclinical tests and clinical trials; activities of the WHO Working Group on Standardization and Control of Nucleic Acid Vaccines; the current status of regulations and guidance; and standardization and nomenclature of gene transfer medicinal products. A draft report of the information presented and discussions held at the meeting was presented to the Committee. The Monitoring Group recommended that WHO should develop guidelines on gene therapy to address manufacture of clinical grade gene therapy products and the initiation and conduct of clinical trials. These guidelines should take account of existing guidance. The Committee also noted that discussions are continuing on reference materials for gene transfer medicinal products but, at present, there is no consensus on the most appropriate approach to standardizing these vectors. Their diversity may even preclude the development of generic reference materials. The Committee endorsed the proposals, noted the information that had been presented, and requested the Secretariat to keep it informed of further developments.

Proposals for discontinuation of international requirements and guidelines

The Committee was informed that the Procedure for approval by WHO of yellow fever vaccines in connection with the issue of international vaccination certificates (Procedure for evaluating the acceptability in principle of vaccines proposed to United Nations agencies for use in immunization programmes (WHO Technical Report Series, No. 786, 1989, Annex 1)) had been superseded by the procedure established for the pre-qualification of vaccines for supply, in principle, to United Nations agencies (WHO Technical Report Series No. 786, 1989). The Committee, therefore, discontinued the Procedure for approval by WHO of yellow fever vaccines in connection with the issue of international vaccination certificates (1981).

International reference materials

Proposals for discontinuation of reference materials

Apart from automatic replacement of reference materials superseded by those adopted during the meeting, the Committee was informed that no other reference materials had been proposed for discontinuation.

Recommendations for the preparation, characterization and establishment of international and other biological reference materials

The Committee was informed that an informal WHO Consultation had been held in Geneva in June 2003, to consider a proposed draft revision of the current Guidelines for the preparation, characterization and establishment of international and other standards and reference reagents for biological substances (WHO Technical Report Series No. 800, 1990). The discussion at the meeting had identified a number of fundamental scientific issues that would require clarification at a further Consultation.

In the establishment of WHO biological reference materials, no restriction has usually been placed on the methods employed in the collaborative study. This is in contrast to established practice in other metrological fields in which a single reference method is used. The Committee affirmed that the methods chosen should take into account the likely use of a given preparation. Where a broad range of uses can be foreseen, it is important to continue to employ a range of methods in collaborative studies. This decision on appropriate methods should be made clearly at the outset of the study. It should also be kept in mind that complex biological materials possess multidimensional properties, which are detected and measured in different ways by different methods that may change with time. The reference method approach therefore has, at best, limited applicability to biological reference materials.

The second issue is the choice of units to be assigned to a particular reference preparation. Other standard-setting bodies such as the International Organization for Standardization (ISO) advocate the use of Système international d'Unités (SI) units as having the highest metrological status. However, biological reference materials are often of complex and unknown composition, and cannot be defined purely in physical and chemical terms or in SI units. For this reason, the activity or potency of such a reference material is determined by biological procedures and stated in arbitrary International Units. The Committee considered that the choice of unit should reflect, and

be based on, the biological and medical as well as the physicochemical information available in each case.

The third and most far-reaching issue is the view of ISO that the uncertainty of the value given to a reference material should be stated. At issue, therefore, is whether, and if so how, uncertainty can be addressed for biological reference materials. Since an arbitrary unitage is assigned to a first WHO standard, no uncertainty is associated with the value. Although, in principle, an uncertainty might be estimated for replacement standards, the overriding concern of WHO is to ensure continuity of the International Unit. For reference materials intended for calibration of analyses of therapeutic and prophylactic products, no statement about uncertainty of an assigned value will be made, consistent with the ISO Guide 35 which states explicitly that reference materials in the pharmacopoeial, and by extension medical regulatory, context have contents stated without any uncertainty because of the circumstances of their use. For reference materials intended for in vitro diagnostics the Committee noted that the European Directive on in vitro diagnostics brings the requirements of ISO 17511 into legal effect. This would require a statement of uncertainty. The Committee was concerned about the scientific basis of attaching an uncertainty to an assigned value for a biological reference material and agreed that further discussions were required. For this purpose, the Committee was informed that several meetings were planned during 2004 including one with ISO and one with regulators concerned with implementation of the European Directive on in vitro diagnostic devices.

The Committee concluded that the current convention of not stating an uncertainty to the value assigned to a WHO biological reference material would remain unchanged. Nevertheless, they also recommended that memoranda accompanying reference materials established during this meeting and in future should contain a statement of the coefficient of variation (CV) of fill of the preparation concerned. This is one factor contributing to uncertainty. The Committee also noted that information about losses on storage is available to users at the time that reference materials are formally established. The Committee supported a proposal to draft additional guidance with regard to stability testing of reference materials.

Antibodies

Anti-toxoplasma serum, human

The Committee was informed that the degree of sensitivity in quantifying and monitoring the immunoglobulin G (IgG) response associated with acute toxoplasmosis is important in supporting appropriate clinical management and that the existing standards are not suitable for calibration of assays to distinguish between background and diagnostic levels of IgG. The Committee noted a proposal to establish a replacement International Standard for anti-toxoplasma serum, human (WHO/BS/03.1971), based on a collaborative study performed by 24 laboratories in 17 countries. The study had been designed to assess the suitability of the candidate preparation for use in cell-killing assays and to calibrate it in terms of the current International Standard, to confirm continuity of unitage, and to assess the reactivity of the candidate in various assays. The candidate preparation appeared to be stable at storage temperatures up to 20 °C. The Committee noted that the value assignment results obtained in the study were based on the currently accepted gold standard method, the dye test, and that the composition of the material means that it is cannot be a direct replacement for the existing anti-toxoplasma standard. In view of these considerations and on the basis of the results obtained, the Committee established the preparation, in ampoules coded 01/600, as the First International Standard for Anti-Toxoplasma IgG, Human, and assigned a potency of 20 IU per ampoule to it.

The Committee noted that it would be useful during the current revision of the Recommendations for Preparation, Characterization and Establishment of International and other Biological Reference Materials to include guidance on the maximum tolerable intra- and inter-laboratory variation during evaluation of results of collaborative studies.

Antigens and related substances

Smallpox reference materials

The Committee was informed that smallpox had been eradicated in 1980, but that there had been a recent revival of interest in smallpox vaccines because of the potential implications of bioterrorism activities. It was considered necessary to review the existing standards and to consider whether new standards were required. Consequently a collaborative study had been performed by 13 laboratories in 10

countries to assess the relative sensitivity of cell culture assays and chorioallantoic membrane (CAM) egg assays for smallpox vaccines and evaluate the suitability of candidates as a replacement for the current International Reference Preparation (WHO/BS/03.1977). The outcomes of the study were that, overall, there were no substantial differences noted in the sensitivity of the assay methods, although some samples performed better in the CAM assays than in the cell culture assays and vice versa. Two suitable candidate preparations have been identified for use as replacements. However, consistent with the proposal from the study participants to continue to use the current reference preparation, the Committee noted that the current International Reference Preparation still has acceptable potency and the existing stocks of this preparation are sufficient for the time being so that its replacement is not urgent. The Committee thus agreed to defer any decision about replacement pending generation of further information, including stability data and information about rate of supply.

Yellow fever vaccine

The Committee was informed that potency determination of yellow fever vaccines has been based historically on mouse LD_{50} assays although in vitro plaque assays have been available and in routine use for some years. The Committee was also informed that the WHO Requirements for yellow fever vaccine (WHO TRS 872, 1998, Annex 2) require determination of a relationship between mouse LD_{50} units and plaque forming units (pfu) in each laboratory. The Committee noted that this relationship is, in practice, often based on values established many years previously and that checks on the continued validity of the relationship may be done infrequently. Moreover, two or more laboratories (e.g. a manufacturer and a national control laboratory) may assay the same batch of vaccine, but apply different conversion factors if the relationship between mouse LD_{50} and pfu had been determined at different times. The standardization of yellow fever potency determinations would benefit from an internationally available reference material.

The Committee noted the report of a collaborative study performed by 13 laboratories in eight countries, intended to assess the suitability of candidate preparations for an international standard and the relationship between the two assay methods (WHO/BS/03.1985 Rev.1). One candidate preparation appeared to be suitable for use in plaque assays. The stability of the preparation had been studied over periods of up to 3 years and appeared satisfactory. On the basis of the results of the collaborative study, the Committee established the prepara-

tion, in ampoules coded 99/616, as the First International Standard for Yellow Fever Vaccine and assigned an activity of $10^{4.5}$ IU per ampoule to it. The data obtained in the study indicated that there was a consistent relationship between mouse LD_{50} and plaque assays. The Committee therefore supported a proposal to encourage manufacturers and control laboratories to include the standard in assays to evaluate its suitability for setting a minimum potency of $10^{4.0}$ IU for yellow fever vaccines. Data should be collated by WHO and analysed to determine whether the potency specification given in the WHO Recommendations (WHO Technical Report Series, No. 872, 1998) should be amended.

Pertussis toxin

The Committee was informed that reference preparations of pertussis toxin are required for the quality control and assessment of pertussis vaccines. Two methods are currently used to assay residual pertussis toxin in both acellular and whole cell pertussis vaccines. The Committee noted a proposal to establish an international standard for pertussis toxin and the report (WHO/BS/03.1978) of a collaborative study performed by six laboratories in six countries using both the histamine sensitizing and Chinese hamster ovary cell assay methods. Although the candidate preparation had been filled some years ago, its stability had been demonstrated by studies following a period of storage at elevated temperatures. On the basis of the results of the collaborative study, the Committee established the preparation, in ampoules coded JNIH-5, as the First International Standard for Pertussis Toxin and assigned an activity of 10 000 IU per ampoule to it. Nominally one IU corresponds to 1 nanogram of protein nitrogen using a conversion factor of 10.

Diphtheria and tetanus reference materials

The Committee was informed that stocks of a number of reference materials for testing diphtheria and tetanus components in vaccines were depleted and that several new reference materials were required for use with methods that would replace tests using animals. The Committee was also informed of the proposed priorities for the replacement and establishment of these materials and other studies on methodology. The Committee recommended that the following studies should be initiated:

— collaborative studies on candidate replacement preparations for use in flocculation assays for diphtheria and tetanus toxoids and on comparison of methods to measure antigenic purity;

— collaborative studies on a candidate replacement for the current International Standard for Diphtheria Toxoid, Adsorbed; and
— collaborative studies intended to establish new WHO standards for diphtheria and tetanus toxins for use in cell culture assays and for human IgG diphtheria antitoxin (for which a candidate preparation is available).

The Committee noted a proposal for collaborative studies on monoclonal antibody panels for diphtheria and tetanus toxoids for use in ELISA assays to monitor product consistency. Although the project was welcomed, the Committee expressed concern about whether sufficient monoclonal material could be made available for long-term use and asked for a more detailed project proposal to be submitted.

Blood products sand related substances

Factor VIII, concentrate

The Committee was informed that stocks of the current (recombinant) standard were likely to be exhausted within 12 months. The Committee was also informed that there had been reports of difficulties in using recombinant material in assaying plasma-derived concentrates. For these reasons and after wide consultation, a decision was taken to use plasma-derived factor VIII as the replacement material. The Committee noted a proposal to establish a replacement International Standard for Factor VIII, Concentrate (WHO/BS/03.1973), based on a collaborative study performed by 38 laboratories in 21 countries in which a candidate material was compared with four existing reference materials for factor VIII (Fifth and Sixth International Standards, USFDA lot 1, EPBRP batch 2). This study was performed in conjunction with CBER and the European Department for the Quality of Medicine (EDQM) with the aim of establishing a common batch of the reference preparation. The participants employed their customary in-house assays which were all either one-stage or chromogenic methods. There were no significant differences in the mean potency obtained by the two methods. The preparation showed adequate stability over 2 years. On the basis of the results obtained, the Committee established the preparation, in ampoules coded 99/678, as the Seventh International Standard for Factor VIII, Plasma-derived, Concentrate and assigned an activity to it of 11.0 IU per ampoule. This value is considered to ensure continuity of values of the International Unit for different preparations in different assay systems.

Factor VIII/von Willebrand factor, plasma

The Committee was informed that stocks of the Fourth International Standard for Factor VIII/von Willebrand Factor, Plasma were almost exhausted. This standard is important for the calibration of both commercial and non-commercial secondary working standards. The Committee noted a proposal to establish a replacement International Standard for Factor VIII/von Willebrand Factor, Plasma (WHO/BS/03.1972), based on the collaborative study performed by 37 laboratories in 13 countries. The candidate preparation showed adequate stability over approximately 1 year. On the basis of the results obtained, the Committee established the preparation, in ampoules coded 02/150, as the Fifth International Standard for Factor VIII/von Willebrand Factor, Plasma and assigned activities to it of 0.68 IU/ampoule for Factor VIII:C; 0.94 IU/ampoule for Factor VIII:antigen; 0.91 IU/ampoule for VWF:antigen; 0.78 IU/ampoule for VWF:ristocetin cofactor; and 0.94 IU/ampoule for VWF:collagen binding. The Committee noted that other studies are in progress that may provide information on the relative clinical values of the different assays for von Willebrand Factor that are performed.

Low-molecular-weight heparin

The Committee was informed that stocks of the current International Standard for low-molecular-weight heparin were almost exhausted. The Committee was also informed that this standard is used in the determination of potency of low-molecular-weight heparin. The Committee noted a proposal to establish a replacement International Standard for low-molecular-weight heparin (WHO/BS/03.1986), based on a collaborative study performed by 30 laboratories in 14 countries, in which both anti-Xa and anti-IIa assays had been employed. Two candidate preparations had performed similarly and that with the lower inter-laboratory variability had been selected. The Committee was informed that this preparation had shown no degradation over 12 months and that the stability study would continue in real time. On the basis of the results obtained, the Committee established the preparation, in ampoules coded 01/608, as the Second International Standard for Low-molecular-weight heparin and assigned to it activities of 1097 IU of anti-Xa per ampoule and 326 IU of anti-IIa per ampoule. The Committee noted that the study had been performed in conjunction with the EDQM with the aim of establishing replacement batches of *European Pharmacopoeia* reference preparations.

Prekallikrein activator

The Committee was informed that stocks of the current International Standard for prekallikrein activator were almost depleted. The Committee was also informed that this standard is used in the determination of the level of prekallikrein activator, an impurity present in preparations of therapeutic blood products, such as albumin. The Committee noted a proposal to establish a replacement International Standard for Prekallikrein Activator (WHO/BS/03.1974 + Add.1), based on a collaborative study performed by 31 laboratories in 17 countries using similar kinetic assays employing a variety of substrates. This study had been carried out in conjunction with the EDQM. The performance of the candidate preparation was satisfactory. The Committee noted that the preparation had shown adequate stability over approximately 1 year. On the basis of the results obtained, the Committee established the preparation, in ampoules coded 02/168, as the Second International Standard for Prekallikrein Activator and assigned an activity of 29 IU per ampoule to it.

Cytokines, growth factors and endocrinological substances

WHO consultation on cytokines, growth factors and endocrinological substances

The Committee was informed that a WHO informal consultation on cytokines, growth factors and endocrinological substances had been held in Potters Bar, England, in October 2003. The attention of the Committee was drawn to the increasing difficulties with nomenclature. An extensive range of titles had been given to reference materials established by WHO and a number of confusions had arisen. The Committee agreed to a recommendation that a comprehensive review of issues of nomenclature should be carried out. The Committee was also informed that the formulation of a policy concerning stability testing of reference materials after their establishment is desirable.

Concerns have been raised relating to the development of unwanted antibodies against some therapeutic biologicals, in addition to interferon alfa or beta (see p.23). The availability of reference human serum preparations containing defined and characterized antibodies against such biologicals was recognized as a potentially valuable resource. The Committee endorsed the provision of such reagents based on prioritization according to need and significance for public health.

A work programme for cytokines, growth factors and endocrinological substances had been agreed and potential new areas of work had been identified. The Committee noted the programme and the advice received from the Consultation.

At the request of WHO, a survey had been carried out to assess the extent of use of the established reference materials in this area. Although demand does not necessarily reflect therapeutic importance, the materials appear to have a significant role and are used in both the developed and developing countries. However, the procedures for obtaining this information are not ideal. Nevertheless, the information on use of these standards is useful for aiding decisions about capacity building.

Human interferon beta

The Committee was informed that the current International Standard for Interferon beta is an impure preparation derived from human fibroblasts containing about 1% interferon, and that other cytokines that are present influence the results of some assays. The Committee was also informed that an extensive collaborative study of new and existing reference preparations for interferon beta had been performed by 16 laboratories in eight countries. The aims of the study were to assess the relative activities of preparations of natural and recombinant interferon beta in a range of bioassays; to compare these activities where possible with those of well-characterized in-house standards; to assess whether the current International Standard remains suitable; to identify candidate replacement preparations; to obtain data about assays in current use and to prepare for a separate study in which a single assay design would be used. A total of eight ampouled preparations of interferon beta were examined. The Committee noted that it is necessary to ampoule interferon beta in the presence of casein to prevent adsorption to the glass. One candidate preparation, consisting of glycosylated interferon beta derived from Chinese hamster ovary cells, gave a smaller inter-laboratory variability than the current standard, with all but one of the samples examined. The Committee reviewed the results of the collaborative study and a proposal to establish a replacement International Standard for Fibroblast Interferon beta (WHO/BS/03.1976). On the basis of the information supplied, the Committee established the preparation, in ampoules coded 00/572, as the Third International Standard for Interferon beta, Human, Recombinant, Glycosylated, and assigned a potency to it of 40 000 International Units per ampoule. This standard replaces the Second International Standard for Interferon, beta, Fibroblast. Since stocks of the current standard remained, the

Committee formally disestablished the Second International Standard for Interferon beta, Fibroblast, Human, code number Gb23-902-531. The material derived from Chinese hamster ovary cells cell is likely to continue to be available and is more suitable for calibration of future therapeutic products than the fibroblast material. However, it is not suitable for assay of the Ser-17 interferon beta analogue and the First International Standard for Interferon-beta Ser 17 mutein, code number Gxb02-901-535, will be retained.

Tumour necrosis factor alpha, human

The Committee was informed that stocks of the current International Standard for Tumour Necrosis Factor Alpha, Human are almost exhausted and the remaining stock is required to ensure traceability in the future. The Committee was also informed that three other candidate preparations had been included in the collaborative study leading to establishment of the current standard in 1991. Stability studies at that time had indicated high thermal stability of the preparations and this has been confirmed by additional studies performed during 2002 and 2003. The Committee noted a proposal to establish a replacement International Standard for Tumour Necrosis Factor, Alpha, Human (WHO/BS/03.1981), based on the results of the earlier collaborative study performed by 20 laboratories in nine countries together with results obtained in recent bridging studies in one laboratory using three bioassays. The proposed standard consists of the full-length 157-amino-acid protein, but is derived from different cells to those used for production of the full-length 157-amino-acid protein in the First International Standard. The other two candidate preparations have different amino acid sequences. On the basis of the information supplied, the Committee established the preparation, in ampoules coded 88/786, as the Second International Standard for Tumour Necrosis Factor, Alpha, Human and assigned a potency to it of 46 500 International Units per ampoule. Since the standard is likely to be used in assays to measure anti-tumour necrosis factor activity, the memorandum dispatched with the standard preparation should draw attention to possible discontinuities in results when compared to those obtained with the International Standard. The recommended International Nonproprietary Name (INN) for the material in the standard should be included in the memorandum distributed with it.

Luteinizing hormone, recombinant

The Committee was reminded that luteinizing hormone (LH) is a two subunit glycoprotein gonadotrophin obtained from pituitary glands,

from urine or by recombinant methods. LH is used for diagnosis and for therapy. Because there are biological, immunological and physicochemical differences between pituitary and urinary hormones, several WHO international standards have been established. The Committee noted that recombinant LH is now available as a therapeutic product and noted a proposal to establish a standard for it for use in bioassays for therapeutic products (WHO/BS/03.1983). When the candidate preparation is calibrated against urinary LH and pituitary LH different values are obtained. The recombinant material most closely resembles pituitary LH, but it is proposed that the value determined relative to the urinary LH standard be adopted to ensure continuity of unitage for therapeutic products. On the basis of the information supplied, the Committee established the preparation, in ampoules coded 96/602, as the First International Standard for Luteinizing Hormone, Recombinant and assigned an activity to it of 189 International Units per ampoule. The Committee recommended that the recommended INN for the material in the standard, lutropin alfa, is included in the memorandum distributed with it.

Thyroid-stimulating hormone for immunoassay

The Committee was reminded that immunoassays for thyroid-stimulating hormone are widely employed in the diagnosis and management of thyroid dysfunction. The Committee was informed that stocks of the current Second International Reference Preparation for Thyroid-Stimulating Hormone are depleted. The Committee was also informed that it had been demonstrated that preparations of recombinant thyroid-stimulating hormone are unsuitable for calibration of diagnostic assays. The Committee noted a proposal to establish a replacement standard for thyroid-stimulating hormone based on a collaborative study carried out by nine laboratories in six countries (WHO/BS/03.1975). The candidate preparation was obtained from the same bulk material of pituitary origin as the current preparation and had been included in the collaborative study when the current preparation was established. The present study was designed to compare the candidate preparation with the current preparation. The study confirmed that its activity and stability have remained unchanged. On the basis of the information supplied, the Committee established the preparation, in ampoules coded 81/565, as the Third International Standard for Thyroid-stimulating Hormone, Human, for Immunoassay and assigned an activity to it of 11.5 milli-International Units per ampoule.

Interferon neutralizing antibody tests

The Committee was informed that interferons alpha and beta can induce antibodies when used therapeutically. Such antibodies may make the patients resistant to further treatment with interferons. However, many different methods are used to determine levels of anti-interferon antibodies and the data obtained in one laboratory cannot necessarily be made use of in another. A standard method for the calculation and reporting of the results of interferon neutralizing antibody tests has been proposed (WHO/BS/03.1980). However, the September 2003 WHO Consultation on cytokines (see p.22) considered that, although the approach proposed was promising, more data should be obtained and the investigators should be encouraged to use WHO reference materials to determine the sensitivity of the assay procedures employed. The Committee endorsed these recommendations.

Diagnostic reagents

WHO consultation on international standards for testing diagnostic kits used in detection of hepatitis B surface antigen and anti-hepatitis C virus antibodies

The Committee was informed of the meeting of a WHO Working Group on International Reference Preparations for testing diagnostic kits used in the detection of hepatitis B surface antigen (HbsAg) and anti-hepatitis C virus (HCV) antibodies held in Geneva in October 2003.

The Working Group reviewed in detail a collaborative study to establish a replacement International Standard for HBsAg (see p.27). Following discussion, the participants agreed that the candidate replacement standard for HbsAg should be assigned a unitage in International Units and that the use of "nanograms" should be discontinued. The Working Group also agreed that it had been shown that the candidate materials were commutable between assays. The WHO International Standard for HBsAg is intended for the calibration of secondary HBsAg reference materials by manufacturers and national regulatory authorities. The Working Group also recommended the establishment of a reference panel for use in the assessment of analytical quantitation of assay kits. The WHO Reference Panel should aid national regulatory authorities in evaluating the analytical sensitivity of rapid tests for HBsAg, particularly where negative diluent is scarce. Nevertheless it was noted that the evaluation of test kits for HBsAg should include performance testing of

seroconversion panels, difficult samples, and local samples representing the prevalent hepatitis B virus genotypes and variants in that target region.

The Group also considered that there is a role for a panel of monospecific anti-HCV sera to define analytical specificities of test kits for anti-HCV antibodies, and that a feasibility study of candidate materials should be performed. In the past the WHO Working Group on Reference Standards for Hepatitis and HIV Diagnostic Kits had designed, produced and tested a candidate anti-HCV genotype 1b reference. However, when the Working Group proposed to the Expert Committee on Biological Standardization that this preparation be used as a WHO Reference Material, the suggestion was not favourably received by the Committee because the characterization of the serum pool was insufficient to permit determination of the analytical sensitivity of kits that detect several antigens. In 2001, the Working Group agreed to prepare a reference material for each of four antibodies deemed appropriate for detection by the commercial kits most frequently manufactured: anti-core, anti-NS3, anti-NS4 and anti-NS5. In early 2003, a potential source of such materials had been identified. The limitations of a sensitivity panel of monospecific antibodies for evaluating diagnostic kits worldwide were discussed. It was agreed that to define kit performance fully, regulatory authorities and manufacturers should use collections of positive specimens from local populations and seroconversion panels when available. However, the participants agreed that a monospecific international reference panel would have unique value in providing a benchmark to evaluate the quality of assay kits worldwide. The value of such a panel should be tested with different genotypes. It was agreed that a protocol should be drafted for the performance of a feasibility study involving the candidate materials identified. The protocol should include the samples to be tested, the test kits with which they are to be evaluated and the responsibility of each collaborating centre in the study.

The Committee endorsed the conclusions from the Working Group.

Hepatitis B surface antigen

The Committee was reminded of the need to replace the current First International Standard for Hepatitis B Surface Antigen. A collaborative study had been performed to assess the suitability of a candidate replacement preparation and to calibrate it (WHO/BS/03.1987). A plasma pool containing HBsAg subtype adw2, genotype A, was inactivated, purified and diluted. Also, a series of four fourfold dilutions of the purified material was prepared. The first preparation was

proposed as a candidate to replace the First WHO International Standard for HBsAg, and the panel of dilutions was proposed as a WHO Reference Panel for HBsAg. The aim of these reference materials is to aid regulatory authorities and manufacturers of HBsAg test kits in measuring the analytical sensitivity of kits by providing a standard with an internationally accepted unitage.

WHO collaborative studies were conducted to characterize and assess the candidate International Standard and the proposed Reference Panel. In order to assign an appropriate unitage, the study analysed the candidate International Standard preparation against the First International Standard and four other widely recognized HBsAg reference standards: the primary and current Paul Ehrlich Institute standards, a French standard and a standard used by an in vitro diagnostics manufacturer (Abbott Laboratories). The assigned values of the different HBsAg reference preparations were found to differ considerably: 1 IU is equivalent to 0.58 Paul Ehrlich Institute (PEI) units (primary) or 0.43 PEI units (current) or 1.9 French "ng" or 5.6 Abbott "ng". However, it is noteworthy that in 1985, the relationship between IS units and the primary PEI units was almost the same as that found in the current study: 1 IU = 0.55 PEI unit. These and related biochemical data indicate that there has been no drift in the IU over 18 years.

The overall mean IU of the candidate International Standard was 33 IU/vial. Because the panel was manufactured in a series of fourfold dilutions of the candidate preparation, panel components A, B, C and D, were estimated to contain 8.25, 2.06, 0.52 and 0.13 IU/vial, respectively. These values were confirmed experimentally for all but panel component A, the values for which were out of the analytical range for the kits used in the quantitative phase of the study and were thus technically invalid. In a second phase of the study, 10 international laboratories tested the candidate International Standard and reference panel using 20 other immunoassays and rapid tests. The study showed that the prediluted panel provides a convenient resource for use by regulatory authorities to assess sensitivity, especially of rapid tests.

On the basis of the results obtained, the Committee established the candidate preparation, in vials coded 00/588, as the Second International Standard for Hepatitis B Surface Antigen with an assigned value of 33 IU per vial. The standard contains antigen subtype adw2, genotype A. The Committee also established panel components A to D, in vials coded 01/400, 01/402, 01/404 and 01/406, which are 1 in 4, 1 in 16, 1 in 64 and 1 in 256 dilutions of the International Standard,

respectively, and panel component E, in vials coded 00/616, which consists of human re-calcified plasma, as a reference panel for HbsAg for use by national regulatory authorities in the assessment of the sensitivity of assay kits for the detection of the surface antigen. The Committee reviewed the memoranda to be supplied with the reference materials, and, after making some changes, approved them.

Lipoprotein (a)

The Committee was informed that the presence of high levels of lipoprotein (a) is a genetically determined marker of predisposition to coronary artery disease. Accurate measurement of lipoprotein (a) is important for diagnosis and a Working Party, established by the International Federation of Clinical Chemistry and Laboratory Medicine, had performed a series of studies to calibrate the lipoprotein (a) content of pooled human serum in terms of purified preparations of lipoprotein (a). The Committee noted a proposal to establish a reference material for lipoprotein (a) (WHO/BS/03.1979), based on a collaborative study performed by 18 laboratories in 10 countries employing two different ELISA methods. The Committee was informed that when the proposal had been presented at its previous (fifty-third) meeting, a request had been made for additional information. This information had now been included in the report. The stability of the preparation had been assessed in various real-time and accelerated studies over 5 years and appeared to be adequate. Monitoring of stability continues. On the basis of the results obtained, the Committee established the preparation, in vials coded SRM 2B, as the First WHO Reference Reagent for Lipoprotein (a) for Immunoassay and assigned a value of 0.107 nanomoles per vial to it.

Miscellaneous

Standardization of human papillomavirus vaccine

The Committee was reminded that human papillomavirus (HPV) is responsible for a variety of diseases and is a major cause of cancer in many women. Four strains of virus have been identified as being associated with cervical cancer. The Committee was also informed that candidate vaccines consisting of papilloma-like virus particles had been developed and studied in clinical trials and appear to be safe, immunogenic and well-tolerated and to offer complete protection against HPV infections. Reference reagents are required to evaluate antibody responses to the vaccines and to monitor incidence of disease. Collaborative studies have been performed to harmonize

diagnostic procedures for HPV DNA type-specific detection and in serological type-specific assays and evaluate the use of reagents for these. The outcome of the studies was reviewed at a WHO Workshop held in Geneva in September 2003 as a result of which it was proposed that international standards and reagents be developed for type-specific assays, beginning with DNA standards for types 16 and 18. It was also proposed that monotypic reference antisera to HPV types 16 and 18 be submitted for adoption as international standards for serological assays. These assays are usually specific for one type, but vary in sensitivity. The Committee agreed that the work proposed on reference materials should proceed and recommended that neutralizing assays should be included in the antisera studies.

Standardization of human immunodeficiency virus neutralizing antibody assays

The Committee was informed that measurement of anti-HIV-1 neutralizing antibodies is important in HIV vaccine research and in clinical trials. To facilitate progress in this field, a WHO–Joint United Nations Programme on HIV/AIDS (UNAIDS) meeting was held in Milan, Italy, in August 2003. This revealed that a wide range of assays were being used. It was agreed that standardization through provision of reference materials and development of assay protocols is required. It was also agreed that a collaborative study should be performed to evaluate candidate neutralization standards using different assays against a panel of viral subtypes, obtained using plasma and/sor sera from different geographical areas, as well as monoclonal antibodies evaluated as potential standards. Detailed proposals will be prepared when details of the availability of material are known. The Committee agreed with the course of action proposed.

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Annex 1
WHO guidelines on nonclinical evaluation of vaccines

This document provides guidance to national regulatory authorities (NRAs) and vaccine manufacturers on the nonclinical evaluation of vaccines by outlining the international regulatory expectations in this area. It should be read in conjunction with the Guidelines on clinical evaluation of vaccines: regulatory expectations (1), in order to complete the understanding of the whole process of vaccine evaluation. Vaccines are a diverse class of biological products and their nonclinical testing programmes will depend on product-specific features and clinical indications. The following text has therefore been written in the form of guidelines rather than recommendations. Guidelines allow greater flexibility than recommendatisons with respect to specific issues related to particular vaccines.

Introduction

Recent progress in biotechnology and basic immunology has led to the development of a broad range of novel vaccines raising exciting possibilities for the prevention of infectious diseases (*2*, *3*). Improvements to already licensed vaccines are also being considered; such improvements will lead to new products as well as to the introduction of new adjuvants. However, the complexity and novelty of these products presents scientific and regulatory challenges because criteria for their safety, potency and quality assessment may not exist. Product diversity and new approaches, technologies and methodologies develop over time; therefore, judgement based on the best science available should always form the basis for deciding on the type and extent of nonclinical evaluation for these products.

Although nonclinical evaluation plays an essential part in the overall development of vaccine candidates, there is at present limited guidance regarding nonclinical evaluation programmes for these products. In this guidance document, the general principles of nonclinical evaluation of vaccines are discussed, with particular attention being given to the regulatory expectations for new and novel vaccines.

Preclinical testing is a prerequisite to moving a candidate vaccine from the laboratory to the clinic and includes all aspects of testing, product characterization, proof of concept/immunogenicity studies and safety testing in animals conducted prior to clinical testing of the product in humans. Nonclinical evaluation, within the context of this document, refers to all in vivo and in vitro testing performed before and during the clinical development of vaccines. For example, nonclinical evaluation may be necessary when changes in the manufacturing process or product formulations are made or to further study potential safety concerns that may have arisen from phase I and II trials or that have been described in the literature for similar products.

1 General remarks

Nonclinical studies are aimed at defining the in vitro and in vivo characteristics of candidate vaccines including those relating to safety and immunogenicity. Nonclinical studies in animals are valuable tools for identifying possible risks to the vaccinees and helping to plan protocols for subsequent clinical studies in human subjects. However, in all cases, when safety testing in animals is performed, there should be a clear rationale for doing so and the study should be performed in

compliance with the national and international laws for the protection of laboratory animals (*4*), biosafety requirements (*5*) and with good laboratory practice (GLP) (*6*). However, there may be situations where full compliance with GLP is not possible. If the study, or part of the study, was not conducted in compliance with GLP, areas of noncompliance should be defined and a statement of the reason for noncompliance should be drawn up.

Potential safety concerns for a vaccine product include those due to inherent toxicities of the product, toxicities of impurities and contaminants, and toxicities that result from interactions between the vaccine components present in the vaccine formulation. In addition, the immune response induced by the vaccine may lead to toxic side-effects.

Despite efforts to maximize the predictive value of nonclinical toxicity studies there is always the possibility that not all risks are identified. The limitations of animal testing in reflecting clinical safety and efficacy in humans should be recognized as pathogenesis and immune responses are frequently species-specific. Moreover, potential safety concerns identified during animal testing may not necessarily indicate a problem in humans. However, any signal observed in nonclinical toxicity studies should be carefully addressed in human clinical trials and may require additional nonclinical testing. It should be noted that the absence of detectable toxicity in animal studies does not necessarily mean a vaccine will be safe in humans. Potential safety concerns related to specific types of vaccine candidate are considered in section 6.

The development and subsequent validation of in vitro tests for use as alternatives to nonclinical evaluation of vaccine candidates in animals is encouraged as it may lead to the improvement of nonclinical testing as well as to a reduction of animal usage.

The need for and extent of nonclinical testing will depend on the product under consideration. For example, for a product for which there is no prior nonclinical and clinical experience, nonclinical testing would be expected to be more extensive than for those vaccines previously licensed and used in humans. In some cases, it may not be necessary to perform preclinical safety studies prior to the initiation of phase 1 clinical trials. For example, in the case of transfer of technology, where access to the database of the originally developed vaccine is available, data from nonclinical bridging studies (e.g. physicochemical characterization and abbreviated in vivo studies) may be an acceptable basis for further development of the product.

Early communication between the vaccine manufacturer and the responsible national regulatory authority to agree on the requirements for and type of nonclinical testing is recommended.

1.1 *Scope*

For the purposes of this document, vaccines are considered to be a heterogeneous class of medicinal products containing immunogenic substances capable of inducing specific, active and protective host immunity against infectious disease.

Although most vaccines are being developed for pre- and post-exposure prophylaxis, in some cases, they may be indicated for therapeutic use against infectious diseases, e.g. human immunodeficiency virus (HIV), and human papillomavirus (HPV). Both prophylactic and therapeutic vaccines for infectious disease indications are considered in this document.

Vaccines for human use include one or more of the following: microorganisms inactivated by chemical and/or physical means that retain appropriate immunogenic properties; living microorganisms that have been selected for their attenuation whilst retaining immunogenic properties; antigens extracted from microorganisms, secreted by them or produced by recombinant DNA technology; chimeric microorganisms; antigens produced in vivo in the vaccinated host following administration of a live vector or nucleic acid or antigens produced by chemical synthesis in vitro. The antigens may be in their native state, truncated or modified following introduction of mutations, detoxified by chemical or physical means and/or aggregated, polymerized or conjugated to a carrier to increase immunogenicity. Antigens may be presented plain or in conjunction with an adjuvant, or in combination with other antigens, additives and other excipients.

Therapeutic vaccines for non-infectious diseases (e.g. certain cancer vaccines) and monoclonal antibodies used as immunogens (e.g. anti-idiotypic antibodies) are *not* considered here.

1.2 *Glossary*

The definitions given below apply to the terms used in these guidelines. They may have different meanings in other contexts.

Adjuvants
Substances that are intended to enhance relevant immune response and subsequent clinical efficacy of the vaccine.

Booster vaccination
Vaccination given at a certain time interval after primary vaccination to enhance immune responses and induce long-term protection.

Combination vaccine
A vaccine that consists of two or more antigens, either combined by the manufacturer or mixed immediately before administration and intended to protect against either more than one disease, or against one disease caused by different strains or serotypes of the same organism.

Genetically modified organism (GMO)
An organism or a microorganism in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination. This definition covers microorganisms including viruses, viroids and cell cultures including those from animals, but does not cover naked recombinant DNA or naked recombinant plasmids.

Good clinical practice (GCP)
A standard for clinical studies that encompasses their design, conduct, monitoring, termination, audit, analyses, reporting and documentation and which ensures that the studies are scientifically and ethically sound and that the clinical properties (diagnostic, therapeutic or prophylactic) of the pharmaceutical product under investigation are properly documented.

Good laboratory practice (GLP)
A quality system concerned with the organizational process and the conditions under which nonclinical health and environmental safety studies are planned, performed, monitored, recorded, archived and reported. GLP principles may be considered as a set of criteria to be satisfied as a basis for ensuring the quality, reliability and integrity of studies, the reporting of verifiable conclusions and the traceability of data.

Good manufacturing practice (GMP)
A part of the pharmaceutical quality assurance which ensures that products are consistently produced and controlled according to the quality standards appropriate to their intended use and as required by the marketing authorization. In these guidelines, GMP refers to the current GMP guidelines published by WHO.

Immunogenicity
Capacity of a vaccine to induce antibody-mediated and/or cell-mediated immunity and/or immunological memory.

Nonclinical evaluation of vaccines
All in vivo and in vitro testing performed before and during clinical development of vaccines. The potential toxicity of a vaccine should be assessed not only prior to initiation of human trials, but throughout clinical development.

Plasmid
Double-stranded circular DNA molecules capable of replicating in bacterial cells.

Potency
The measure of biological activity, using a suitable quantitative biological assay, based on the attribute of the product that is linked to the relevant biological properties.

Preclinical evaluation of vaccine
All in vivo and in vitro testing carried out prior to the first testing of vaccines in humans. This is a prerequisite to the initiation of clinical trials and includes product characterization, proof of concept/immunogenicity studies and animal safety testing.

Preclinical toxicity study
A study designed with the primary purpose of demonstrating the safety and tolerability of a candidate vaccine product. The design of the preclinical toxicity study should meet the criteria outlined in the section on study design to be considered supportive of the intended clinical trial.

Primary vaccination
First vaccination or series of vaccinations given within a predefined period, with an interval of less than 6 months between doses, to induce clinical protection.

Product characterization
A full battery of physical, chemical and biological tests conducted for a particular product. These tests include, but are not limited to, in-process control testing, testing for adventitious agents, testing process additives and process intermediates, and lot release.

Protocol or study plan
A document that states the background, rationale and objectives of the nonclinical studies and describes its design, methodology and organization, including statistical considerations, and the conditions under which it is to be performed and managed.

Relevant animal model
An animal that develops an immune response similar to the expected human response after vaccination. It is acknowledged that species-specific differences in immune responses are likely. Ideally, the animal species chosen should be sensitive to the pathogenic organism or toxin under consideration.

Route of administration
The means by which the candidate vaccine product is introduced to the host. Possible routes of administration include the intravenous, intramuscular, subcutaneous, transcutaneous, intradermal, transdermal, oral, intranasal, intranodal, intravaginal and intrarectal routes.

Seroconversion
Predefined increase in antibody concentration, considered to correlate with the transition from seronegative to seropositive, providing information on the immunogenicity of a vaccine. If there are pre-existing antibodies, seroconversion is defined as a transition from a predefined low level to a significantly higher defined level, such as a fourfold increase in geometric mean antibody concentration.

Validation
The action of proving, in accordance with the principles of good manufacturing practice, that any procedure, process, equipment (including the computer software or hardware used), material, activity or system actually leads to the expected results.

2 Characterization of candidate vaccines

2.1 *Vaccine production*

The biological nature of the starting materials, the manufacturing process and the test methods needed to characterize batches of the product are important elements to be considered in the design and the interpretation of nonclinical testing of vaccines. Many vaccines are produced using prokaryotic or eukaryotic microorganisms and subtle changes in these organisms may radically affect the vaccine product. Therefore, the establishment of a seed-lot system is essential for vaccine production. Moreover, the quality, safety and potency of

these products are usually sensitive to changes in manufacturing conditions. The quality and safety of vaccine preparations cannot be assured solely by testing of the end-product, but depends on the strict control of the manufacturing process following the principles of good manufacturing practice (GMP) (*7*). This includes demonstration of the purity and quality of the starting material (raw materials and seeds), in-process control testing, testing for process additives and process intermediates and the development and establishment of lot release tests. Moreover, as the relationship between physical and chemical characteristics, and the immunogenicity and efficacy of these products is frequently not completely understood, biological characterization through the use of biological assays should always complement the physical and chemical product characterization. The development of appropriate laboratory methods to characterize a vaccine formulation with respect to its components, as well as its safety and potency, is a prerequisite to the clinical use of any new or novel vaccines against bacteria, viruses or parasites.

Consistency of production is essential, and the demonstration that the product does not differ from vaccine lots that have been shown to be safe and adequately immunogenic and protective in clinical studies is a crucial component of vaccine evaluation, licensing and batch release. For this reason, manufacturers should make every effort to characterize these clinical lots and if possible to keep some of these lots for future reference.

Where no appropriate animal model exists for testing potency or where direct serological or immunological correlates of clinical protection are not available, the challenge is to ensure that each production batch has the same protective efficacy as those batches shown to be protective in clinical trials. In such cases, emphasis is increasingly being placed on assuring the consistency of production using modern physical, chemical and immunological methods that enable characterization of some products to a degree of precision not previously possible.

The vaccine lots used in preclinical studies should be adequately representative of the formulation intended for use in the clinical investigation and, ideally, preclinical testing should be done on the same lot as that proposed for the clinical trials. If this is not feasible, then the lots studied should be comparable with respect to physicochemical data, stability and formulation.

At a minimum, candidate vaccines for clinical trials should be prepared under conditions of good manufacturing practice (GMP) for

clinical trial material (*8*). However full GMP will be required at the later stages of clinical development (*7*, *9*).

Any change proposed to the manufacturing process during vaccine development should be considered carefully to evaluate its impact on the quality, safety and efficacy of the vaccine and the possible need for additional nonclinical and clinical investigations.

Subsequent changes in production methods or scale-up following product licensure will necessitate further product characterization to demonstrate comparability with the original lot(s) used to demonstrate safety and efficacy of the product. The extent of comparability testing needed depends on the nature of the changes implemented (*10*). These changes should be documented and the national regulatory authority consulted. Regulatory authorities should clearly define and implement in their regulations what changes require only a notification and which changes require formal approval before implementation (*11*).

The procedures used in the characterization and control of existing licensed traditional vaccines are not likely to be applicable to newer products developed using state-of-the-art technology to protect against the same infection. For example, specific guidelines have been developed for the production and control of acellular pertussis vaccines that differ from those applied to whole cell pertussis vaccine (*12*). Likewise, the tests applied to the characterization and control of traditional inactivated cholera vaccine for parenteral use are not necessarily applicable to the new inactivated whole-cell cholera vaccine intended for oral administration, and an appropriate potency test for the oral vaccine needs to be developed.

2.2 *Potency*

Potency tests measure the biological activity of a vaccine but do not necessarily reflect the mechanism of protection in humans. Potency measurement is often used to verify the consistency of the manufacturing process. The initial concept of potency testing for vaccines was to quantify the biological activity of the vaccine in comparison with a reference preparation of known bioactivity, where the antigenic component(s) were not well-defined.

Classical challenge studies in animals immunized with the vaccine under consideration have been developed into routine potency assays (e.g. for diphtheria and tetanus toxoids). In the case of the whole-cell pertussis potency assay, which consists of intracerebral challenge of immunized and nonimmunized animals, a correlation was established with clinical protection in humans (*11*). Where no suitable animal

challenge model exists, potency is often based on measurement of immune responses, usually serological (e.g. influenza and hepatitis B vaccines).

More recently, recombinant DNA methodology and modern physicochemical techniques have resulted in the manufacture of highly purified products that can be better characterized than the classic biologicals. However, the ability to measure the "relevant" biological activity for such products may still be lacking. For these products, characterization using physicochemical parameters, such as amount of antigen, size of the antigen, protein content and others can be used as a measure of consistency, but not necessarily of the potency of a vaccine.

For live attenuated vaccines, the approach to potency measurement is generally different. The potency of live viral vaccines is usually based on titration of the minimum infective dose in cell culture or chicken embryos, which may be considered as a surrogate marker of potency, but not as a measure of potency itself. A similar approach is taken to the potency measurement of live attenuated bacterial vaccines, bacille Calmette–Guérin (BCG), and typhoid vaccine (live Ty21A oral), where the number of live organisms present is the measure of potency.

For vaccines that express inserts encoding heterologous vaccine antigens (vaccines based on viral or bacterial vectors), it is not sufficient to determine the "biological activity" of the entire construct by measuring colony forming units (CFU) or infectious titre. For these vaccines, the use of other methods such as the quantitation of the expression of the insert, or the evaluation of the effective dose (ED_{50}) of the vectored vaccine should be considered.

2.3 *Stability*

The evaluation of vaccine stability is complex, as they are very susceptible to inactivation by environmental factors. Potency, as defined in the glossary, should be measured as a part of the stability testing, except in those cases where potency testing based on biological activity is not possible. Physical and chemical product characterization should be included in the stability evaluation. For a product entering human clinical trials, sufficient data should be collected to support the stability of the product for the duration of the preclinical and clinical trial. In certain cases, accelerated stability data may be used to support preliminary data obtained at the normal storage temperature. Stability data to support licensure should be obtained under the proposed storage conditions and should be based on long-term,

real-time stability studies. Finally, the stability of standards and reference materials also needs to be considered to ensure that the procedures used to measure relevant parameters are reliably standardized.

2.4 *International and national guidelines*

The World Health Organization (WHO), through considerable international consultation, develops Recommendations and Guidelines on the production and control of vaccines and other important biologicals (*13*), and these form the basis for assuring the acceptability of products globally. These documents specify the need for appropriate starting materials, including seed lot system and cell banks; strict adherence to established protocols; tests for purity, potency, and safety at specific steps during production; and the keeping of proper records. Guidelines allow greater flexibility than Recommendations with respect to specific issues related to particular vaccines.

WHO also provides Guidelines on manufacturing establishments involved in vaccine production. Recommendations can be found in the WHO document on good manufacturing practice for biologicals (*7*). Particular attention should be given to developing documented standard operating procedures for both production processes and testing procedures. These should be introduced as early as possible during the development of a vaccine and be well established by the time phase III clinical studies are undertaken and an application for marketing authorization is filed. The basic principles for the production and control of vaccines are published in the WHO Technical Report Series (*7*, *14–18*). Specific WHO guidelines and recommendations for particular vaccines are also available and should be consulted where appropriate.

WHO Recommendations and Guidelines are intended to be scientific and advisory in nature and to provide guidance for national regulatory authorities and for vaccine manufacturers. These documents may be adopted by national health authorities as definitive national regulations or used as the basis of such regulations. They are also used as the basis for deciding the acceptability of vaccines for purchase by United Nations agencies such as the United Nations Children's Fund (UNICEF) for use in global immunization programmes. Regulatory requirements for vaccines and other biologicals are also produced by other bodies, such as the European Agency for the Evaluation of Medicinal Products (EMEA) and the US Center for Biologics Evaluation and Research (CBER) (*19*); these documents can be found on the appropriate web sites (www.emea.eu.int and www.fda.gov/cber). In addition, pharmacopoeial requirements, such as those of the

European Pharmacopoeia, are also established for vaccines and are available at www.pheur.org.

For newly developed products, specific WHO, national or pharmacopoeial requirements may not be available and a national regulatory authority will need to agree on specifications with the manufacturer on a case-by-case basis during the evaluation of products for clinical trials and for licensing. For some of these novel products general guidance on production and control from WHO can be found in relevant documents, such as those describing DNA and peptide vaccines (*14*, *16*), as well as recommendations on animal cell substrates used for production of biologicals (*14*).

In addition, information on how to assure the quality of biologicals in general and on procedures for approving manufacture and for setting up a national control laboratory, can be found in the relevant WHO guidelines (*17*, *18*). For a vaccine intended to be marketed worldwide, the development of which also involves much international collaboration, it will be essential to ensure consistency of a regulatory approach for novel products such as vaccines for HIV prevention (*19*).

2.5 *Batch release and independent laboratory evaluation*

The potential variability of methods for the production of biologicals has led to the establishment of national and international requirements to define procedures for assuring the quality of vaccines and for assessing consistency both among manufacturers and over long periods of time. Licensed vaccines are subject to independent batch release (review, testing and authorizing release of a batch of vaccine independent of the manufacturer) by a national regulatory authority or national control laboratory, before release on to the market. Independent evaluation entails at least an evaluation of a manufacturer's batch release data (protocol review), but in many instances it also includes independent laboratory testing in addition to that carried out by the manufacturer.

Batch or lot release tests are those tests chosen during full product characterization to demonstrate the purity, safety and potency of the product. Lot release testing provides one measure of assurance that a lot can be manufactured consistently. Validation and establishment of lot release tests and specifications is a process that continues throughout product development and should be finalized prior to licensure.

In some countries, samples of vaccine for clinical trials are required by the national regulatory authority, as a part of the approval process for clinical trials. Vaccine developers are encouraged to consult the

appropriate regulatory agency early on during the development of a vaccine.

2.6 *Standards and reference materials*

Standards and reference materials play a vital part in the licensing and quality control process, their role ranging from use in specific antigen recognition tests to assays of vaccine toxicity, immunogenicity and potency. The standardization of the methods used to evaluate vaccines, as well as those used to evaluate immune responses to vaccine antigens, is also vital so that results may be compared directly between laboratories both within and between countries, and between clinical trials.

WHO International Biological Standards and Reference Reagents are the primary standards in use worldwide. In addition, national regulatory authorities and manufacturers may establish secondary (regional, national), working standards for the purpose of testing vaccine quality on a lot-to-lot basis. Such standards should be calibrated against International Standards, when they exist. There is concern that different secondary standards may result in "drifting" from the International Standard. Production of secondary standards on a large scale (e.g. on a regional basis) reduces the number of secondary standards in use, and should improve accuracy of testing vaccine quality. For example, the European Department for the Quality of Medicines of the Council of Europe, has been active in establishing working standards for vaccines that are calibrated against the WHO International Standards, where appropriate. The complete list of WHO International Standards and Reference Reagents can be found on the WHO web site at: www.who.int/ biologicals.

3 Immunogenicity and other pharmacodynamic studies

A pharmacodynamic study for a vaccine product is generally conducted to evaluate the immunogenicity. However, a pharmacodynamic study may also extend to include the pharmacology of an adjuvant.

Immunization studies in animal models should be conducted because they may provide valuable "proof of concept" information to support a clinical development plan. In addition, immunogenicity data derived from appropriate animal models are useful in establishing the immunological characteristics of the product and may guide selection of the doses, schedules and routes of administration to be evaluated in clinical trials. Nonclinical immunogenicity studies should assess the relevant immune response, e.g. humoral and/or cell-mediated

immune response, induced in the vaccinated animals. Depending on the immune response induced, such studies may include an evaluation of seroconversion rates, geometric mean antibody titres, or cell-mediated immunity in vaccinated animals. Nonclinical studies should, where possible, be designed to assess relevant immune responses, including functional immune response (e.g. neutralizing antibodies, opsonophagocytic activity, etc.) leading to protection. These studies may also be designed to address interference between antigens and/or live viruses. If a vaccine consists of more than one defined antigen (e.g. acellular pertussis vaccine consisting of 3–5 protein products) the response to each antigen should be evaluated. Where appropriate, challenge/protection studies with the corresponding infectious agent may be conducted to confirm the relevance of the animal models. A primary concern in interpreting the data obtained from such studies should be to determine how closely the animal model resembles the disease and immune response in humans. It should be recognized that animal models frequently fail to predict immunogenicity and efficacy in humans.

4 Toxicity assessment

The nonclinical safety assessment of vaccines needs to be viewed in the context of the evolving field of vaccine development. Thus, judgement based on the best science available should always form the basis for any decisions regarding the need for nonclinical safety studies, types of study and study designs. Similarly, scientific judgement should be applied to the interpretation of data from preclinical studies, regarding the risk–benefit ratio, animal model, dosing etc. For example, the observation of hypersensitivity reactions in an animal model may not necessarily preclude proceeding to clinical trials, but may indicate the necessity for careful monitoring of a particular clinical parameter.

Section 4.1 provides a general framework for designing a preclinical toxicity study for a vaccine. The parameters set out in this section are considered the minimum necessary for a safety assessment prior to the initiation of clinical trials in humans, in situations where preclinical safety studies are deemed necessary. As the design of any toxicity study is product-specific and based on indications, modifications to the framework outlined below may be necessary in response to particular product features, availability of animal models, methodologies, etc.

Section 4.2 provides additional considerations for performing special toxicity assessments that may be required on a case-by-case basis.

4.1 *Basic toxicity assessment*

4.1.1 *Study design*

The preclinical toxicity study should be adequate to identify and characterize potential toxic effects of a vaccine to allow investigators to conclude that it is reasonably safe to proceed to clinical investigation. The parameters to be considered in designing animal toxicology studies are the relevant animal species and strain, dosing schedule and method of vaccine administration, as well as timing of evaluation of end-points (e.g. sampling for clinical chemistry, antibody evaluation and necropsy). The route of administration should correspond to that intended for use in the clinical trials. When the vaccine is to be administered in human clinical trials using a particular device, the same device should be used in the animal study, where feasible (e.g. measles aerosol vaccine in the monkey model). Potential toxic effects of the product should be evaluated with regard to target organs, dose, route(s) of exposure, duration and frequency of exposure, and potential reversibility. The toxicity assessment of the vaccine formulation can be done either in dedicated-stand alone toxicity studies or in combination with studies of safety and activity that have toxicity end-points incorporated into the design. The study should also include an assessment of local tolerance.

4.1.2 *Animal species, sex, age and size of groups*

Data to be recorded on the animals used for toxicity testing should include information on the source, species and animal husbandry procedures (e.g. housing, feeding, handling and care of animals). In general, the use of outbred animals is recommended. The health of the animal will need to be evaluated in accordance with acceptable veterinary medical practice to ensure that animals are free of any condition that might interfere with the study. For instance, individual housing of laboratory animals may be required to minimize the risk of cross-infection.

Where possible, the safety profile of a product should be characterized in a species sensitive to the biological effects of the vaccine being studied. Ideally, the species chosen should be sensitive to the pathogenic organism or toxin. The animal species used should develop an immune response to the vaccine antigen. In general, one relevant animal species is sufficient for use in toxicity studies to support initiation of clinical trials. However, there may be situations in which two or more species may be necessary to characterize the product, for example where the mechanism of protection induced by the vaccine is not well understood (for example, intranasal influenza vaccine and intranasal measles vaccine).

In addition, when species-specific or strain-specific differences in the pharmacodynamics of the product are observed, it may be necessary to address the nonclinical safety of the product in more than one safety study and in more than one animal model.

The size of the treatment group depends on the animal model chosen. The number of animals used in studies using non-human primates would be expected to be less than that in studies that used rodents. For small animal models, e.g. rats and mice, it is recommended that approximately 10 males + 10 females per group be studied.

In general, the approximate age at the start of the study for rodents is 6–8 weeks, and for rabbits, 3–4 months.

4.1.3 *Dose, route of administration and control groups*

The toxicity study should be performed using a dose that maximizes exposure of the animal to the candidate vaccine and the immune response induced, for example, peak antibody response. In general, an evaluation of the dose–response is not required as part of the basic toxicity assessment and the lethal dose does not have to be determined. However, pilot dose–response studies may be conducted to determine which dose induces the highest antibody production in the animal model. If feasible, the highest dose (in absolute terms) to be used in the proposed clinical trial should be evaluated in the animal model. However, the dose is sometimes limited by the total volume that can be administered in a single injection, and guidelines on animal welfare should be followed. In such cases, the total volume may be administered at more than one site using the same route of administration. Alternatively, a dose that exceeds the human dose on a mg/kg basis and that induces an immune response in the animal model may be used. In such cases, the factor between human and animal dose should be justified.

The number of doses administered to the test animals should be equal to or more than the number of doses proposed in humans. To better simulate the proposed clinical usage, vaccine doses should be given at defined time intervals rather than as daily doses; the dosing interval used in the toxicity study may be shorter (e.g. an interval of 2–3 weeks) than the proposed interval in clinical trials in humans. The dosing interval in nonclinical trials may be based on the kinetics of the primary and secondary antibody responses observed in the animal model. A single-dose study may be performed in situations in which vaccine-induced antibodies are expected to neutralize a live viral vector, thus limiting the expression of the gene of interest (e.g. anti-adenovirus immune response), or when immune responses induced in

animals are expected to react with species-specific proteins present in the vaccine formulation (e.g. human recombinant cytokines used as adjuvants).

The route of administration should correspond to that intended for use in the human clinical trials. If toxic effects are observed in safety studies using a particular route of administration (e.g. intranasal), further toxicity studies using a different route of administration (e.g. intravenous) may be helpful in understanding the full spectrum of toxicity of the product.

The study design should include a negative control group(s) to evaluate a baseline level of treatment. If appropriate, active control groups (e.g. vaccine formulation without antigen) may also be included in the study. The study should include an additional treatment group of animals to be killed and evaluated as described below at later timepoints after treatment, to investigate the reversibility of any adverse effects observed during the treatment period and to screen for possible delayed adverse effects.

4.1.4 *Parameters monitored*

Toxicity studies should address the potential of the product for causing local inflammatory reactions, and possible effects on the draining lymph nodes, systemic toxicity and on the immune system. A broad spectrum of information should be obtained from the toxicity studies. Parameters to be monitored should include daily clinical observations, weekly body weights and weekly food consumption. During the first week of administration frequent measurements of body weight and food consumption are recommended, if feasible, as these are sensitive parameters indicating "illness". Interim analysis of haematology and serum chemistry should be considered approximately 1–3 days following the administration of the first and last dose and at the end of the recovery period. Haematology and serum chemistry analyses should include, at the minimum, an evaluation of relative and absolute differential white blood cell counts (lymphocytes, monocytes, granulocytes, abnormal cells) and albumin/globulin ratio, enzymes and electrolytes. In some cases, it may also be useful to evaluate coagulation parameters, urine samples and serum immunoglobulin classes. Data should be collected not only during treatment, but also following the recovery phase (e.g. 2 weeks or more following the last dose) to determine persistence, and look at exacerbation and/or reversibility of potential adverse effects.

At study termination, final body weights (after a period of fasting) should be measured. Terminal blood samples should be collected and

serum chemistry, haematology and immunological investigations should be done as described in the preceding paragraph. The immune response induced by the candidate vaccine should be assessed in order to confirm that the relevant animal model has been selected. A complete gross necropsy should be conducted and tissues collected and preserved, gross lesions should be examined and organ weights recorded (*23*). Histopathological examinations of tissues should be performed and special attention paid to the immune organs, i.e. lymph nodes (both local and distant from site of administration), thymus, spleen, bone marrow and Peyer's patches or bronchus-associated lymphoid tissue, as well as organs that may be expected to be affected as a result of the particular route of administration chosen. Histopathological examinations should always include pivotal organs (e.g. brain, kidneys, liver and reproductive organs) and the site of vaccine administration. The choice of tissues to be examined (ranging from a short list limited to immune and pivotal organs to a full list as provided in the Appendix) will depend on the vaccine in question, and the knowledge and experience obtained from previous nonclinical and clinical testing of the vaccine components. For example, full tissue examination will be required in the case of novel vaccines for which no prior nonclinical and clinical data are available. Therefore, the list of tissues to be tested should be defined on a case-by-case basis, following consultation with the relevant regulatory authority. Data should be reported in full listing the original collection of values, and summarized.

4.1.5 *Local tolerance*

The evaluation of local tolerance should be conducted either as a part of the repeated dose toxicity study or as a stand-alone study. Tolerance should be determined at those sites that come into contact with the vaccine antigen as a result of the method of administration, and also at those sites inadvertently exposed (e.g. eye exposure during administration by aerosol) to the vaccine. More details have been published elsewhere (*24*).

If abnormalities are observed in the basic toxicity study outlined in section 4.1., further studies may be necessary to evaluate the mechanism of the toxic effect.

4.2 ***Additional toxicity assessments***

4.2.1 *Special immunological investigations*

In certain cases, the results from evaluations of immune response from nonclinical and clinical studies, or from data on natural disease, may indicate immunological aspects of toxicity, e.g. precipitation of

immune complexes, humoral or cell-mediated immune response against antigenic determinants of the host itself as a consequence of molecular mimicry or exacerbation of the disease (e.g. inactivated measles vaccine). In such cases, additional studies to investigate the mechanism of the effect observed might be necessary.

Great similarity of vaccine determinants and host molecules could cause autoimmune reactions induced by molecular mimicry (*26*). Therefore, any vaccine antigen whose characteristics might mimic those of a host antigen should be treated with caution, even though it is recognized that molecular mimicry does not necessarily predispose to autoimmunity.

Because considerable efforts may be required in selecting and developing relevant animal models to address the above issues, caution should be exercised and a strong rationale provided when developing vaccines for diseases associated with autoimmune pathology.

If data suggest that the pathogen against which the vaccine is directed may cause autoimmune pathology, studies may be needed to address this concern on a case-by-case basis, if an appropriate animal model exists.

It should be noted that observations of biological markers for autoimmune reactions are not necessarily linked to pathogenic consequences. For instance, the presence of autoimmune antibodies does not necessarily indicate the induction of autoimmune disease (*25*).

When hypersensitivity reactions induced by the antigen(s), adjuvants, excipients or preservatives are of concern, additional investigations may be warranted.

4.2.2 *Developmental toxicity studies*

Developmental toxicity studies are usually not necessary for vaccines indicated for immunization during childhood. However, if the target population for the vaccine includes pregnant women and women of childbearing potential, developmental toxicity studies should be considered, unless a scientific and clinically sound argument is put forward by the manufacturer to show that conducting such studies is unnecessary. For a preventive vaccine, reproductive toxicity assessments are generally restricted to prenatal and postnatal developmental studies, because the primary concern is any potential untoward effect on the developing embryo, fetus or newborn. The need to conduct fertility and post-weaning assessments should be considered on a case-by-case basis. The animal model chosen should develop

an immune response to the vaccine, which is usually determined by serum antibody measurements. In addition, it is important to evaluate maternal antibody transfer by measuring vaccine-induced antibody in cord or fetal blood to verify exposure of the embryo or fetus to maternal antibody. The route of administration should mimic the clinical route of administration. Ideally, the maximal human dose should be administered to the test animal. If it is not possible to administer the full human dose, e.g. limitations on the total volume that can be administered, or if local toxicity is observed that may result in maternal stress, a dose that exceeds the human dose on a mg/kg basis and is able to induce an immune response in the animal should be used.

To assess any potential adverse effects of the vaccine during the period of organogenesis, the gestating animal is usually exposed to the vaccine during the period from implantation until closure of the hard palate and end of gestation defined as stages C, D and E in the ICH S5a document (*27*). Because of the relatively short gestation period of most animal models used, pre-mating treatment is frequently required to ensure maximal exposure of the embryo or fetus to the vaccine-induced immune response. For a preventive vaccine, the number of doses administered depends on the time of onset and duration of the response. Booster immunizations may be necessary at certain times during the period of gestation to maintain a high level of antibody throughout the gestation period and to expose the developing embryo to the components of the vaccine formulation. End-points include, but are not limited to, viability, resorptions, abortions, fetal body weight and morphology. The reader is referred to other publications for guidance on end-points used to evaluate potential toxic effects of the product on development of the embryo or fetus (*27*). It is also recommended that a period of postnatal follow-up of pups from birth to weaning be incorporated in the study design to assess normality of growth, body weight gain, suckling activity and viability. Studies should therefore be designed so that test groups are divided into subgroups. Half of the animals should be delivered by Caesarean section and the other half allowed to deliver their pups without surgical intervention.

4.2.3 *Genotoxicity and carcinogenicity studies*

Genotoxicity studies are normally not needed for the final vaccine formulation. However, they may be required for particular vaccine components such as novel adjuvants and additives. If needed, the in vitro tests for mutations and chromosomal damage should be done prior to first human exposure. The full battery of tests for genotoxicity may be performed in parallel with clinical trials (*28*).

Carcinogenicity studies are not required for vaccine antigens. However, they may be required for particular vaccine components such as novel adjuvants and additives.

4.2.4 *Safety pharmacology*

The purpose of safety pharmacology is to investigate the effects of the candidate vaccine on vital functions. If data from nonclinical and/or human clinical studies suggest that the vaccine (e.g. one based on specific toxoids) may affect physiological functions (e.g. central nervous system, respiratory, cardiovascular and renal functions) other than those of the immune system, safety pharmacology studies should be incorporated into the toxicity assessment. Useful information on this topic can be found in the *Note for Guidance on safety pharmacology studies for human pharmaceuticals* (*29*).

4.2.6 *Pharmacokinetic studies*

Pharmacokinetic studies (e.g. for determining serum or tissue concentrations of vaccine components) are normally not needed. The need for specific studies should be considered on a case-by-case basis (e.g. when using novel adjuvants or alternative routes of administration) and may include local deposition studies that would assess the retention of the vaccine component at the site of injection and its further distribution (e.g. to the draining lymph nodes). Distribution studies should be considered in the case of new formulations, novel adjuvants or when alternative routes of administration are intended to be used (e.g. oral or intranasal).

5 Special considerations

5.1 *Adjuvants*

Adjuvants may be included in vaccine formulations or co-administered with vaccines to enhance the immune responses to particular antigen(s), or to target a particular immune response. It is important that the adjuvants used comply with pharmacopoeial requirements where they exist, and that they do not cause unacceptable toxicity.

Adjuvant activity is a result of many factors and the immune response obtained with one particular antigen/adjuvant formulation cannot, as a rule, be extrapolated to another antigen. Individual antigens vary in their physical and biological properties and antigens may interact differently with an adjuvant. Adjuvants must be chosen according to the type of immune response desired and they must be formulated with the antigen in such a way that distribution of both is optimized to ensure availability to the relevant lymphatic tissues. The route of

administration of the vaccine is also an important factor influencing the efficacy and safety of an adjuvant.

The effect of the adjuvant should be demonstrated in preclinical immunogenicity studies. If no toxicological data exist for a new adjuvant, toxicity studies of the adjuvant alone should first be performed. In general, assessment of new or novel adjuvants should be undertaken as required for new chemical entity (*30–32*). These data may be obtained by the vaccine manufacturer or by the producer of the adjuvant. In addition to assessing the safety of the adjuvant by itself it is also important to assess whether the combination of antigen and adjuvant exerts a synergistic adverse effect in the animal model (*33*, *34*). When species-specific proteins (e.g. cytokines) are used as novel adjuvants, the issue of species-specific response should be considered.

When evaluating the safety profile of the combination of adjuvant and vaccine, the formulation proposed for clinical use should be used.

Compatibility of the adjuvant(s) (e.g. lack of immune interference) with all antigenic components present in the vaccine should be evaluated.

If applicable, adsorption of all antigenic components present in the vaccine should be shown to be consistent on a lot-to-lot basis. Potential desorption of antigen during the shelf-life of the product should be performed as a part of stability studies, the results reported and specifications set, as this may affect not only immunogenicity, but also the toxicity profile of the product.

It should be noted that no adjuvant is licensed in its own right, but only as a component of a particular vaccine.

5.2 *Additives (excipients and preservatives)*

Where a new additive is to be used, for which no toxicological data exist, toxicity studies of the additive alone should first be performed and the results documented according to the guidelines for new chemical entities (*31*). The compatibility of a new additive with all vaccine antigens should be documented together with the toxicological profile of the final vaccine formulation under consideration in animal models as outlined in section 4.

5.3 *Vaccine formulation and delivery device*

The vaccine formulation (i.e. liquid form, capsules or powder), as well as the delivery device, may have an impact on the uptake of

the vaccine, its effectiveness and safety. Ideally, the delivery device and vaccine formulation tested in an animal safety study should be identical to those intended to be used clinically. However, animal models in which delivery devices intended for clinical use can be tested may not be available. In these instances, in order to develop an appropriate animal model, it may be necessary to conduct pilot studies to define and optimize the conditions for drug delivery in the animal model before it can be used to assess the preclinical safety of the product.

5.4 *Alternative routes of administration*

When using a vaccine formulation administered by alternative routes (e.g. intranasal, oral, intradermal, rectal and intravaginal routes), it can be assumed that their potency, relevant immunogenicity, tolerability, toxicity, and long-term safety may differ from that of products delivered by the parenteral route. Thus, when different routes of administration are proposed, nonclinical safety studies may have to be conducted using vaccine formulation and/or adjuvant alone in a suitable animal model to address the specific safety concerns associated with vaccine administration by these routes. Particular issues relevant to vaccines administered using alternative routes that may need to be considered are discussed below.

5.4.1 *Animal models*

A special consideration for vaccines administered by alternative routes should be the anatomy and physiology of the site of vaccine administration of the particular animal model chosen and its accessibility for the administration of the vaccine. For example, for intranasally administered products, the species chosen should ideally be receptive to spray administration of the product. In general, rabbits and dogs are useful test models for use of spray devices; however, their olfactory bulbs are highly protected and special techniques would be required to ensure that the test product reached this organ. Although mice and rats are useful models, intranasal administration to these species presents technical difficulties. Intranasal administration to non-human primates may be preferable, if they are susceptible to the infectious agent in question.

Depending on the level of concern regarding a particular route of administration or when there are species-specific differences between the animal models in their sensitivity to the candidate vaccine, it may be necessary to address the preclinical safety of the product in more than one safety study and in more than one animal model.

5.4.2 *Dose*

As the optimal dose derived from studies using the parenteral route of administration may differ from the dose used for alternative route(s) of administration, dose-finding studies may need to be conducted for a particular route of administration. Also, consideration should be given to the total volume of the vaccine administered as it may affect the outcome of the safety study. For example, intranasal administration of more than 5 µl of test preparation per nostril to a mouse would result in the test preparation being swallowed, rather than being adsorbed by the nasal mucosa.

5.4.3 *End-points*

The toxicity end-points would include those described in section 4 and may include additional outcome measures that would depend on the route of administration and specific concerns associated with the particular route and target organ. For example, if there is concern about the potential passage of vaccine components to the brain following intranasal administration, immunohistology and "in situ" methods and/or neurological assays and examinations may be necessary. For vaccines administered by inhalation, outcome measures may include pulmonary function tests and data on histopathology of the lungs. Considerable efforts may be required to develop appropriate methods to address potential safety concerns associated with the use of new routes of administration.

5.4.4 *Immunogenicity assessment*

The development of appropriate assays for measuring mucosal immune responses is critical for vaccines that are expected to function as mucosal immunogens because serological assays alone may not reflect the relevant immune response for a mucosal vaccine. Thus, in addition to measuring serological responses, it may be necessary to evaluate T cell responses, antibody-secreting cells and cytokine production. In addition, assays may need to be developed to assess the induction of local and systemic responses at sites distant from administration of the vaccine antigen.

6 Specific considerations for particular types of vaccines

In addition to the testing strategies outlined in sections 3, 4 and 5, studies may be necessary to address specific safety concerns associated with particular product types using suitable in vitro and in vivo test methods. The specific testing requirements for live attenuated and combination vaccines are discussed below. Detailed information regarding the production and control of other types of vaccine is available in the WHO guidance documents for production and con-

trol (13), and should be consulted. For example, in the recently developed guidelines for DNA (16) and synthetic peptide vaccines (18, 35), as well as for particular vaccines such as Hib conjugated vaccine (26), the issues relevant for nonclinical testing are discussed and should be considered in the development of an appropriate design for the nonclinical study of the vaccine in question.

6.1 *Live attenuated vaccines*

An assessment of the degree of attenuation, and the stability of the attenuated phenotype, are important considerations for the nonclinical testing programme of a live attenuated vaccine. Laboratory markers of attenuation are invaluable for this purpose. These markers should be capable of distinguishing the attenuated vaccine from fully virulent wild-type strains and, ideally, of detecting partial reversion to full virulence. To assess the stability of the attenuation phenotype, the vaccine may be passaged under production conditions beyond the maximum passage number to be used for production. Stability of attenuation may also be assessed by passage under conditions that are outside the conditions to be used for vaccine production. For example, higher or lower temperatures may exert selection pressure for reversion to virulence. The marker(s) of attenuation may subsequently be used to qualify new vaccine seed preparations and to monitor the effect of any significant changes in production conditions of the attenuated phenotype.

If the wild-type organism is neurotropic, or if passages through neural tissue have been used in the attenuation of a virus vaccine, then a test for neurovirulence should be performed at least at the level of the vaccine seed. A neurovirulence test is not necessarily required for all live attenuated vaccines. The specifications for an appropriate neurovirulence test depend on the organism under test and should be capable of distinguishing the attenuated vaccine from fully virulent wild-type strains and, ideally, of detecting partial reversion to full virulence. Specific reference preparations may be needed for this purpose. Neurovirulence tests in small animal models may be acceptable.

If the live attenuated vaccine is based on a genetically modified organism, then an environmental risk assessment may be required as part of the preclinical evaluation. An investigation into the possible shedding of vaccine organisms following administration contributes to the environmental risk assessment. For all live attenuated vaccines, information on the likelihood of exchange of genetic information with non-vaccine strains may be required and suitable nonclinical tests may be designed to provide data for this purpose.

6.2 *Combined vaccines*

New combinations produced either by formulation or at the time of reconstitution of antigens or serotypes should be studied for appropriate immunogenicity in an animal model, if available, before initiation of human clinical trials (*36*, *37*). Combined antigens should be examined by appropriate physicochemical means to evaluate possible changes to antigen properties on combination, such as degree of adsorption to aluminium adjuvants, as well as stability of the combination.

The immune response to each of the antigens in the vaccine should be assessed, including the quality of response and any potential interference and incompatibilities between combined antigens. It is preferable to study a new combination in comparison with the individual antigens in animals to determine whether augmentation or diminution of response occurs.

The need to evaluate the safety of the new combination in an animal model should be considered on a case-by-case basis. Such evaluation is likely to be necessary if there is concern that combining antigens and/or adjuvants may lead to problems of toxicity (e.g. novel adjuvant).

Similar consideration for nonclinical testing will also apply to cases where a new candidate single-component vaccine is developed from an already licensed combined vaccine (e.g. monovalent oral polio vaccine versus trivalent oral polio vaccine).

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Appendix

List of tissues to be collected in a repeated dose toxicity study

adrenal glands

aorta

bone (femur) and articulation

bone (sternum) with bone marrow

bone marrow smears[1]

brain

bronchi (main-stem)

caecum

colon

duodenum

epididymides

eyes

heart

ileum

injection site(s) (a sample should be taken from the area of injection)

jejunum

kidneys and ureters

larynx

liver

lungs

lymph node (mandibular)

lymph node (mesenteric)

mammary gland

oesophagus

optic nerves

[1] Bone marrow smears should be prepared at the scheduled necropsy for all animals including any moribund animals killed during the study. The smears should be fixed in methanol and then stained by the May-Grunwald-Giemsa method.

ovaries and oviducts
pancreas
parathyroid glands
Peyer's patches
pituitary gland
prostate
rectum
salivary glands (mandibular, parotid, sublingual)
sciatic nerves
seminal vesicles
skeletal muscle
skin
spinal cord (cervical, thoracic, lumbar)
spleen
stomach
testes
thymus
thyroid glands
tongue
trachea
ureters
urinary bladder
uterus (horns + cervix)
vagina
all gross lesions

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Annex 2
Recommendations for the production and control of pneumococcal conjugate vaccines

Recommendations published by WHO are intended to be scientific and advisory in nature. The parts of each section printed in type of normal size have been written in a form, such that, should a national regulatory authority so desire, they may be adopted as they stand as definitive national requirements or used as the basis of such requirements. Those parts of each section printed in small type are comments and recommendations for guidance for those manufacturers and national regulatory authorities which may benefit from additional information.

It is recommended that modifications be made only on condition that the modifications ensure that the vaccine is at least as safe and efficacious as that prepared in accordance with the recommendations set out below.

The terms "national regulatory authority" and "national control laboratory" as used in these recommendations, always refer to the country in which the vaccine is manufactured.

Introduction

Recommendations (formerly known as Requirements) for pneumococcal polysaccharide vaccines were drafted in 1980 but were never adopted by the WHO Expert Committee on Biological Standardization (*1*). Vaccines based on the capsular polysaccharides of the 23 serotypes of *Streptococcus pneumoniae* most commonly associated with human disease have been licensed in many countries (*2*). These vaccines have been shown to be efficacious against invasive pneumococcal disease and have proved to be effective for the protection of individuals who are at particular risk of infection. Nevertheless, their inability to elicit protective responses in young infants or to induce good immunological memory has prevented their inclusion in national infant immunization schedules.

The development of bacterial capsular polysaccharide–protein conjugates represents a major advance in prophylaxis against bacterial infections (*3*). Following the successful introduction of the *Haemophilus influenzae* type b conjugate (Hib) and meningococcal C conjugate (MenC) vaccines into paediatric vaccination schedules, considerable progress has been made in the development of similar conjugate vaccines based on pneumococcal capsular polysaccharides. Glycoconjugate vaccines are both physically and immunobiologically distinct from their unconjugated counterparts, emphasizing the need for recommendations specifically for these products.

General considerations

Infections caused by *S. pneumoniae*, the pneumococcus, are responsible for substantial morbidity and mortality, particularly in the very young and in the elderly (*2*, *4*, *5*). Pneumococci are grouped into serotypes on the basis of their chemically and serologically distinct capsular polysaccharides. Of the 90 pneumococcal serotypes (*6*), the capsular polysaccharides of the 23 most commonly associated with disease are included in the polysaccharide vaccines produced by various manufacturers. These vaccines are effective in individuals from about 2 years of age but, as they elicit T-cell independent immunity, they are not effective in younger children. In addition, they fail to induce boostable immunity and have little or no impact on nasopharyngeal carriage (*7*). In contrast, polysaccharide–protein conjugates have been shown to be highly immunogenic in infants and to induce T-cell dependent immunity. Several pneumococcal conjugate vaccines are now available or are at an advanced stage of development (*8–10*). The results of controlled clinical trials of these vaccines have

demonstrated that such conjugates are both safe and highly immunogenic, T-cell dependent antigens (*11*, *12*). They have been shown to induce high levels of serum antibody and to offer protective immunity against invasive pneumococcal disease (*13*). They are effective in young children, induce immunological memory and reduce nasopharyngeal carriage of the pneumococcal serotypes included in the formulation (*14*). A 7-valent conjugate, manufactured using diphtheria protein CRM197 as the carrier protein for all seven serotypes, was first licensed in the USA in 2000 and has become increasingly available worldwide.

Protective levels of antibody elicited by the CRM197 conjugated vaccines against invasive pneumococcal disease have been estimated using the data from three clinical efficacy trials: one in Northern California; one among Navajo Indians and one in Soweto, South Africa. The aggregate efficacy for the seven serotypes these vaccines had in common was 93.0% (95% confidence interval, 81.0–98.2%). Using the data from enzyme-linked immunosorbent assays (ELISA) for anti-capsular polysaccharide antibody, an estimate of 0.35 µg/ml aggregated across the serotypes was associated with the point-estimate of clinical efficacy against invasive disease (see Appendix for additional details). However, this reference value is neither applicable to the determination of the protective status of the individual nor to protection against other disease end-points, e.g. pneumonia or otitis media. Practical or ethical considerations may make it impossible to perform protective efficacy trials of most new vaccine formulations. Therefore, this reference value will be important for the licensure of future products using data from immunogenicity trials.

Differences in the incidence of serotypes causing disease from one continent to another have led to the development of pneumococcal vaccine formulations consisting of increasing numbers of conjugated components (*15*). Recently clinical trials of 7-valent and 9-valent formulations have been completed in Finland and South Africa respectively (*16–18*), and further formulations with potentially greater coverage are under development (*9*). From a practical perspective, however, it is evident that there is a limit to the number of serotypes that can be included in such conjugate vaccine formulations and the incidence of disease-causing serotypes in the target population should be taken into consideration before vaccine development. Although geographical and temporal factors undoubtedly contribute to differences in the incidence rates between regions, the impact of differences between national epidemiological surveillance systems on case ascertainment may also prove to be a critical factor in the assessment

of pneumococcal vaccine coverage (*19*). The serotype composition of pneumococcal vaccines should be agreed with the national regulatory authority based on appropriate epidemiological data on the target population. The superiority of a vaccine should not be assumed on the basis of the number of serotypes included unless there is evidence that the inclusion of additional serotypes is likely to enhance its effectiveness in a particular epidemiological setting.

Special considerations

The production and control of conjugate vaccines is more complex than that of their unconjugated capsular polysaccharide counterparts. Polysaccharide vaccines consist of defined chemical substances that, if prepared to the same specifications, can reasonably be expected to have comparable potencies. Although only the 7-valent conjugate formulation has been licensed to date, experience with *H. influenzae* type b and meningococcal conjugate vaccines suggests that effective pneumococcal vaccines may be developed that differ both in the nature of the saccharide and the carrier protein employed. Vaccines are under development that utilize carrier proteins other than CRM197 and vaccine formulations could be developed in which more than one carrier is employed. The manufacturer has a choice of possible carrier proteins providing that the resulting vaccine is safe and elicits a T-cell dependent, protective immune response.

Unfortunately, the lack of a suitable animal model for all pneumococcal serotypes makes it impossible to assess the potency of these vaccines for humans on the basis of studies in animals. Consequently, it is important that new pneumococcal vaccine formulations are evaluated in humans for immunogenicity by monitoring the production of serotype-specific immunoglobulin G (IgG). Immune responses to pneumococcal vaccines have been measured using methods that determine either the total amount of antibody binding to capsular polysaccharide or the amount of functional antibody present in serum. Antibody binding is typically evaluated by the use of ELISAs or radioimmunoassays (*20–22a*), whereas the opsonophagocytic assay is used to measure functional antibodies (*23–25*). Clinical studies of conjugate vaccines have shown a good association between antibody levels measured by ELISA and protection (see Appendix). However, such studies also usually include an analysis of a subset of sera to confirm their functional (e.g. opsonophagocytic) activity. Whichever assay is used, it should be standardized so as to ensure comparability of data both between laboratories and between different clinical

studies. A set of calibration sera is available to help establish comparability between laboratories (*26*). As conjugate vaccines should induce a T-cell dependent immune response, this should also be evaluated during clinical trials. Indicators of T-cell dependent immunity include the production of predominantly high-avidity IgG antibody and the demonstration of a good booster response in children who have already had the primary vaccination (*14*).

Given the lack of a suitable animal model that will predict the potency of all pneumococcal serotypes, the strategy for the control of the vaccine is dominated by the use of tests for molecular characterization and purity. These tests focus on physicochemical criteria to ensure each vaccine lot is consistent with the specification of the vaccine lots used in the definitive clinical trials that confirmed their safety and efficacy. Animal studies form an essential part of the development of these vaccines to provide evidence that they induce T-cell dependent immunity and to characterize the immunogenicity of the vaccine during stability studies. However, an immunogenicity test in animals is not necessary for routine lot release when vaccine consistency has been assured by alternative means.

Combined vaccines containing pneumococcal polysaccharide conjugate components

The introduction of Hib and MenC conjugates as additional elements of infant immunization programmes has served to highlight the need to combine paediatric vaccines for effective vaccine delivery (*27*). Vaccine formulations with multiple components that include pneumococcal conjugates are likely to be developed within the next decade. If one or more conjugated pneumococcal components are indicated for co-administration with other vaccines, the possible effects on the clinical performance of each component in the vaccine, including the pneumococcal conjugate components, should be evaluated in terms of their safety and immunogenicity. Similarly, the clinical effect of concomitant administration of a pneumococcal conjugate vaccine with other vaccines at different sites should be evaluated. Because of the problems associated with performing physicochemical analyses on complex vaccine formulations, the manufacturer should consider which batch release tests are appropriate to perform on final bulks of a particular product and which tests should be performed on final lots of such vaccines. The tests should be agreed with the national regulatory authority.

Part A. Manufacturing recommendations

A.1 Definitions

A.1.1 *Proper name*

The proper name of the vaccine should be "Pneumococcal conjugate vaccine" translated into the language of the country of use. The serotypes included in the vaccine should be associated with the name of the vaccine and listed in the packaging material. The use of this proper name should be limited to vaccines that satisfy the recommendations formulated below.

A.1.2 *Descriptive definition*

Multivalent pneumococcal conjugate vaccine is a preparation of capsular polysaccharide from specific serotypes of *Streptococcus pneumoniae* that are covalently linked to carrier protein.

A.1.3 *International reference materials*

No formally established international reference materials that would allow the standardization of immune responses to pneumococcal conjugate vaccines are currently available.

The following reagents are available through the courtesy of individuals, manufacturers and national regulatory or reference laboratories:

C-polysaccharide (Statens Serum Institute, Copenhagen, Denmark)
Capsular polysaccharides (American Type Culture Collection (ATCC), Manassas, Virginia, USA)
89-SF reference serum (Dr Carl Frasch, Center for Biologics Evaluation and Research, US Food and Drug Administration (CBER/FDA), Rockville, MD, USA) (*22*)
96DG secondary reference serum (provided by Dr David Goldblatt and distributed by National Institute for Biological Standards and Control (NIBSC), Potters Bar, Herts., England)
ELISA calibration sera (provided by Dr David Goldblatt and distributed by NIBSC, Potters Bar, Herts., England) (*26*)
Pneumococcal serotyping reagents (Statens Serum Institute, Copenhagen, Denmark)
HL-60 cells (ATCC, Manassas, Virginia, USA)

A.1.4 *Terminology*

Master seed lot. A bacterial suspension of *S. pneumoniae* derived from a strain that has been processed as a single lot and is of uniform composition. It is used for the preparation of the working seed lots.

Master seed lots shall be maintained in the freeze-dried form or be frozen below –45 °C.

Working seed lot. A quantity of live *S. pneumoniae* organisms derived from the master seed lot by growing the organisms and maintaining them in aliquots in the freeze-dried form or the frozen state at or below –45 °C. The working seed lot is used, when applicable, after a fixed number of passages, for the inoculation of production medium.

Single harvest. The material obtained from one batch of cultures that have been inoculated with the working seed lot (or with the inoculum derived from it), harvested and processed together.

Purified polysaccharide. The material obtained after final purification. The lot of purified polysaccharide may be derived from a single harvest or a pool of single harvests processed together.

Modified polysaccharide. Purified polysaccharide that has been modified by chemical reaction or physical process in preparation for conjugation to the carrier.

Carrier. The protein to which the polysaccharide is covalently linked for the purpose of eliciting a T-cell dependent immune response to the pneumococcal polysaccharide.

Monovalent bulk conjugate. A conjugate prepared from a single lot or pool of lots of polysaccharide and a single lot or a pool of lots of protein. This is the parent material from which the final bulk is prepared.

Final bulk conjugate. The blend of monovalent conjugates present in a single container from which the final containers are filled, either directly or through one or more intermediate containers derived from the initial single container.

Final lot. A number of sealed, final containers that are equivalent with respect to the risk of contamination during filling and, when it is performed, freeze-drying. A final lot must therefore have been filled from a single container and freeze-dried in one continuous working session.

A.2 General manufacturing recommendations

The general manufacturing recommendations contained in good manufacturing practices for pharmaceuticals (*28*) and biological products (*29*) should apply to establishments manufacturing pneumococcal conjugate vaccines with the addition of the following:

Details of standard operating procedures for the preparation and testing of pneumococcal conjugate vaccines adopted by the manufacturer together with evidence of appropriate validation of each production step should be submitted for the approval of the national regulatory authority. All assay procedures used for quality control of the conjugate vaccines and vaccine intermediates must be validated. Proposals for the modification of manufacturing and control methods should also be submitted for approval to the national regulatory authority.

Streptococcus pneumoniae is a Biological Safety Level (BSL) 2 pathogen and represents a particular hazard to health through infection by the respiratory route. The organism should be handled under conditions appropriate for this class of pathogen (*30*). Standard operating procedures need to be developed for dealing with emergencies arising from the accidental spillage, leakage or other dissemination of pneumococcal organisms. Personnel employed in the production and control facilities should be adequately trained and appropriate protective measures including vaccination with a pneumococcal vaccine licensed for use in adults should be implemented. Adherence to current good manufacturing practices is important to the integrity of the product, to protect workers and to protect the environment.

A.3 Production control

A.3.1 *Control of polysaccharide*

A.3.1.1 *Strains of Streptococcus pneumoniae*

The strains of *S. pneumoniae* used for preparing the polysaccharide should be agreed with the national regulatory authority. Each strain should have been shown to be capable of producing polysaccharide of the appropriate serotype. Each master seed lot should be identified by a record of its history, including the source from which it was obtained and the tests done to determine the characteristics of the strain.

> The cultures may be examined for the following characteristics: microscopically, stained smears from a culture should appear typical of *S. pneumoniae*; the organism should grow at 37 °C , but not at 25 °C, and should have characteristic smooth alpha haemolytic colonies; the organism should have the ability to ferment insulin; the organism should be lysed in the bile solubility test and be sensitive to optochin; a suspension of the culture should be agglutinated or give a positive Quellung reaction with the appropriate serotyping serum.
>
> Nuclear magnetic resonance spectrometry (either ^{1}H or ^{13}C) is a suitable method for the confirmation of identity of purified polysaccharide.

A.3.1.2 *Seed lot system*

The production of pneumococcal polysaccharide should be based on a working seed lot system. Cultures derived from the working seed

lots should have the same characteristics as the cultures of the strain from which the master seed lot was derived (A.3.1.1). If materials of animal origin are used in the medium for seed production, preservation of strain viability for freeze-drying or for frozen storage, then they should comply with the guidance given in the *Guidelines on Transmissible Spongiform Encephalopathies in Relation to Biological and Pharmaceutical Products* (*31*) and should be approved by the national regulatory authorities.

Manufacturers are encouraged to avoid wherever possible the use of materials of animal origin.

A.3.1.3 *Culture media for the production of pneumococcal polysaccharide*

The liquid culture medium used for vaccine production should be free from ingredients that will form a precipitate upon purification of the capsular polysaccharide. If materials of animal origin are used then they should comply with the guidance given in the *Guidelines on Transmissible Spongiform Encephalopathies in Relation to Biological and Pharmaceutical Products* (*31*) and should be approved by the national regulatory authorities.

Manufacturers are encouraged to avoid wherever possible the use of materials of animal origin.

A.3.1.4 *Single harvests*

Consistency of growth of *S. pneumoniae* should be demonstrated by monitoring growth rate, pH and the final yield of polysaccharide.

A.3.1.5 *Control of bacterial purity*

Samples of the culture should be taken before killing and be examined for microbial contamination. The purity of the culture should be verified by suitable methods, which should include inoculation on to appropriate culture media, including plate media that do not support growth of *S. pneumoniae*. If any contamination is found, the culture or any product derived from it should be discarded. The killing process should also be adequately validated.

A.3.1.6 *Purified polysaccharide*

Each lot of pneumococcal polysaccharide should be tested for identity, purity and molecular size. A number of approaches to determining polysaccharide identity and purity give complementary but incomplete information, so a combination of methods should be employed to provide all necessary data and should be agreed by the national regulatory authority. The purity limits given below are expressed with reference to the polysaccharide in its salt form (sodium or calcium), corrected for moisture. Variations in these specifications

that may be appropriate if unusual salt forms are present should be agreed by the national regulatory authority.

Generally, after killing the organism, the culture is harvested and the polysaccharide isolated and purified by techniques such as fractional precipitation, chromatography, enzyme treatment and ultrafiltration. The polysaccharide is partially purified by fractional precipitation, washed, and dried to a residual moisture content shown to favour the stability of the polysaccharide. Methods used for the purification of bulk polysaccharide should be approved by the national regulatory authority. Purified pneumococcal polysaccharide and, when necessary, partially purified intermediates, are usually stored at or below –20 °C to ensure stability.

A.3.1.6.1 *Polysaccharide identity*

A test should be performed on the purified polysaccharide to verify its identity. In cases where other polysaccharides are produced at the same manufacturing site, the method should be validated to show that it distinguishes the desired polysaccharide from all other polysaccharides produced at that manufacturing site.

A serological method such as countercurrent immunoelectrophoresis and/or nuclear magnetic resonance spectrometry (either ^{1}H or ^{13}C) is convenient for this purpose (*32*–*34*). In some cases the identity of the polysaccharide can be deduced from its composition if appropriate analytical methods are employed.

A.3.1.6.2 *Polysaccharide composition*

The composition of the polysaccharide provides information on its purity, identity and the amount of specific impurities, such as pneumococcal C-polysaccharide, that are present. Analyses should be based on the dry weight of the polysaccharide. The composition of the polysaccharide can be defined in a number of ways depending on the methodology employed and the salt form present. The specifications used should be agreed by the national regulatory authority.

Chemically, the composition of pneumococcal polysaccharides can be defined by the percentage of total nitrogen, phosphorus, uronic acid, hexosamine, methyl pentose and *O*-acetyl groups. These are usually determined by a combination of simple wet chemical tests with colorimetric read outs. Typical specifications are listed in Table 1 (*35*).

Other methods, such as high performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection (HPAEC–PAD) applied to hydrolysates of the polysaccharide, may be used to defineaspects of the quantitative composition of certain polysaccharide types, but the method should be validated for the purpose (*36*). ^{1}H nuclear magnetic resonance spectrometry also provides a convenient approach for quantitation of the composition of the purified polysaccharide if an internal reference compound is included (*33*, *34*). The proportion of pneumococcal C polysaccharide may be determined by a combination of ^{1}H and ^{31}P nuclear magnetic resonance spectrometry (*37*, *38*) or HPAEC–PAD (*39*).

Table 1
Theoretical composition of pneumococcal polysaccharides

Serotype	Total nitrogen (%) (range)	Phosphorus (%) (range)	Uronic acid[1] (%)	Hexosamines[1] (%)	Methyl pentose[1] (%)	*O*-acetyl groups[1] (%)
1	3.56 (3.5–6)	0 (0–1.5)	55.17 (≥45)	0	0	5.47 (≥1.8)
2	0 (0–1)	0 (0–1.0)	22.59 (≥15)	0	50.58 (≥38)	0
3	0 (0–1)	0 (0–1.0)	60.23 (≥40)	0	0	0
4	4.95 (4–6)	0 (0–1.5)	0	71.84 (≥40)	19.11 (≥10)	0
5	3.04 (2.5–6)	0 (<2)	23.59 (≥12)	44.14 (≥20)	35.22 (≥25)	0
6B	0 (0–2)	4.38 (2.5–5.0)	0	0	22.86 (≥15)	0
7F	2.28 (1.5–4.0)	0 (0–1.0)	0	33.09	26.40 (≥13)	3.5 (present)
8	0 (0–1)	0 (0–1.0)	31.70 (≥25)	0	0	0
9N	3.09 (2.2–4.0)	0 (0–1.0)	23.96 (≥20)	44.82 (≥28)	0	0
9V	1.44 (0.5–3)	0 (0–1.0)	22.33 (≥15)	20.89 (≥13)	0	8.85 (present)
10A	1.12 (0.5–3.5)	2.48 (1.5–3.5)	0	16.21 (≥12)	0	0
11A	0 (0–2.5)	3.25 (2.0–5.0)	0	0	0	13.54 (≥9)
12F	3.82 (3–5)	0 (0–1.0)	19.73 (≥15)	55.36 (≥25)	14.73 (≥10)	0
14	2.03 (1.5–4)	0 (0–1.0)	0	29.44 (≥20)	0	0
15B	1.31 (1–3)	2.89 (2.0–4.5)	0	18.94 (≥15)	0	4.01 (present)
17A	0 (0–1.5)	0 (0–3.5)	16.16 (≥10)	0	24.12 (≥20)	3.2 (present)
17F	0 (0–1.5)	2.93 (0–3.5)	0	0	30.60 (≥20)	4.06 (present)
18C	0 (0–1)	3.05 (2.4–4.9)	0	0	15.96 (≥14)	4.24 (present)
19A	2.27 (0.6–3.5)	5.04 (3.0–7.0)	0	32.98 (≥12)	26.32 (≥20)	0
19F	2.27 (1.4–3.5)	5.04 (3.0–5.5)	0	32.98 (≥12.5)	26.32 (≥20)	0
20	1.28 (0.5–2.5)	0 (1.5–4.0)	0	18.49 (≥12)	0	7.83 (present)
22F	0 (0–2)	0 (0–1.0)	21.30 (≥15)	0	31.80 (≥25)	4.22 (present)
23F	0 (0–1)	3.90 (3.0–4.5)		0	40.77 (≥37)	0
33F	0 (0–2)	0 (0–1.0)	0	0	0	4.24 (present)

[1] Typical specifications (*35*) are given in brackets.
The values are calculated using broad definitions of the classes of sugars, so, for example, "hexosamines" include 2-acetamido-2,6-dideoxyhexoses and 2-acetamido-2-deoxyuronic acids, "methylpentose" includes 2-acetamido-2,6-dideoxyhexoses and "uronic acid" includes 2-acetamido-2-deoxyuronic acids. It is not certain that such sugars would give an identical response in chemical tests used to determine the composition. The values are cited as equivalents of compounds used as references in the relevant tests. The values assume complete *O*-acetylation at each distinct site for *O*-acetylation, using published and unpublished data.

A.3.1.6.3 *Moisture content*

If the purified polysaccharide is to be stored as a lyophilized powder, the moisture content should be determined by suitable methods approved by the national regulatory authority and shown to be within agreed limits.

A.3.1.6.4 *Protein impurity*

The protein content should be determined by the method of Lowry *et al*., using bovine serum albumin as a reference (*1*, *40*), or another suitable validated method. Sufficient polysaccharide should be assayed to detect 1% protein contamination accurately.

Each lot of purified polysaccharide should typically contain not more than 3% by weight of protein. However, this will vary depending upon the serotype and an acceptable level of protein contamination should be agreed with the national regulatory authority.

A.3.1.6.5 *Nucleic acid impurity*

Each lot of polysaccharide should contain not more than 2% by weight of nucleic acid as determined by ultraviolet spectrophotometry, on the assumption that the absorbance of a 1 g/l nucleic acid solution contained in a cell of 1 cm path length at 260 nm is 20 (*1*) or by another validated method.

Sufficient polysaccharide should be assayed to detect 2% nucleic acid contamination accurately.

A.3.1.6.6 *Pyrogen content*

The pyrogen content of the purified polysaccharide should be determined and shown to be within acceptable limits agreed by the national regulatory authority.

A recognized pyrogenicity test can be performed in rabbits. Alternatively, the *Limulus* amoebocyte lysate test can be performed.

A.3.1.6.7 *Molecular size distribution*

The molecular size of the purified polysaccharide in each lot provides an indication of the manufacturing consistency. An acceptable level of consistency should be agreed with the national regulatory authority and can be established either by process validation or measurement on each lot.

The distribution constant (K_D) can be determined by measuring the molecular size distribution of the polysaccharide at the main peak of the elution curve obtained by a suitable chromatographic method. The K_D value and/or the mass distribution limits should be established.

Methods such as gel filtration through Sepharose CL-4B or CL-6B (or similar) in a 0.2 molar buffer using either a refractive index detector or colorimetric assay for the detection of the polysaccharide; and high performance size-exclusion chromatography (HPSEC) with refractive index detectors either alone or in combination with light scattering (e.g. multiple angle laser light scattering (MALLS)) are suitable for this purpose (*34*, *41*). The methodology and column used should be validated to demonstrate sufficient resolution in the appropriate molecular weight range.

A.3.1.7 *Modified polysaccharide*

Modified polysaccharide preparations may be partially depolymerized either before or during the chemical modification. The registered pneumococcal conjugate vaccines and several of the candidate vaccines use polysaccharides and oligosaccharide chains.

A.3.1.7.1 ***Chemical modification***

Several methods are available for the chemical modification of polysaccharides before conjugation. The chosen method should be approved by the national regulatory authority.

The current methods used are similar to those employed in the production of conjugate vaccines against *Haemophilus influenzae* type b. For example, polysaccharide may be oxidized with periodate and the periodate-activated polysaccharide attached to free amino groups on the carrier protein by reductive amination. Alternatively, the polysaccharide can be randomly activated by cyanogen bromide, or a chemically similar reagent, and a bifunctional linker added, which then allows the polysaccharide to be attached to the carrier protein directly, or through a secondary linker.

A.3.1.7.2 ***Extent of modification of the polysaccharide***

The manufacturer should demonstrate consistency of the degree of modification of the polysaccharide, either by an assay of each batch of the polysaccharide or by validation of the manufacturing process.

A.3.1.7.3 ***Molecular size distribution***

The degree of size reduction of the polysaccharide will depend upon the manufacturing process. The average size distribution (degree of polymerization) of the modified polysaccharide should be determined by a suitable method and shown to be consistent. The molecular size distribution should be specified for each serotype, with appropriate limits for consistency, as the size may affect the reproducibility of the conjugation process.

The molecular size may be determined by gel filtration on soft columns or by HPSEC using refractive index alone, or in combination with laser light scattering (e.g. MALLS) (*34*, *41*).

A.3.2 ***Control of the carrier protein***

A.3.2.1 *Microorganisms and culture media for production of carrier protein*

Microorganisms to be used for the production of the carrier protein should be grown in media free from substances likely to cause toxic or allergic reactions in humans. If any materials of animal origin are used in seed preparation or preservation or in production, they should comply with the guidance given in the *Guidelines on Transmissible Spongiform Encephalopathies in Relation to Biological and Pharmaceutical Products* (*31*) and should be approved by the national regulatory authority.

Production should be based on a seed lot system with the strains identified by a record of their history and of all tests made periodically to verify strain characteristics. Consistency of growth of the microorganisms used should be demonstrated by monitoring the growth rate, pH and final yield of appropriate protein(s).

A.3.2.2 *Characterization and purity of the carrier protein*

Potentially there are many proteins that could be used as carriers in pneumococcal conjugate vaccines. The principal characteristics of the carrier protein should be that it is safe and, in the conjugate, elicits a T-cell dependent immune response against the polysaccharide. Test methods used to characterize such proteins, to ensure that they are non-toxic and to determine their purity and concentration, should be approved by the national regulatory authority.

Proteins and purification methods that might be used include:

1. *Tetanus or diptheria toxoid*. This must satisfy the relevant requirements p ublished by WHO (*42*) and be of high purity (*43*).
2. *Diphtheria CRM 197 protein*. This is a non-toxic mutant of diphtheria toxin, isolated from cultures of *Corynebacterium diphtheriae* C7/β197 (*44*). Protein purity should be greater than 90% as determined by an appropriate method. When produced in the same facility as diphtheria toxin, methods must be in place to distinguish the CRM 197 protein from the active toxin.

The protein carrier should also be characterized. The identity may be determined serologically. Physicochemical methods that may be used to characterize protein include sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE), isoelectric focusing, high-performance liquid chromatography (HPLC), amino acid analysis, amino acid sequencing, circular dichroism, fluorescence spectrophotometry (fluorimetry), peptide mapping and mass spectrometry as appropriate (*34*).

A.3.3 ***Control of monovalent bulk conjugates***

There are a number of possible conjugation methods that might be used for vaccine manufacture; all involve multi-step processes. Both

the method and the control procedures used to ensure the reproducibility, stability and safety of the conjugate should be established for licensing. The derivatization and conjugation process should be monitored by analysis for unique reaction products or by other suitable means. The conditions used in the conjugation process may affect the structure of the polysaccharide chain by causing the loss of labile substituents. Unless the combination of tests used to characterize the bulk monovalent conjugates confirm that the structure is maintained, a test to demonstrate identity of the intact polysaccharide should be performed.

Residual activated functional groups potentially capable of reacting in vivo may be present following the conjugation process. The manufacturing process should be validated to show that the activated functional groups do not remain at the conclusion of the manufacturing process and any residual groups are below a limit approved by the national regulatory authority.

After the conjugate has been purified, the tests described below should be performed in order to assess consistency of manufacture. The tests are critical for assuring lot-to-lot consistency.

A.3.3.1 *Identity*

A test should be performed on the monovalent bulk to verify its identity. The method should be validated to show that it distinguishes the desired monovalent material from all other polysaccharides and conjugates produced at that manufacturing site.

A.3.3.2 *Residual reagents*

The conjugate purification procedures should remove residual reagents used for conjugation and capping. The removal of reagents and reaction by-products such as cyanide, 1-ethyl-3,3-(3-dimethylaminopropyl)-carbodiimide (EDAC) and others, depending on the conjugation chemistry, should be confirmed by suitable tests or by validation of the purification process.

A.3.3.3 *Polysaccharide–protein ratio and conjugation markers*

For each batch of the bulk conjugate of each serotype, the ratio of polysaccharide to carrier protein should be determined as a marker of the consistency of the conjugation chemistry. For each conjugate, the ratio should be within the range approved for that particular conjugate by the national regulatory laboratory and should be consistent with vaccine shown to be effective in clinical trials.

For pneumococcal conjugate vaccines the ratio is typically in the range of 0.3–3.0 but varies with the serotype. The ratio can be determined either by independent measurement of the amounts of protein and polysaccharide

present (corrected for unbound protein and unbound polysaccharide), or by methods that give a direct measure of the ratio. Methods include ^{1}H nuclear magnetic resonance spectroscopy or the use of HPSEC with dual monitoring (e.g. refractive index and ultraviolet, for total material and protein content, respectively).

If the chemistry of conjugation results in the creation of a unique linkage marker (e.g. a unique amino acid), each batch of the bulk conjugate of that serotype should be assessed to quantify the degree of substitution of the carrier protein by covalent reaction with the modified pneumococcal polysaccharide.

The structural complexity and structural differences between the pneumococcal serotypes are such that in most cases a simple conjugation marker will not be able to be identified.

A.3.3.4 *Capping markers*

Each batch should be shown to be free of activated functional groups on either the chemically modified polysaccharide or carrier protein. Alternatively, the product of the capping reaction can be monitored, or the capping reaction can be validated to show removal of unreacted functional groups. Validation of the manufacturing process during vaccine development can eliminate the need to perform this analysis for routine control.

A.3.3.5 *Conjugated and unbound (free) polysaccharide*

Only the pneumococcal polysaccharide that is covalently bound to the carrier protein, i.e. conjugated polysaccharide, is immunologically important for clinical protection. Each batch of conjugate should be tested for unbound or free polysaccharide in order to ensure that the amount present in the purified bulk is within the limits agreed by the national regulatory authority based on lots shown to be clinically safe and efficacious.

Methods that have been used to separate unbound polysaccharide prior to assay, that are potentially applicable to pneumococcal conjugates, include hydrophobic chromatography, acid precipitation, precipitation with carrier-protein-specific antibodies, gel filtration and ultrafiltration. The amount of unbound polysaccharide can be determined by specific chemical or immunological tests, or by HPAEC after hydrolysis.

A.3.3.6 *Protein content*

The protein content of the conjugate should be determined by means of an appropriate validated assay and should comply with limits for the particular product. Each batch should be tested for conjugated and unbound protein.

If possible, the unconjugated protein should also be measured. Appropriate methods for the determination of conjugated and unconjugated protein include HPLC or capillary electrophoresis.

A.3.3.7 *Molecular size distribution of polysaccharide–protein conjugate*

The molecular size of the polysaccharide–protein conjugate is an important parameter in establishing consistency of production and in studying stability during storage.

The relative molecular size of the polysaccharide–protein conjugate should be determined for each bulk using a gel matrix appropriate to the size of the conjugate. The method should be validated with an emphasis on having sufficient specificity to distinguish the polysaccharide–protein conjugate from other components that may be present, e.g. unbound protein or polysaccharide. The size-distribution specifications will be vaccine-specific and should be consistent with lots shown to be immunogenic in clinical trials.

> Typically the size may be determined by gel filtration on Sepharose CL-2B, or by HPSEC on an appropriate column. Because the polysaccharide–protein ratio is an average value, characterization of this ratio over the conjugates with their size distribution (e.g. by dual monitoring of the column eluent) can be used to provide further proof of manufacturing consistency (*46*).

A.3.3.8 *Sterility*

The bulk purified conjugate should be tested for bacterial and mycotic sterility in accordance with the recommendations of Part A, sections 5.1 and 5.2, of the revised Requirements for the Sterility of Biological Substances (*47*) or by a method approved by the national regulatory authority. If a preservative has been added to the product, appropriate measures should be taken to prevent it from interfering with the test.

A.3.3.9 *Specific toxicity of carrier protein*

The bulk conjugate should be tested for the absence of specific toxicity of the carrier protein where appropriate (e.g. when tetanus or diphtheria toxoids have been used). Absence of specific toxicity of the carrier protein may also be assessed through validation of the production process.

A.3.3.10 *Endotoxin content*

To ensure an acceptable level of endotoxin in the final product, the endotoxin content of the monovalent bulk may be determined and shown to be within acceptable limits agreed by the national regulatory authority.

A.3.4 ***Final bulk***

A.3.4.1 *Preparation*

To formulate the final bulk, monovalent conjugate bulks may be mixed together and an adjuvant, a preservative and/or stabilizer is

added before final dilution. Alternatively, the monovalent conjugate bulks may be adsorbed to adjuvant individually before mixing them to formulate the final vaccine.

A.3.4.2 *Sterility*

Each final bulk should be tested for bacterial and mycotic sterility as indicated in section. A.3.3.7.

A.3.5 *Filling and containers*

The recommendations concerning filling and containers given in Annex 1, Section 4 of Good Manufacturing Practices for Biological Products (*39*) should be applied (*29*).

A.3.6 *Control tests on final product*

A.3.6.1 *Identity*

An identity test should be performed that demonstrates that all of the intended pneumococcal polysaccharide serotypes are present in the final product, unless this test has been performed on the final bulk.

> A serological test, using antibodies specific for the purified polysaccharide may be used.

A.3.6.2 *Sterility*

The contents of final containers should be tested for bacterial and mycotic sterility as indicated in section A.3.3.8.

A.3.6.3 *Pneumococcal polysaccharide content*

The amount of each pneumococcal polysaccharide in the final containers should be determined, and shown to be within the specifications agreed by the national regulatory authority.

> The conjugate vaccines produced by different manufacturers differ in formulation. A quantitative assay for each of the pneumococcal polysaccharides in the final container should be carried out. The assays used are likely to be product-specific and might include chromatographic or serological methods. Immunological assays such as rate nephelometry (*48*) or ELISA inhibition may be used.

A.3.6.4 *Residual moisture*

If the vaccine is freeze-dried, the average moisture content should be determined by methods accepted by the national regulatory authority. Values should be within limits for the preparations shown to be adequately stable in the stability studies of the vaccine.

> The test should be performed on 1 vial per 1000 up to a maximum of 10 vials but on no less than 5 vials taken at random from throughout the final lot. The average residual moisture content should generally be no greater than 2.5% and no vial should be found to have a residual moisture content of 3% or greater.

A.3.6.5 *Endotoxin content*

The vaccine in the final container should be tested for endotoxin content by a *Limulus* amoebocyte lysate (LAL) test. Endotoxin content or pyrogenic activity should be consistent with levels found to be acceptable in vaccine lots used in clinical trials and approved by the national regulatory authority.

A.3.6.6 *Adjuvant content*

If an adjuvant has been added to the vaccine, its content should be determined by a method approved by the national regulatory authority. The amount and nature of the adjuvant should be agreed with the national regulatory authority. If aluminium compounds are used as adjuvants, the amount of aluminium should not exceed 1.25 mg per single human dose.

A.3.6.7 *Preservative content*

The manufacturer has a choice of possible preservatives. Consideration should be given to the stability of the chosen preservative and possible interactions between the vaccine components and the preservative. If a preservative has been added to the vaccine, the content of preservative should be determined by a method approved by the national regulatory authority. The amount of preservative in the vaccine dose should be shown not to have any deleterious effect on the antigen or to impair the safety of the product in humans. The preservative and its concentration should be approved by the national regulatory authority.

A.3.6.8 *General safety test (innocuity)*

The requirement to test lots of pneumococcal conjugate vaccine for unexpected toxicity (abnormal toxicity) should be agreed with the national regulatory authority.

> Such a test may be omitted for routine lot release once consistency of production has been well established to the satisfaction of the national regulatory authority and when good manufacturing practice is in place.

A.3.6.9 *pH*

If the vaccine is a liquid preparation, the pH of each final lot should be tested and shown to be within the range of values found for vaccine lots shown to be safe and effective in the clinical trials and in stability studies. For a lyophilized preparation, the pH should be measured after reconstitution with the appropriate diluent.

A.3.6.10 *Inspection of final containers*

Each container in each final lot should be inspected visually (manually or with automatic inspection systems), and those showing abnor-

malities, lack of integrity and, if applicable, clumping or the presence of particles should be discarded.

A.4 Records

The recommendations in section 8 of Good manufacturing practices for biological products (*39*, Annex 1) should be applied (*29*).

A.5 Retained samples

The recommendations in section 9.5 of Good manufacturing practices for biological products (*39*, Annex 1) should be applied (*29*).

A.6 Labelling

The recommendations in section 7 of Good manufacturing practices for biological products (*39*, Annex 1) should be applied with the addition of the following (*29*).

The label on the carton or the leaflet accompanying the container should indicate:

— the pneumococcal serotype and carrier protein present in each single human dose;
— the amount of each conjugate present in a single human dose;
— the temperature recommended during storage and transport;
— if the vaccine is freeze-dried, that after its reconstitution it should be used immediately unless data have been provided to the licensing authority that it may be stored for a limited time; and
— the volume and nature of the diluent to be added in order to reconstitute a freeze-dried vaccine, specifying that the diluent should be supplied by the manufacturer and approved by the national regulatory authority.

A.7 Distribution and transport

The recommendations in section 8 of Good manufacturing practices for biological products (*39*, Annex 1) should be applied (*29*).

A.8 Stability, storage and expiry date

A.8.1 *Stability testing*

Adequate stability studies form an essential part of the vaccine development process. The stability of the vaccine in its final form and at the recommended storage temperatures should be demonstrated to the satisfaction of the national regulatory authority with final containers from at least three lots of final product made from different independent bulk conjugates.

Given the complexity of these multivalent vaccines, other approaches may be used, with the approval of the national regulatory authority.

The polysaccharide component of conjugate vaccines may be subject to gradual hydrolysis at a rate that may vary depending upon the type of conjugate, the type of formulation or adjuvant, the types of excipient and conditions of storage. The hydrolysis may result in reduced molecular size of the pneumococcal polysaccharide component, a reduction in the amount of the polysaccharide bound to the protein carrier and in a reduced molecular size of the conjugate.

The structural stability of the oligosaccharide chains and of the protein carrier vary between different conjugate vaccines.

Tests should be conducted before licensing to determine the extent to which the stability of the product has been maintained throughout the proposed validity period. The vaccine should meet the specifications for final product up to the expiry date.

Molecular sizing of the final product may be carried out to ensure the integrity of the conjugate. The antigen content of each serotype conjugate may be determined by a quantitative serological assay.

The desorption of antigen from aluminium-based adjuvants, if used, may take place over time. The level of adsorption should be shown to be within limits agreed by the national regulatory authority, unless data are available to show that the immunogenicity of the final product is not dependent upon adsorption of the antigen to the adjuvant.

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

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

The statements concerning storage temperature and expiry date appearing on the label should be based on experimental evidence, which should be submitted for approval to the national regulatory authority.

A.8.2 *Storage conditions*

Storage conditions should be based on stability studies and approved by the national regulatory authority.

Storage of both liquid and freeze-dried vaccines at a temperature of 2–8 °C has been found to be satisfactory. The stability of pneumococcal conjugate components varies with serotype of the capsular polysaccharide.

A.8.3 *Expiry date*

The expiry date should be approved by the national regulatory authority and based on the stability of the final product as well as the results of the stability tests referred to in section A.8.1.

Part B. Requirements for national regulatory authorities

B.1 General

The general recommendations for control laboratories contained in the Guidelines for National Authorities on Quality Assurance for Biological Products (*29*) should be applied.

B.2 Official release and certification

A vaccine lot should be released only if it fulfils national requirements and/or Part A of these Recommendations.

A statement signed by the appropriate official of the national regulatory authority should be provided at the request of the manufacturing establishments and should certify that the lot of vaccine in question satisfies all national requirements as well as Part A of these Recommendations. The certificate should state the number under which the lot was released by the national controller, and the number appearing on the labels of the containers. Importers of pneumococcal conjugate vaccines should be given a copy of the official national release document. The purpose of the certificates is to facilitate the exchange of vaccines between countries.

B.3 Reactogenicity and immunogenicity of vaccine in humans

The national regulatory authority should satisfy itself that adequate control of the pneumococcal conjugate vaccine has been achieved. Clinical data supporting consistency of vaccine production should be obtained prior to registration of the product. Several different lots of the product should be used during the clinical studies and shown to give similar immune responses. Such studies may need to be repeated if changes in production are made, or when the pneumococcal conjugate is intended to be part of a new combination vaccine formulation. The national regulatory authority should ensure that the studies include an adequate number of subjects to provide statistically valid data on reactivity and immunogenicity. The pneumococcal conjugate vaccines are manufactured from purified components by a clearly defined chemical process. Any changes in production or formulation of the vaccine should be reported to the national regulatory authority,

which will decide whether additional clinical data are required on a case-by-case basis. Such a review should take into account the likelihood of such changes affecting the quality, the consistency, the structural integrity and the immunogenicity of the product, and consider the possible cumulative effect of multiple modifications that individually may be regarded as minor.

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Appendix

Serological criteria for evaluation and licensure of new pneumococcal conjugate vaccine formulations for use in infants

The lack of a definitive serological correlate of protection and the multiplicity of antigens involved, especially because the clinical efficacy of several of the individual serotypes represented in the only licensed vaccine has not been established, have been an obstacle for licensure of new formulations or combinations of pneumococcal conjugate vaccines.

WHO undertook a series of consultations to develop serological criteria for the evaluation and licensure of new formulations, combinations or different vaccination schedules for pneumococcal conjugate vaccines. At a consultation held in Alaska in May 2002, a preliminary analysis of data from the efficacy trial in northern California was presented. The results of the analysis showed that a threshold antibody concentration for protection against invasive disease could be estimated using a few simplifying assumptions, and the following relationship between the point estimate of clinical efficacy (VE) and a protective antibody concentration:

$$VE = 1 - \frac{\text{Probability of disease in Vax group}}{\text{Probability of disease in control group}}$$

$$\therefore VE = 1 - \frac{\text{\% of Vax subjects with [Ab]} < Ab_{protective}}{\text{\% of control subjects with [Ab]} < Ab_{protective}}$$

where Vax group is the vaccinated group and [Ab] is concentration of antibody.

The threshold antibody concentration of 0.20 µg/ml thus derived was supported by a number of other observations. These included:

— the threshold corresponded with the threshold opsonophagocytic antibody titre of 1:8;
— it predicted age-specific disease rates;
— it was consistent with available data from passive immunization using bacterial polysaccharide immune globulin (BPIG) to prevent pneumococcal otitis media and invasive pneumococcal disease;
— it appeared to discriminate clearly between vaccinees who had received conjugate and controls in immunogenicity studies;

— infants with antibody above the threshold showed evidence of priming and a booster response to a subsequent dose of vaccine. The rationale for selecting the threshold antibody concentration is described in more detail in the proceedings of a WHO meeting (*1*).

On the recommendations arising from this consultation, this analysis was repeated using the pooled immunogenicity and efficacy data from all the completed trials of pneumococcal conjugate vaccines to narrow the confidence limits around the point-estimate of efficacy and to allow additional populations to be represented. The threshold antibody concentration derived from the pooled analysis using the methods described previously was 0.35 µg/ml. Opsonophagocytic antibody titres were available from two of the three studies and analysis of the data showed that antibody concentrations in the range of 0.20–0.35 µg/ml correlated best with an opsonophagocytic antibody titre of 1:8, which in turn correlates best with protective efficacy. The results of the pooled analysis were presented at a second consultation held in June 2003, which was attended by experts in pneumococcal epidemiology and vaccine evaluation, as well as representatives of regulatory agencies. On the basis of the data presented at this consultation, the criteria listed in the following section were recommended for use as a relevant value to establish non-inferiority of a new vaccine when compared to a vaccine against invasive pneumococcal disease that is already licensed. These criteria should not be used to evaluate vaccines against other clinical end-points, e.g. pneumonia and otitis media. It should be noted that immunological responses to pneumococcal conjugate vaccines may vary significantly by population, and a new candidate vaccine shown to be inferior to the licensed vaccine in one population may nevertheless be non-inferior in a second population and may therefore be acceptable in the second population.

The development of standardized assays to evaluate serological responses to new pneumococcal conjugate vaccines has been long pursued by WHO through many consultations. Agreement was reached at a WHO Workshop held in Geneva in 2000 to select one well-characterized pneumococcal ELISA protocol as a reference or benchmark assay for laboratories evaluating serological responses to pneumococcal vaccines and to make the link with the pivotal clinical protection studies carried out during the licensure of the first seven-component conjugate vaccine. Two WHO reference laboratories have been established to help other laboratories set up and standardize their own pneumococcal ELISA and to ensure the comparability and acceptability of the serological data. These reference laboratories

are located at the Institute of Child Health, London, England , and at the Bacterial Respiratory Pathogen Reference Laboratory, The University of Birmingham, Alabama, USA. The detailed protocol for the pneumococcal ELISA, developed with technical assistance from Wyeth Vaccines, Rochester, New York, USA, is available through the Internet site at: www.vaccine.uab.edu.

Primary end-point

The following criteria are recommended for use as the primary end-point for demonstration of non-inferiority against a registered vaccine:

- IgG antibody concentration, as measured by ELISA, in sera collected 4 weeks after a three-dose primary series is considered to be the optimal primary end-point and main licensing parameter.
- A single threshold or reference antibody concentration is recommended for use for all pneumococcal serotypes. A reference antibody concentration of 0.35 μg/ml, that has been determined through a pooled analysis of data from the efficacy trials with invasive disease end-points that have been completed to date, is recommended (*1*, *2*). This threshold does not necessarily predict protection in an individual subject.
- The reference value is defined on the basis of data obtained using ELISA without pre-adsorption with serotype 22F. Antibody concentrations determined using an alternative method will need to be bridged to this method to derive an equivalent threshold concentration. It is recommended that the assay used be calibrated against a reference assay (*3*).
- Direct clinical comparison of the registered (established) vaccine with the new one is the preferred method for evaluating new vaccine formulations.
- The percentage of responders (those in whom post-immunization antibody concentration is above the threshold) should be used as the criterion to determine non-inferiority.
- For the serotypes present in a registered vaccine, the percentage of responders to each serotype in the new formulation or combination should be compared with the percentage of responders to the same serotype in the registered vaccine in the same population. Non-inferiority to antibody response for each of the serotypes in the registered vaccine is desirable, but not an absolute requirement. Registration of products in which one or more serotypes do not meet non-inferiority criteria would have to be decided on an individual basis.

- Serotypes not contained in a registered formulation may be evaluated for non-inferiority to the aggregate response to the serotypes in the registered vaccine. Failure of one or more new serotypes to meet this criterion may be considered on an individual basis (see example given above).

Additional criteria that must be met to support registration

In addition to showing non-inferiority with respect to the primary end-point, additional data to demonstrate the functional capacity of the antibody and induction of immunological memory in a subset of the sera are required for registration.

Functional antibodies

- Opsonophagocytic activity as measured by opsonophagocytic assay after a three-dose priming series is required to demonstrate the functionality of antibodies.
- The method used to demonstrate opsonophagocytic activity should be comparable to the reference assay (*4*).

Immunological memory

- Evidence of memory should be demonstrated. One possible method is to administer a booster dose of pneumococcal polysaccharide vaccine and to compare concentrations between age-matched unprimed and primed individuals; data from non-concurrent controls may be sufficient for the purposes of comparison.
- A full dose of polysaccharide vaccine should be used at this stage because the use of a reduced dose of the polysaccharide vaccine as a booster has not been sufficiently tested.
- Avidity of antibodies is also a useful marker for immunological memory.

The following reference reagents and quality control and reference materials are available for the serological assays (also available at: http://www.vaccine.uab.edu/information.htm) (see also Tables A1, A2a and A2b).

Pneumococcal ELISA calibration sera: To obtain an aliquot of each of the 12 sera please contact (email preferred):

Dr David Goldblatt
Email: d.goldblatt@ich.ucl.ac.uk
WHO Pneumococcal Reference Laboratory
Institute of Child Health

Table A.1
Materials for pneumococcal assays

Name	Provider	Address	Fax Number	E-mail/web site
C-polysaccharide	Statens Serum Institute	5 Artillerivej DK-2300 Copenhagen S Denmark	45-3268-3167	Serum@ssi.dk
Capsular polysaccharide	American Type Culture Collection (ATCC)	10801 University Boulevard Manassas, VA, 20110-2209 USA	1-703-365-2750	http://www.atcc.org/
89-SF (standard)	Dr Carl Frasch	Center for Biologics Evaluation and Research, US Food and Drug Administration Bethesda, MD USA	1-301-402-2776	Frasch@cber.fda.gov
ELISA Calculation program	Mr Brian Plikaytis	Centers for Disease Control and Prevention Atlanta, GA USA	1-404-639-2780	http://www.cdc.gov/ncidod/dbmd/bimb/elisa.htm

30 Guilford Street
London WC1N 1EH, England

Sera are stored at, and will be distributed by, the National Institute of Biological Standards and Control, Potters Bar, Herts., England.

Table A.2a
Values assigned to 89-SF (reference serum)

Type[a]	Total antibody (mg/l)	IgG (mg/l)[b]	IgM (mg/l)	IgA (mg/l)
1	10.7	6.3	1.7	1.4
3	7.9	2.4	0.6	4.3
4	7.0	4.1	1.4	1.2
5	10.0	5.8	4.2	1.2
6B	24.3	16.9	3.0	1.5
7F	7.3	5.2	1.9	1.1
9V	10.2	6.9	1.6	1.7
14	37	27.8	1.2	1.9
18C	6.7	4.5	1.3	0.8
19F	18.8	13.0	3.2	2.02
23F	11.9	8.1	0.7	1.3

[a] Serotypes 1 and 5 are included in a typical 9-valent vaccine and serotypes 3 and 7F are included in a typical 11-valent vaccine.
[b] Source: reference 5, confirmed by CBER, USFDA. The value assigned for 19F is subject to further confirmation.

Table A.2b
Values assigned to 89-SF for additional serotypes

Type	Total Ig (mg/l)	IgG (mg/l)	IgM (mg/l)	IgA (mg/l)
2	21.4	12.2	5.1	3.9
8	11.5	5.1	2.0	2.0
9N	12.7	7.8	2.4	2.1

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Annex 3
Recommendations for the production and control of influenza vaccine (inactivated)

Recommendations published by WHO are intended to be scientific and advisory in nature. The parts of each section printed in type of normal size have been written in a form, such that, should a national regulatory authority so desire, they may be adopted as they stand as definitive national requirements or used as the basis of such requirements. Those parts of each section printed in small type are comments and recommendations for guidance for those manufacturers and national regulatory authorities which may benefit from additional information.

It is recommended that modifications be made only on condition that the modifications ensure that the vaccine is at least as safe and efficacious as that prepared in accordance with the recommendations set out below. In order to facilitate the international distribution of vaccine made in accordance with these recommendations, a summary protocol for the recording of results of the tests is given in Appendix 1.

The terms "national regulatory authority" and "national control laboratory" as used in these recommendations, always refer to the country in which the vaccine is manufactured.

Introduction

Influenza is a significant cause of morbidity and mortality and has a major social and economic impact throughout the world. During major epidemics many people require medical treatment or hospitalization. Excess mortality often accompanies influenza epidemics, the vast majority of those affected being elderly. Because the elderly constitute the most rapidly increasing sector of the population in many countries, the epidemiology of influenza can be expected to change accordingly, especially in the developed countries. At present, the only means of influenza prophylaxis generally available is vaccination.

In 1967, a group of experts formulated requirements for inactivated influenza vaccine and these were published as an annex to the twentieth report of the Expert Committee on Biological Standardization (*1*). During the following 5 years, technical developments in the purification of the virus suspensions from which vaccines were made, as well as in the measurement of the virus content, were such that the potency of whole virus vaccines could be expressed in international units. Accordingly, an addendum to the requirements was annexed to the twenty-fifth report of the Expert Committee on Biological Standardization (*2*). In its twenty-ninth report (*3*), the Committee recognized that technical developments had completely altered the method of measurement of the haemagglutinin content of the vaccines and that the International Reference Preparation of Influenza Virus Haemagglutinin (Type A) established in 1967 was no longer appropriate for controlling the haemagglutinin content of inactivated influenza vaccines because it no longer represented the haemagglutinin of the prevalent strains. Accordingly, the International Reference Preparation was withdrawn, and the Committee recommended that the requirements for inactivated influenza vaccine should be revised. Revised requirements were approved by the WHO Expert Committee on Biological Standardization in 1978 (*4*) and modified in 1990 (*5*).

Since 1990, there have been significant new developments in methods of influenza vaccine production resulting from: increased development of mammalian cell lines for vaccine production (*6*); increased experience in use of adjuvants; and rapid development of reverse genetics technologies for generation of vaccine viruses. There has also been considerable effort directed to pandemic planning to ensure that safe, effective vaccines can be quickly produced in response to a pandemic emergency. Consequently it has become necessary to revise the requirements to reflect these new developments. In accordance

with current WHO policy, the revised document is renamed as "Recommendations".

General considerations

Inactivated influenza vaccines have been in widespread use for nearly 60 years. The efficacy of immunization has varied according to circumstances, but protection rates of 75–90% have been reported. Differences in protective efficacy may result from continuing antigenic variation in the prevalent epidemic strains. Because of this variation, the composition of inactivated influenza virus vaccine, unlike that of most viral vaccines, must be kept constantly under review. Accordingly, WHO publishes recommendations concerning the strains to be included in the vaccine twice annually.

Influenza vaccines usually contain one or more influenza A viruses. However, because influenza A viruses undergo frequent and progressive antigenic drift in their haemagglutinin and neuraminidase antigens, vaccines containing formerly prevalent viruses are expected to be less protective against virus variants showing antigenic drift than against the homologous virus. When a new subtype of influenza A virus bearing new haemagglutinin (and neuraminidase) antigen(s) appears, it is likely that vaccine containing the antigen(s) of the influenza A subtype(s) formerly prevalent will be ineffective, so that a vaccine containing the new pandemic virus will be required.

Changes in the structure of the haemagglutinin and neuraminidase molecules, which result in changes in antigenicity as new epidemic strains appear, involve surface residues in the region of the molecule furthest from the viral envelope. Prediction of future variations is not possible because the mechanism of selection of antigenic variants, antigenic drift, is not known and several evolutionary pathways appear possible. Antigenic shift (i.e. the appearance of epidemic strains with a new haemagglutinin subtype) is also unpredictable.

Antigenic drift in influenza B virus strains is less frequent than that in the A strains and antigenic shift is unknown. Although distinct lineages of influenza B may occasionally co-circulate, it is usual for influenza vaccines to contain only one influenza B strain.

In addition to antigenic drift and shift, there is another type of variation among influenza viruses, namely the preferential growth of certain virus subpopulations in different host cells in which the virus is cultivated. Influenza viruses grown in embryonated eggs often exhibit antigenic and biological differences from those isolated and

maintained in mammalian cells. Sequence analysis of the haemagglutinin gene of such variants has shown that, typically, virus grown in mammalian cells differs from virus from the same source cultivated in eggs only by the substitution of a single amino acid in the haemagglutinin molecule.

The WHO Expert Committee on Biological Standardization recommended in its twenty-ninth report (*3*) that the potency of influenza vaccines should be expressed in μg of haemagglutinin per ml (or dose), as determined by suitable immunodiffusion methods. In order to standardize these methods, reference antigen (calibrated in μg of the haemagglutinin per ml) and specific anti-haemagglutinin serum, suitable for use in the assay of the haemagglutinin content of each component of inactivated vaccines, are prepared and distributed by reference laboratories (Appendix 2). A new reference antigen and antiserum is prepared each time it is necessary to introduce a new virus strain into the vaccines, and these are standardized by international collaborative study.

Over the past 20 years there have been many clinical trials of whole virus, split and subunit influenza vaccines. This has led to the generally accepted view that one dose of vaccine containing 15 μg of haemagglutinin per strain per dose, will stimulate haemagglutination-inhibition antibody levels consistent with immunity in most primed individuals (*7*).

There has been much progress in developing influenza vaccines with adjuvant and some such vaccines are now licensed. The main issues for vaccine quality are: demonstration of compatibility of the adjuvant with the antigenic components of the vaccine; proof of consistent association with vaccine antigens (if appropriate) at time of production and throughout shelf-life; effect of adjuvant on vaccine potency assays; and biochemical purity of adjuvant.

There is a long history of safety for egg-grown vaccines. However it is known that influenza viruses cultivated in eggs can be contaminated with other viral agents and there has been a recent example of contamination of a candidate pandemic vaccine virus with avian adenovirus. These recommendations have therefore been revised in view of the findings with egg-grown viruses, the increasing use of mammalian cells for virus isolation and vaccine production, and the improved methods for detecting extraneous agents.

Influenza pandemic alerts occurred in 1997 (H5N1 virus), 1999 (H9N2 virus) and 2003 (H5N1 and H7N7 viruses), when avian influenza viruses caused serious illness and, on occasion, death in humans.

Experience gained from these events has illustrated that different strategies for the production and clinical use of vaccine may be needed in response to a pandemic.

- It may be necessary to generate a vaccine virus from a highly pathogenic virus by use of reverse genetics.
- Initially, reference reagents for testing vaccine potency may not be available;
- A vaccine with an adjuvant may be desirable.
- Monovalent vaccine may be preferred to conventional trivalent vaccine.
- Whole virus vaccine may be preferred to a split or subunit preparation.
- A different dosing strategy may be needed (i.e. two doses).

It is important to develop WHO recommendations for production and quality control of the vaccine that reflect the special needs of a pandemic.

Although the technology is still under development, the use of reverse genetics for vaccine virus development is likely to affect interpandemic and pandemic vaccines alike. This technology involves transfecting mammalian cells with plasmids coding for influenza virus genes in order to produce a virus reassortant. Production of reassortants by reverse genetics is similar in concept to traditional methods, but there are some quality issues which should be taken into account.

- The influenza virus haemagglutinin and neuraminidase genes may be derived from a variety of sources (an egg isolate, an isolate in cells approved or not approved for human vaccine production, virus present in clinical specimens).
- The reassortant virus will have been generated in mammalian cells.
- In some countries, reassortants produced by reverse genetics may be classified as "genetically modified organisms" and vaccine production should comply with national Contained Use regulations (although the final inactivated product will not be a genetically modified organism).

Part A. Manufacturing recommendations

A.1 Definitions

A.1.1 *Proper name*

The proper name shall be "influenza vaccine (whole virion, inactivated)" or "influenza vaccine (split virion, inactivated)",

"influenza vaccine (surface antigens, inactivated)" or "influenza vaccine (inactivated, adjuvanted)" translated into the language of the country of use.

The use of the proper name should be limited to vaccines that satisfy the recommendations specified below.

A.1.2 *Descriptive definition*

Influenza vaccine is a sterile, aqueous suspension of a strain or strains of influenza virus, type A or B, or a mixture of these two types, which have been grown individually in embryonated hen's eggs or in mammalian cells. Four types of influenza vaccine are available:

(i) a suspension of whole virus particles inactivated by a suitable method;
(ii) a suspension treated so that the virus particles have been partially or completely disrupted by physicochemical means (split vaccine);
(iii) a suspension treated so that the preparation consists predominantly of haemagglutinin and neuraminidase antigens (subunit vaccine);
(iv) a suspension of inactivated influenza virus particles, split or subunit components formulated with an adjuvant.

The preparation should satisfy all the requirements formulated below.

A.1.3 *Choice of vaccine strain*

The World Health Organization reviews the world epidemiological situation twice annually and if necessary recommends new vaccine strain(s) in accordance with the available evidence.

Such strains, or those antigenically related to them, should be used in accordance with the regulations in force in the country concerned.

It is now common practice to use reassortant strains that give high yields of the appropriate surface antigens. Reassortant strains for vaccine production have the surface glycoproteins (haemagglutinin and neuraminidase) of the recommended reference virus and the internal proteins of a high-growth donor virus.

These recommendations shall also apply to the subsequent production and quality control of reassortant vaccine viruses produced by reverse genetics.

The passage history of the parent and reassortant virus strains should be approved by the national regulatory authority.

A.1.4 *Reference reagents*

WHO reference antigens for strain characterization of influenza virus are preparations that are antigenically representative of viruses isolated throughout the world. They may be obtained from one of the WHO Collaborating Centres for Reference and Research on Influenza (see Appendix 2).

Candidate influenza vaccine viruses are preparations antigenically representative of a virus strain likely to be included in a current vaccine. They may be wild-type viruses or reassortant viruses with surface antigens appropriate for the current recommendations for vaccine strains. They are distributed on demand when a new virus appears and the likelihood of its spreading throughout the world makes its inclusion in a vaccine desirable. These preparations may be obtained from one of the custodian laboratories listed in Appendix 2.

Antigen reagents for standardization of vaccine potency contain a calibrated quantity of haemagglutinin antigen of influenza virus measured in µg/ml. The calibrations are performed by international collaborative study using single radial immunodiffusion tests (*8*) with purified virus of known haemagglutinin antigen concentration. The reference antigen and antiserum reagents are used to calibrate the haemagglutinin content of inactivated influenza vaccines by an in vitro immunodiffusion test. These reference haemagglutinin antigens, together with the specific antihaemagglutinin sera, may be obtained for the purpose of such tests from one of the custodian laboratories listed in Appendix 2.

A.1.5 *Terminology*

Master seed lot: A quantity of virus, antigenically representative of a WHO-recommended strain, that has been processed at one time to assure a uniform composition and is fully characterized. It is used for the preparation of working seed lots. The master seed lot and its passage level are approved by the national authority.

Working seed lot: A quantity of fully characterized virus of uniform composition that is derived from a master seed lot by a number of passages that does not exceed the maximum approved by the national regulatory authority. The working seed lot is used for the production of vaccines.

Cell seed: A quantity of well-characterized cells of human or animal origin stored frozen in liquid nitrogen 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 or animal origin derived from the cell seed stored frozen in liquid nitrogen in aliquots of uniform composition, one or more of which may be used for the production of a manufacturer's working cell bank. The testing performed on a replacement master cell bank (derived from the same 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 (WCB): A quantity of cells of uniform composition derived from one or more ampoules of the master cell bank, which may be used for the production cell culture. In normal practice, a cell bank is expanded by serial subculture up to a passage number (or population doubling, as appropriate) selected by the manufacturer, at which point the cells are combined to give a single pool and preserved cryogenically to form the manufacturer's WCB. One or more of the ampoules from such a pool may be used for the production cell culture.

Production cell culture: A cell culture derived from one or more ampoules of the manufacturer's WCB and used for production of the live influenza virus.

Single harvest: A quantity of virus suspension derived from either a group of embryonated eggs or a culture of mammalian cells that were inoculated with the same virus working seed lot, incubated together and harvested together in one session.

Monovalent virus pool: A pool of a number of single harvests of a single virus strain processed at the same time.

Final bulk: The finished vaccine prepared from one or more monovalent pools present in the container from which the final containers are filled. It may contain one or more virus strains.

Final lot: A collection of sealed final containers that are homogeneous with respect to the risk of contamination during filling. A final lot must therefore have been filled in one working session from a single final bulk.

A.2 General manufacturing requirements

The general requirements for manufacturing establishments contained in good manufacturing practices for pharmaceuticals (*9*) and biological products (*10*) should apply to establishments manu facturing inactivated influenza vaccine, with the addition of the following:

Details of standard operating procedures for the preparation and testing of influenza vaccines adopted by a manufacturer together with evidence of appropriate validation of the production process, should be submitted for approval to the national regulatory authority. Proposals for modification of the manufacturing/control methods should also be submitted for approval by the national regulatory authority.

Personnel employed in the production and control facilities should be adequately trained and protected against accidental infection with influenza virus (*11*).

High levels of biological containment are likely to be required for the production of vaccines for use in pandemic or potential pandemic situations. WHO will provide advice where appropriate (*12*) and national or regional safety guidelines must be followed.

Standard operating procedures need to be developed for dealing with emergencies involving accidental spillage, leakage or other dissemination of influenza virus.

The areas where processing of inactivated influenza vaccine takes place shall be separate from those where work with live influenza virus is performed.

A.3 Production control

A.3.1 *Control of source materials*

A.3.1.1 *Eggs used for seed virus growth*

If the vaccine is to be produced in embryonated eggs, the eggs to be used should be from closed, specific-pathogen-free, healthy flocks.

> The flock should be monitored at regular intervals for specific agents. The agents monitored may include *Mycobacterium avium*, fowlpox virus, avian leukosis virus (ALV) and other avian retroviruses, Newcastle disease virus and other avian parainfluenza viruses, avian encephalomyelitis virus, infectious laryngotracheitis virus, avian reticuloendotheliosis virus, Marek's disease virus, infectious bursal disease virus, *Haemophilus paragallinarum*, *Salmonella gallinarum*, *Salmonella pullorum*, *Mycoplasma gallisepticum* and *Mycoplasma synoviae*.
>
> In some countries, all birds are bled when a colony is established, and thereafter 5% of the birds are bled each month. The resulting serum samples are screened for antibodies to the relevant pathogens. Any bird that dies should be investigated to determine the cause of death.

A.3.1.2 *Eggs used for vaccine production*

If the vaccine is to be produced in embryonated eggs, the eggs should be from healthy flocks, which are monitored by methods approved by local animal health authorities.

As large numbers of eggs are needed for vaccine production, it is not feasible to use eggs from specific-pathogen-free flocks.

Monitoring of flocks for avian influenza viruses is performed in some countries.

In both situations (production of vaccine seed and production of vaccine), the flock must not have been vaccinated with live Newcastle disease virus vaccine. It is also recommended that eggs be obtained from young birds.

In countries where use of live Newcastle disease vaccine is mandatory, vaccination should take place during the first few weeks of the chickens' life and well before the use of flocks for supply of eggs.

A.3.1.3 *Master cell bank and manufacturer's working cell bank*

If a cell line is used for the manufacture of influenza vaccines, it should be based on the cell bank system. The national regulatory authority should approve the master cell bank and should establish the maximum number of passages (or population doublings) by which the manufacturer's WCB is derived from the master cell bank and the maximum number of passages of the production cultures.

WHO has established a reference cell bank of Vero cells[1] characterized in accordance with the requirements produced in 1996 (*13*) as modified by Annex 4 of this report (*14*). This should not be considered as a master cell bank for direct use in vaccine production, but may be used to develop a master cell bank by thorough requalification.

A.3.1.3.1 ***Identity test***

The master cell bank should be characterized according to the WHO Requirements for the use of animal cells as in vitro substrates for the production of biologicals (*13*, *14*) as they relate to continuous cell lines, or to human diploid cells, as appropriate.

The manufacturer's WCB should be identified by means, inter alia, of biochemical (e.g. isoenzyme analysis), immunological and cytogenetic marker tests, and DNA fingerprinting, approved by the national regulatory authority.

A.3.1.4 *Cell culture medium*

Serum used for the propagation of cells should be tested to demonstrate freedom from bacteria, fungi and mycoplasmas, according to the requirements given in sections A.5.2 and A.5.3 of the revised

[1] Available to manufacturers on application to Quality Assurance and Safety of Biologicals: Vaccines and Biologicals, World Health Organization, 1211 Geneva 27, Switzerland.

Requirements for Biological Substances No. 6 (*15*) and from infectious viruses. Suitable tests for detecting viruses in bovine serum are given in Appendix 1 of the Recommendations for Poliomyelitis Vaccine (Oral) (*16*).

Where approved by the national regulatory authority, alternative tests for bovine viruses may be used.

As an additional monitor of quality, sera may be examined for freedom from phage and endotoxin.

Irradiation may be used to inactivate potential contaminant viruses.

The sources(s) of serum of bovine origin should be approved by the national regulatory authority. The serum should comply with current guidelines in relation to animal transmissible spongiform encephalopathies (*17*).

Human serum should not be used. If human albumin is used, it should meet the revised Requirements for Biological Substances No. 27 (Requirements for the Collection, Processing and Quality Control of Blood, Blood Components and Plasma Derivatives) (*18*), as well as current guidelines in relation to human transmissible encephalopathies (*17*).

Manufacturers are encouraged to explore the possibilities of using serum-free media for production of inactivated influenza vaccine.

Penicillin and other β-lactams should not be used at any stage of the manufacture.

Other antibiotics may be used at any stage in the manufacture, provided that the quantity present in the final product is acceptable to the national regulatory authority. Nontoxic pH indicators may be added, e.g. phenol red in a concentration of 0.002%. Only substances that have been approved by the national regulatory authority may be added.

Trypsin used for preparing cell cultures should be tested and found free of cultivable bacteria, fungi, mycoplasmas and infectious viruses, especially parvoviruses appropriate to the species of animal used. The methods used to ensure this should be approved by the national regulatory authority.

The source(s) of trypsin of bovine origin, if used, should be approved by the national regulatory authority. Bovine trypsin, if used, should comply with current guidelines in relation to animal transmissible spongiform encephalopathies (*17*).

A.3.1.5 *Virus strains*

Strains of influenza virus used in the production of inactivated influenza vaccine should be identified by historical records, which should

include information on the origin of the strains and their subsequent manipulation.

Only strains that have been isolated in embryonated hen's eggs, in cells derived from eggs, or in mammalian cells approved for human vaccine production under validated laboratory conditions should be used. The national regulatory laboratory should approve the virus strain. It is now common practice to use reassortant strains giving high yields of the appropriate surface antigens. However, it has been noted that antigenic changes may occur during the development of high-yielding reassortants, and the absence of such changes should be shown by haemagglutination-inhibition tests using antibodies to the haemagglutinin of the reassortant and of wild type-viruses.

Where reassortant strains are used, the parent high-yield strain and the method of preparing the reassortant should be approved by the national regulatory authority.

> Where reverse genetics is used to generate the reassortant vaccine virus, the influenza haemagglutinin and neuraminidase genes may be derived from a variety of sources (egg isolate, mammalian cell isolate or virus in clinical specimen). The haemagglutinin and neuraminidase genes are expected to be free of extraneous agents associated with the wild-type virus by virtue of the recombinant DNA technology employed. The cell substrate used for transfection to generate the reassortant virus should be approved for human vaccine production. The derivation of the reassortant virus should be approved by the national regulatory authority.
>
> Strains of virus suitable for manufacture of a vaccine for use in a pandemic or potential pandemic should be supplied by procedures agreed by the WHO Collaborating Centres for Reference and Research on Influenza and the custodian laboratories for supply of candidate strains (Appendix 1) and approved by the national regulatory authority.

If any materials of animal (non-avian) origin are used in production, they should comply with the guidance given in the report of a WHO Consultation on medicinal and other products in relation to human and animal transmissible spongiform encephalitis (*17*) and should be approved by the national regulatory authority.

> Reference strains for antigenic analysis may be obtained from the WHO Collaborating Centres for Reference and Research on Influenza, or other custodian laboratories (Appendix 2).

A.3.1.5.1 *Seed lot system*

The production of vaccine should be based on a seed lot system. Each seed lot should be identified as influenza virus of the appropriate strain by methods acceptable to the national regulatory authority (section A.1.3). The maximum number of passages between a master seed lot and a working seed lot should be approved by the national

regulatory authority. The vaccine should be not more than one passage from the working seed lot.

Each manufacturer should identify the haemagglutinin and neuraminidase antigens of the vaccine virus strains by suitable tests capable of detecting biologically significant variation, as well as cross-contamination during manipulation.

A.3.1.5.2 ***Tests on seed lots***

Either the master or working seed virus should be shown to be free from relevant extraneous agents by tests or procedures approved by the national regulatory authority in accordance with the requirements of Part A, sections 5.2 and 5.3, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (*15*).

Strategies to ensure freedom from extraneous agents in the final vaccine may involve a combination of testing the seed virus and validation of the production process, depending on the substrate used for production. For egg-derived vaccines, the emphasis should be on a validation process, whereas for cell-derived vaccines, the emphasis may be on a testing strategy. The national regulatory authority should approve the strategy chosen.

Validation strategy

The production process should be validated to demonstrate removal and/or inactivation of likely potential contaminating agents. Validation may be performed using appropriate model agents.

If removal or inactivation cannot be demonstrated for a potential contaminant, a testing strategy should be implemented.

Testing strategy

Cell-derived vaccine

The susceptibility of mammalian cells to various human pathogens should be taken into account and this information should be used in considering a list of potential human pathogens to be included in testing for extraneous agents in seed virus. Pathogens to be considered could include adenovirus, parainfluenza virus, respiratory syncytial virus, coronavirus, rhinovirus, enterovirus, *human herpesvirus* 4 (Epstein–Barr virus), herpes simplex virus, cytomegalovirus and mycoplasmas.

It is recognized that when a vaccine strain changes, there may be time constraints that make testing seed viruses for extraneous agents problematic, and the full results of such testing may not always be available before further processing. The use of rapid assays (e.g. multiplex polymerase chain reaction (PCR)) which could be applied within these time constraints is encouraged.

If an extraneous agent is detected in a seed virus and the mammalian cells used for production are shown to be susceptible to this agent, the seed virus should not be used for vaccine production.

If an extraneous agent is detected in a seed virus and the mammalian cells are not susceptible to the agent, steps should be in place to ensure that the contaminating agent is removed and/or inactivated by the production process. If removal or inactivation of the agent cannot be demonstrated, appropriate and specific downstream testing at the level of each inactivated monovalent bulk should be implemented to demonstrate that any contaminant identified in the seed virus is absent from the vaccine.

Egg-derived vaccine

A strategy for testing for specific potential contaminating agents may be needed if removal or inactivation of the agent by the production process cannot be demonstrated. The use of rapid tests (e.g. PCR) is encouraged. If the agent is detected, the seed virus should not be used for vaccine production.

The seed lot should be stored at a temperature lower than –60 °C, unless it is in the lyophilized form, in which case it should be stored at a temperature lower than –20 °C.

A.3.2 ***Production precautions***

The manufacture of inactivated influenza vaccines should follow the relevant guidance on good manufacturing practice (*10*, Annex 1) and quality assurance (*10*, Annex 2) for biological products with the addition of the following:

- for egg-derived vaccines, only allantoic and amniotic fluids may be harvested.
- β-Lactam antibiotics should not be used at any stage in the manufacture of the vaccine.

Minimal concentrations of other suitable antibiotics may be used.

If vaccines are produced by the splitting of the virus by chemical means, the splitting conditions and the concentration of the chemicals used should be approved by the national regulatory authority. If an adjuvant is used, the concentration of adjuvant should be approved by the national regulatory authority.

A.3.3 ***Production of monovalent virus pools***

A.3.3.1 *Single harvests*

For egg-derived vaccine, each strain of virus should be grown in the allantoic cavity of embryonated hen's eggs derived from healthy flocks. After incubation at a controlled temperature, both the allantoic and amniotic fluids may be harvested. For mammalian-cell derived vaccine, each strain of virus should be grown in cells approved for human vaccine production.

For both cell-derived and egg-derived vaccines, a number of single harvests of the same strain of virus may be combined to give a monovalent virus pool. Cell-derived monovalent virus pools should not be mixed with egg-derived monovalent virus pools.

A.3.3.2 *Inactivation of monovalent virus pools*

Time of inactivation. To limit the possibility of contamination, monovalent virus pools should be inactivated as soon as possible after their preparation. However, if delay is unavoidable, the temperature and duration of the storage should be validated with respect to bioburden and quality of the haemagglutinin and neuraminidase antigens.

Validation with respect to bioburden may be omitted for cell-culture-derived monovalent virus pools, with the agreement of the national regulatory authority.

Before monovalent virus pools are inactivated, samples shall be taken and tested for bacterial and fungal contamination. Limits for bioburden should be approved by the national regulatory authority.

Inactivation procedure. The virus in the monovalent virus pools should be inactivated by a method that has been demonstrated to be consistently effective in the hands of the manufacturer and has been approved by the national regulatory authority. For egg-derived vaccine, the inactivation process should also have been shown, to the satisfaction of the national regulatory authority, to be capable of inactivating avian leukosis viruses and mycoplasmas. If the virus pool is stored after inactivation, the temperature and duration of the storage should be validated. The inactivation procedure should have been shown to be capable of inactivating influenza viruses without destroying their antigenicity.

The usual storage temperature is 5 °C ± 3 °C.

Consideration should be given to investigating whether influenza virus inactivation also inactivates human or avian pathogens capable of becoming extraneous agents. These investigations may be performed using appropriate model agents e.g.

— *egg-derived vaccines:* avian leukosis virus, mycoplasma, avian adenovirus;
— *cell-derived vaccines:* poliovirus, human immunodeficiency virus, human adenovirus, parainfluenza virus, minute virus of mice.

If formalin (40% formaldehyde) or β-propiolactone (2-oxetanone) is used, the concentration by volume should not exceed 0.1% at any time during inactivation. Other suitable inactivating agents can also be used.

Consideration should be given to strategies to limit the entry into the manufacturing process of potential adventitious agents that may not be inactivated by the influenza inactivation conditions.

A.3.3.3 *Testing of control cells*

A cell sample equivalent to at least 500 ml of the cell suspension, at the concentration employed for vaccine production cultures, should be used to prepare control cell cultures. In countries with the technology for large-scale production, the national regulatory authority should determine the size of the sample of cells to be examined, the time at which the control cells should be taken from the production culture, and how the control cells are maintained.

These control cell cultures should be incubated for at least 2 weeks and should be examined during this period for evidence of cytopathic changes. For the test to be valid, not more than 20% of the control cell cultures may have been discarded for nonspecific, accidental reasons.

If this examination or any of the tests required in this section show evidence of the presence in a control culture of any adventitious agent, the influenza virus grown in the corresponding inoculated cultures should not be used for vaccine production.

Samples not tested immediately should be stored at –60 °C or below.

A.3.3.3.1 ***Tests for haemadsorbing viruses***

At the end of the observation period or at the time the virus is harvested from the production cultures, whichever is the later, at least 25% of the control cells should be tested for the presence of haemadsorbing viruses using guinea-pig red blood cells. If the cells have been stored, the duration of storage should not have exceeded 7 days, and the storage temperature should have been in the range of 2–8 °C. In tests for haemadsorbing viruses, calcium and magnesium ions should be absent from the medium.

This test is usually done using guinea-pig red cells. However, in some countries the national regulatory authority requires that additional tests for haemadsorbing viruses should be made in other types of red cell, including those from humans (blood group O), monkeys and chickens (or other avian species).

The results of all tests should be read after incubation for 30 min at 0–4 °C and again after a further incubation for 30 minutes at 20–25 °C. A further reading for the test with monkey red blood cells should also be taken after another incubation for 30 min at 34–37 °C.

In some countries the sensitivity of each new batch of red blood cells is demonstrated by titration against a haemagglutinin antigen before use in the haemadsorbtion test.

A.3.3.3.2 *Tests on supernatant fluids*

A sample of at least 10 ml of the pooled supernatant fluid from the control cultures collected at the end of the observation period should be tested in the same cell substrate, but not the same batch, as that used for production. Additional samples of at least 10 ml should be tested in both human and monkey cells. The samples should be inoculated into bottles of these cell cultures, in such a way that the dilution of the supernatant fluid in the nutrient medium does not exceed 1 in 4. The area of the cell sheet should be at least 3 cm^2/ml of supernatant fluid. At least one bottle of each of the cell cultures should remain uninoculated and serve as a control.

The cultures should be incubated at a temperature of 35–37 °C and should be observed for a period of at least 2 weeks.

> The use of rapid assays (e.g. multiplex PCR), which could be conducted within the time constraints of the procedure are encouraged.

A.3.3.3.3 *Identity test*

For vaccines produced in continuous cell culture the control cells should be identified by means, inter alia, of biochemical (e.g. isoenzyme analysis), immunological and cytogenetic marker tests approved by the national regulatory authority.

A.3.3.4 *Concentration and purification*

The monovalent material should be concentrated and purified by high-speed centrifugation or other suitable methods approved by the national regulatory authority, either before or after the inactivation procedure.

> The aim is to separate the virus or viral components from other constituents in either the allantoic and amniotic fluids or the mammalian cell culture fluids as efficiently as possible. It is advisable to concentrate and purify the virus under optimum conditions to preserve its antigenic properties.
>
> Consideration should be given to investigating whether the concentration and purification steps remove potential extraneous agents.

A.3.4 *Control of monovalent virus pools*

A.3.4.1 *Effective inactivation*

The inactivated and purified monovalent virus pool should be shown not to contain viable influenza virus when tested by a method approved by the national regulatory authority. Tests for viable virus should be conducted in eggs for egg-derived vaccine and in the mammalian cells used for vaccine production for cell-derived vaccine.

> A suitable method for egg-derived vaccine consists of inoculating 0.2 ml of undiluted monovalent pool and 1 : 10 and 1 : 100 dilutions of the monovalent pool into the allantoic cavities of groups of fertilized eggs (ten eggs in each

group), and incubating the eggs at 33–37 °C for 3 days. At least eight of the ten embryos should survive at each dosage level.

A volume of 0.5 ml of allantoic fluid is harvested from each surviving egg. The fluid harvested from each group is pooled and 0.2 ml of each of the three pools is inoculated, undiluted, into a further group of ten fertile eggs. Haemagglutinin activity should not be detected in these new groups of eggs.

In some countries, alternative methods are used.

In some countries, the requirement that 80% of the embryos should survive during incubation may be impossible to satisfy. The national regulatory authority should then specify the requirement to be satisfied.

For mammalian-cell-derived vaccine, methods could be modelled on those used for egg-derived vaccines with the exception that a mammalian cell substrate with validated sensitivity should be used. The national regulatory authority should approve the method used and specify the requirement to be satisfied.

As testing of residual virus infectivity after inactivation may be problematic due to aggregation, validation of test sensitivity should be performed.

A.3.4.2 *Haemagglutinin content*

The content of haemagglutinin in the monovalent virus pool should be determined by a suitable and approved technique, such as single radial immunodiffusion. In the test, an influenza reference haemagglutinin antigen reagent or a national preparation calibrated against it should be used for purposes of comparison (see section A.1.4).

For adjuvanted vaccines it should be established whether the adjuvant is compatible with the antigenic components of the vaccine and whether the presence of adjuvant interferes with the test for haemagglutinin content. If there is likely to be interference, this test may be performed before the addition of adjuvant. The test should be fully validated.

There is evidence to suggest that when cell-derived vaccines are produced from viruses isolated in eggs, the conventional reference antigen reagents (egg-derived) are suitable for measurement of haemagglutinin content. However the use of conventional antigen reagents may not be suitable to measure the haemagglutinin content of mammalian cell-derived vaccines when viruses isolated in cells are used for production. As further information becomes available, advice will be provided by WHO.

During the early stages of production of vaccines for pandemics, there may be no reference reagents to measure vaccine haemagglutinin content by conventional methods. It may be necessary to use alternative estimates of antigen content as advised by WHO and national regulatory authorities.

A.3.4.3 *Presence of neuraminidase*

Vaccine should be prepared under conditions that allow retention of detectable levels of viral neuraminidase for each strain.

In some countries, a test is included for the presence of neuraminidase enzymatic or antigenic activity. The ratio of haemagglutinin to neurami-

nidase should be consistent for the particular virus strain and method of vaccine production used, but the neuraminidases of different strains vary markedly in their stability during processing.

A.3.4.4 *Virus disruption (split vaccines)*

Monovalent pools in which the virus has been split by chemical means should be shown by procedures approved by the national regulatory authority to consist predominantly of disrupted virus particles.

This test need be performed on only three samples of monovalent pool for each vaccine strain provided that the test result is satisfactory.

A.3.4.5 *Surface antigens (subunit vaccines)*

The purity of monovalent pools intended for the preparation of subunit vaccine shall be determined by polyacrylamide gel electrophoresis or by other suitable techniques approved by the national regulatory authority. Mainly haemagglutinin and neuraminidase antigens for each strain should be present.

This test need be performed on only three samples of monovalent pool for each vaccine provided that the test result is satisfactory.

A.3.4.6 *Identity*

Antigenic specificity may be confirmed by an immunodiffusion or haemagglutination-inhibition technique using appropriate specific immune sera. The tests for haemagglutinin content (A.3.4.2) and presence of neuraminidase (A.3.4.3) also serve as identity tests. Reference viruses for identity tests may be obtained from reference laboratories (Appendix 2).

Alternatively antigenic identity may be confirmed by:

— injection of vaccine into mice, chickens or other suitable animals and demonstration of the production of antibodies to the haemagglutinin of the influenza virus used to produce the vaccine. In addition, demonstration of production of antibody to neuraminidase may also be performed; or
— suitable genetic tests.

With split and subunit vaccines, the identity test may be performed before virus disruption.

A.3.4.7 *Extraneous agents*

Cell-derived vaccines

If a contaminating agent is found in the working seed, mammalian cells are not susceptible to infection by the agent (A.3.1.5.2) and removal and/or inactivation of the agent by the production process cannot be demonstrated, monovalent bulks should be tested to ensure freedom from the agent. The data should be approved by the national regulatory authority.

Egg-derived vaccines

If removal and/or inactivation of a potential contaminating agent by the production process cannot be demonstrated, monovalent bulks should be tested to ensure freedom from the agent. The data should be approved by the national regulatory authority.

A.3.4.9 *Purity of cell-derived vaccines*

To monitor consistency in purity, monovalent virus pools derived from mammalian cell cultures should be tested for the ratio of haemagglutinin content: total protein. This ratio should be within the limits approved by the national regulatory authority.

For viruses grown in continuous cell culture, the purified monovalent pool should be tested for residual cellular DNA. The purification process should be shown to consistently reduce the level of cellular DNA to less than 10 ng per human dose. This test may be omitted, with the agreement of the national regulatory authority, if the manufacturing process is validated as achieving this specification.

A.3.4.9 *Tests for chemicals used in production*

The concentration of each detergent, organic solvent and inactivating agent remaining in the final vaccine should be determined using methods approved by the national regulatory authority. These concentrations should not exceed the upper limits specified by the national regulatory authority. For preservatives, both the method of testing and the concentration should be approved by the national regulatory authority.

Alternatively, tests for chemicals may be performed on the final bulk.

A.3.5 *Control of final bulk*

Final bulks are prepared by mixing and diluting monovalent pools of the relevant strains. In the preparation of the final bulk, only preservatives or other substances, including diluents, approved by the national regulatory authority should be added. Such substances should have been shown by appropriate tests not to impair the safety or effectiveness of the product in the concentrations used, and should not be added before samples have been taken for any tests that would be affected by their presence.

Vaccines for use during pandemics are likely to contain only one strain.

A.3.5.1 *Test for content of haemagglutinin antigen*

The haemagglutinin concentration in the final bulk should be determined as described in section A.3.4.2.

This test may be omitted if such a test is performed on each final lot.

A.3.5.2 *Sterility tests*

Each bulk should be tested for sterility by a method approved by the national regulatory authority.

Many countries have regulations governing sterility testing. Where these do not exist, the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (*15*) should be satisfied. If a preservative has been added to the vaccine, appropriate measures should be taken to prevent it from interfering with the sterility test.

A.3.5.3 *Total protein*

The total protein content should be not more than six times the total haemagglutinin content of the vaccine, as determined in the test for haemagglutinin content, but in any case not more than 100 µg of protein per virus strain per human dose and not more than a total of 300 µg of protein per human dose.

> In some countries, protein stabilizers are added to vaccine. The total protein content should reflect such additions. For subunit vaccines, a lower protein content is achievable, i.e. not more than 40 µg of protein per virus strain per human dose and not more than a total of 120 µg of protein per human dose.

A.3.5.4 *Ovalbumin (egg-derived vaccines)*

The ovalbumin content should be not more than 5 µg per human dose. The amount of ovalbumin should be determined by a suitable technique using a suitable reference preparation of ovalbumin.

> Values of less than 1 µg of ovalbumin per human dose are attainable and lower limits may be set.

A.3.5.4 *Adjuvant content*

If an adjuvant has been added to the vaccine, its content should be determined by a method approved by the national regulatory authority. The amount and nature of the adjuvant should be within the range shown to be clinically effective and should be approved by the national regulatory authority.

> The formulation of adjuvant and antigen should be stable and consistent. The purity of the adjuvant should be demonstrated to be within the range found for vaccine lots shown to be clinically effective.

A.4 Filling and containers

The requirements concerning filling and containers given in *Good manufacturing practices for biological products* (*10*, annex 1, section 4) should apply. Single- and multiple-dose containers may be used. If the latter are used, a suitable preservative, approved by the national regulatory authority, should be incorporated.

A.5 Control tests on final lot

A.5.1 *Identity test*

An identity test should be performed by a method approved by the national regulatory authority on at least one container from each final lot.

The identity of the haemagglutinins in the vaccine should be determined by an immunological technique, such as immunodiffusion or haemagglutinin inhibition, using the appropriate specific immune serum.

> In some countries, a test to identify the specific neuraminidase antigens is also included.

A.5.2 *Sterility test*

Final containers should be tested for sterility as described in section A.3.5.2.

A.5.3 *Haemagglutinin content*

The test for haemagglutinin antigen concentration is performed as described in section A.3.4.2.

The vaccine should contain in each human dose at least 15 µg of haemagglutinin of each strain used in the preparation.

> In some countries, lower limits may be set, based on clinical experience.
>
> Expression of haemagglutinin antigen content can also reflect uncertainty of measurement by stipulating that the lower confidence interval ($P = 0.95$) of the assay should be not less than 12 µg of haemagglutinin of each strain per dose.
>
> It may be necessary to formulate vaccine for use in a pandemic to contain a different haemagglutinin antigen concentration. Advice will be provided by WHO and the national regulatory authority.

A.5.4 *General safety (innocuity) tests*

Each filling lot should be tested for unexpected toxicity (sometimes called abnormal toxicity) using a general safety (innocuity) test approved by the national regulatory authority.

> This test may be omitted for routine lot release once consistency of production has been well established to the satisfaction of the national regulatory authority and when good manufacturing practices are in place. Each lot, if tested, should pass a test for abnormal toxicity.

A.5.5 *Endotoxin*

A test for endotoxin should be included, e.g. the *Limulus* amoebocyte lysate test.

The permissible level of endotoxin is determined by the national regulatory authority. It is likely that the permissible level of endotoxin for mammalian-cell-derived vaccine will be lower than that for egg-derived vaccine.

A.5.6 *Inspection of final containers*

Each container in each final lot shall be inspected visually, and those showing abnormalities such as lack of integrity shall be discarded.

A.6 Records

The requirements given in section 8 of Good manufacturing practices for biological products (*10*, annex 1) should apply.

A.7 Retained samples

The requirements given in section 9 of Good manufacturing practices for biological products (*10*, annex 1) should apply.

A.8 Labelling

The requirements given in section 7 of Good manufacturing practices for biological products (*10*, annex 1) should apply, with the addition of the following information.

The label on the carton, the container or the leaflet accompanying the container should state:

- that the vaccine has been prepared from virus propagated in embryonated hen's eggs or in mammalian cells;
- the type of cell line i.e. monkey, dog, etc. (if appropriate);
- the strain or strains of influenza virus present in the preparation;
- the haemagglutinin content in μg per virus strain, expressed as μg of haemagglutinin per dose;
- the number of doses, if the product is issued in a multiple-dose container;
- the influenza season for which the vaccine is intended;
- the method used for inactivating the virus;
- the name and maximum quantity of any antibiotic present in the vaccine;
- the name and concentration of any preservative added;
- the name and concentration of any adjuvant added;
- the temperature recommended during storage and transport;
- the expiry date; and
- any special dosing schedules (e.g. for a pandemic vaccine).

For a pandemic vaccine — special dosing schedules (e.g. two doses).

A.9 Distribution and transport

The requirements given in section 8 of Good manufacturing practices for biological products (*10*, annex 1) should apply.

A.10 Stability testing, storage and expiry date

A.10.1 *Stability testing*

Adequate stability studies form an essential part of vaccine development. The stability of the vaccine in its final form and at the recommended storage temperatures should be demonstrated to the satisfaction of the national regulatory authority on final containers from at least three lots of final product.

Stability data may be presented to the national regulatory authority after use of vaccine. Accelerated stability studies may be used.

In some countries, vaccine haemagglutinin content should comply with final product specifications (see A.5.3) at the expiry date.

The formulation of vaccine antigens and adjuvant (if used) must be stable throughout its shelf-life. Acceptable limits for stability should be agreed with national authorities.

When any changes are made in the production process that may affect stability of the products, the vaccine produced by the new method should be shown to be stable.

A.10.2 *Storage conditions*

Inactivated influenza vaccine should be stored at a temperature of 2–8 °C.

If other storage conditions are used, they should be fully validated and approved by the national regulatory authority.

A.10.3 *Expiry date*

The expiry date should be fixed with the approval of the national regulatory authority, and should take account of the experimental data on stability of the vaccine.

In general, the expiry date should not exceed 1 year from the date of issue by the manufacturer because the strains used in one year's vaccine may not be appropriate the next year.

Part B. Requirements for national control authorities

B.1 General

The general recommendations for control laboratories, contained in the Guidelines for national authorities on quality assurance of biological products should apply (*10*, Annex 2).

The national regulatory authority should give directions to manufacturers concerning the influenza virus strains to be used, the

haemagglutinin content, whether or not neuraminidase is present, and the recommended human dose.

B.2 Release and certification

A vaccine lot shall be released only if it fulfils national requirements and/or Part A of these Recommendations. A statement signed by the authorized official of the national regulatory authority should be provided at the request of the manufacturing establishment and should certify that the lot of vaccine in question satisfies all national requirements as well as Part A of these Recommendations. The certificate should state the number under which the lot was released by the national controller, and the number appearing on the labels of containers. Importers of influenza vaccine (inactivated) should be given a copy of the official national release document. The purpose of the certificate is to facilitate the exchange of inactivated influenza vaccine between countries.

An example of a suitable certificate is given in Appendix 3.

B.3 Clinical evaluation of influenza vaccines

In the case of a new manufacturer, the national regulatory authority should assess the safety and immunogenicity of the vaccine by arranging for studies in human volunteers of one or more of the lots of vaccine that have satisfied the above-mentioned requirements. Such studies shall include the assessment of the immune responses and adverse reactions in various age groups.

In the case of a significant change in the manufacturing process, clinical studies may also be required by the national regulatory authority.

Some national authorities require a limited clinical evaluation for licensing purposes whenever a new vaccine strain is introduced.

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Appendix 1

Summary protocol for influenza vaccine (inactivated) (master/working seed lot Type A or Type B)

The model summary protocol that follows is provided as general guidance to manufacturers. It is not intended to constrain them in the presentation of data relevant to the complete review of the quality control tests performed on the vaccine. It is important to note that satisfactory test results do not necessarily imply that the vaccine is safe and effective, since many other factors must be taken into account, including the characteristics of the manufacturing facility.

Name and address of manufacturer

Laboratory reference no. of lot

Date when the processing was completed

Information on manufacture

Virus used to inoculate eggs or cells for the manufacture of the lot:

(a) strain and substrain
(b) passage level
(c) source and reference no.
(d) remarks

Results of sterility test

Results of tests for extraneous agents

Results of tests on adjuvant (if any)

Conditions of storage

Monovalent virus pool Type A or Type B

Name and address of manufacturer

Laboratory reference no. of virus pool

Virus used to inoculate eggs or cells.

(a) master seed strain and source
(b) passage level of master seed
(c) working seed lot, reference no. and source

Date of inoculation

Date of harvesting allantoic or amniotic fluids or cell culture fluids

Storage conditions before inactivation

Date of inactivation

Time of inactivation

Method of inactivation

Concentration of inactivating agent

Storage conditions after inactivation

Concentration/purification procedure

Antibiotics used during preparation, if any

Identification of adjuvant added, if any

Tests on monovalent pool[1]

Test for absence of viable influenza virus

No. of eggs or cell culture vessels inoculated

Incubation time and temperature

Date of test

Results

Determination of haemagglutinin content

Method

Date of determination

Results

Tests for presence of neuraminidase (if performed)

Method

Date of test

Results

Virus disruption (for split vaccine)

Method

Date

Results

[1] If there are more than four virus pools in the monovalent pool, the relevant data should be given on a separate sheet.

Purity (for subunit vaccine)

Method

Date

Results

Purity (for cell-derived vaccine)

Method

Date

Results

Identity tests

Method

Date of test

Results

Test for extraneous agents (if performed)

Method

Date

Results

Final bulk

Name and address of manufacturer

Identification of final bulk

Identification of monovalent virus pool used to prepare final bulk

Date of manufacture

Control of final bulk

Preservative(s) added and concentration

Any other substances added and concentration

Determination of haemagglutinin content

Method

Date of determination

Results

Sterility

Date of test

Results

Total protein content

Method

Date of test

Results

Ovalbumin content (egg-derived vaccines)

Method

Date of test

Results

Test for residual DNA (if performed)

Method

Date

Results

Test for adjuvant (if performed)

Method

Date

Results

Tests for chemicals used

Date of tests

Results

Final lot

Identity test

Method

Date of test

Results

Sterility

Method

Date of test

Results

Determination of haemagglutinin content

Method

Date of determination

Results

Innocuity (if performed)

No. and species of animals

Doses injected

Period of observation

Date of test

Results

Endotoxin content

Method

Date of test

Results

Inspection of final container

Results

Other tests

Additional comments (if any)

A sample of a completed final container label and package insert should be attached.

Certification by producer

Name of head of production of the final vaccine

Certification by head of the quality assurance department taking overall responsibility for production and control of the final vaccine:

I certify that lot no . . . of influenza vaccine (inactivated), whose number appears on the label of the final container, meets all national

requirements[1] and satisfies Part A of the Requirements for Biological Substances No. 17, revised 1990.

Signature: _____
Name (typed): _____
Date: _____

Certification by the national controller

If the vaccine is to be exported, provide a copy of the certificate from the national regulatory authority as described in section B.2, a label of a final container, and a leaflet of instructions to users.

[1] If any national requirement(s) is (are) not met, specify which one(s) and indicate why release of the lot has nevertheless been authorized.

Appendix 2

Reference laboratories

WHO collaborating centres for reference and research on influenza

Centers for Disease Control, Atlanta, GA, USA

National Institute for Medical Research, Mill Hill, London, England

National Institute for Infectious Disease, Tokyo, Japan

WHO Collaborating Center for Reference and Research on Influenza, Melbourne, Australia

Custodian laboratories for candidate influenza vaccine viruses and antigen reagents for vaccine potency

National Institute for Biological Standards and Control, Potters Bar, Herts., England

Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD, USA

Therapeutic Goods Administration, Canberra, Australia

National Institute for Infectious Diseases, Tokyo, Japan

Centers for Disease Control, Atlanta, GA, USA

National Institute for Medical Research, Mill Hill, London, England

WHO Collaborating Centre for Reference and Research on Influenza, Melbourne, Australia

Laboratories performing calibration of haemagglutinin content

National Institute for Biological Standards and Control, Potters Bar, Herts., England

Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD, USA

Therapeutic Goods Administration, Canberra, Australia

National Institute for Infectious Diseases, Tokyo, Japan

Appendix 3

Model certificate for the release of influenza vaccine (inactivated)[1]

The following lots of influenza vaccine (inactivated) produced by _____[2] in _____[3] whose numbers appear on the labels of the final containers, meet all national requirements,[4] Part A of the Recommendations for Influenza Vaccine (Inactivated) (revised 2003)[5] and the recommendatons for good manufacturing practice and quality assurance for biological products.[6]

Lot Number	Date of last potency test by manufacturer	Expiry lot number

As a minimum, this certificate is based on examination of the manufacturing protocol.

The number of this certificate is: _____

The Director of the National Control Laboratory (or Authority as appropriate):[7]

Name (typed): _____

Signature: _____

Date: _____

[1] To be provided by the national regulatory authority of the country where the vaccines have been manufactured, on request by the manufacturer.
[2] Name of manufacturer.
[3] Country.
[4] If any national requirement(s) is (are) not met, specify which one(s) and indicate why release of the lot(s) has nevertheless been authorized by the national regulatory authority.
[5] Published in WHO Technical Report Series, No. 927, 2005, Annex 3 and with the exception of the provisions on shipping, which the national regulatory authority may not be in a position to control.
[6] Published in WHO Technical Report Series, No. 822, 1992, Annexes 1 and 2.
[7] Or his or her representative.

WHO Technical Report Series, No. 927, 2005

Annex 4
Requirements for the use of animal cells as in vitro substrates for the production of biologicals (Addendum 2003)

Introduction

World Health Organization (WHO) Requirements for the use of animal cells as in vitro substrates for the production of biologicals (*1*) provide information on a WHO cell bank of Vero cells. These cells were developed in 1987 and designated as a Master Cell Bank in 1998. Producers of biologicals and national control authorities can obtain cultures of these Vero cells (free of charge), as well as additional information, from WHO.

At its fifty-third meeting in February 2003, the Expert Committee on Biological Standardization was informed of the outcome of a meeting of a WHO Monitoring Group on Cell Banks held in Potters Bar, England, in October 2002 (*2*). The Monitoring Group noted that significant changes in regulatory expectations and technological advances had occurred in the requirements of cell bank operation and testing since the development of the WHO Vero bank 10-87 in 1987 and its designation as a Master Cell Bank in 1998. The Committee endorsed a recommendation from the Monitoring Group that the 10-87 bank should not be considered as a Master Cell Bank for direct use in manufacturing processes. Rather, the 10-87 bank should be regarded as a Cell Seed qualified by scientific analytical consensus from which Master Cell Banks may be established for thorough requalification. The Committee noted (*3*) that it would be necessary to revise the Requirements for the use of animal cells as in vitro substrates for the production of biologicals (WHO Technical Report Series, No. 878, 1998) to accommodate this change.

Manufacturers testing regimes for Master Cell Banks derived from the WHO Vero 10-87 Cell Seed will need to extend beyond the tests used to establish the WHO Vero 10-87 bank to include techniques such as product enhanced reverse transcriptase (PERT) assays (*2*). Furthermore, manufacturers should be continually aware of current developments regarding adventitious agents and ensure that data

from safety testing on banks of cells used in manufacturing processes are regularly reviewed and updated where appropriate.

The redesignation of the WHO Vero cell bank 10-87 as a Cell Seed may lead to investigations into the use of cells for manufacturing processes at higher population doublings than have previously been recommended. The potential for increased tumorigenicity at higher population doublings, among other issues, must therefore be considered (*2*). This assessment of tumorigenicity should take into account the variation that may occur in assessment of population doublings (both between and within laboratories), the potential for variability of in vivo tumorigenicity tests and the variation between different cell-culture processes. The establishment of arbitrary passage limits for the use of cells in a manufacturing process may be less important than careful process validation and testing of cells passaged beyond the process limits.

The amendments to the 1998 Requirements apply to the general considerations section and are listed below.

General considerations

Continuous-cell-line substrates

The last paragraph on page 23 of WHO Technical Report Series No. 878 currently reads:

"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."

This paragraph should be replaced by the following:

"The WHO 10-87 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. This cell bank should be regarded as a Cell Seed qualified by scientific analytical consensus from which Master Cell Banks may be established for thorough re-qualification. Producers of biologicals and national regulatory authorities can obtain cultures of these Vero cells (free of charge), as well as additional background information, from Quality Assurance and Safety of Biologicals, World Health Organization, 1211 Geneva 27, Switzerland."

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WHO Technical Report Series, No. 927, 2005

Annex 5
Recommendations for diphtheria, tetanus, pertussis and combined vaccines (Amendments 2003)

Introduction

These amendments should be read in conjunction with the introduction to the Requirements for diphtheria, tetanus, pertussis and combined vaccines published in WHO Technical Report Series, No. 800, 1990 (Annex 2).

Diphtheria and tetanus vaccines are among the most frequently used vaccines worldwide and have been remarkably successful products. Their use has resulted in a significant decrease in the incidence of these diseases in both the industrialized world and in developing countries. Nevertheless, some difficulties exist in the global harmonization of potency testing procedures, even when International Standards are used, and different approaches have been taken by different countries. Some follow WHO and *European Pharmacopoeia* procedures, whereas others follow the National Institutes of Health (NIH) procedures used in the USA, with or without modifications.

The approach taken by the *European Pharmacopoeia*, like that of WHO, is based on the determination of the immunizing potency of each final bulk by comparison with an appropriate reference material calibrated against the International Standard for Diphtheria Toxoid (adsorbed) or the International Standard for Tetanus Toxoid (adsorbed), as appropriate (*1*, *2*). There has been much activity in recent years aimed at simplifying the current tests, reducing the number of animals used and refining the end-point used in potency testing. Some studies have also considered the possibility of using the same animals to test the potency of several antigens.

The approach taken by the USA is based on the NIH assays (*3–6*) where the minimal acceptable potency is defined as the capacity of a test vaccine to induce an antibody response that reaches or surpasses the threshold of 2 units per ml. A suitable reference antitoxin, to which "units/ml" have been assigned, is used to express antibody concentration in relative terms, as measured by an in vivo toxin neutralization assay.

The inclusion of a control vaccine in the NIH test is being considered and would, in principle, improve control of the variations in the immune response induced in animals. The application of the Vero cell assay for the detection of anti-diphtheria toxin neutralizing antibodies is also being considered in the USA. Also, the expression of antibody levels in International Units could be achieved by calibration of the reference antitoxin against the International Standard for antiserum (see section A.1.3).

Despite many attempts to harmonize potency requirements globally, there are still no universally accepted methods. This leads to problems in international exchange of these vaccines arising from difficulties in the mutual recognition of the results of testing. The development of new combination vaccines, has led to an increased need for harmonization of the diphtheria and tetanus potency tests, creating a unique opportunity to resolve this long-standing issue.

The purpose of the potency test is to assess in a suitable animal model the capacity of the product being tested to induce a protective response analogous to that of toxoids shown to be efficacious in humans. The potency test has two stages. During the first stage a protective response is induced in mice or guinea-pigs, and during the second stage the protective response is measured by direct or indirect methods.

Considerable international consultation has identified the need to clarify the current WHO text relating to the introduction and use of simplified potency assays for the purpose of routine lot release. This should be seen as a first step towards the revision of the whole text of the current WHO Requirements (Recommendations) for Diphtheria, Tetanus, Pertussis and Combined Vaccines. The following amendments have thus been made to Annex 2, WHO Technical Report Series, No. 800, 1990. These include:

1. The updating of sections on International Reference Preparations for Diphtheria vaccine (adsorbed) and Tetanus vaccine (adsorbed);

2. The division of the sections on potency for Diphtheria vaccine (adsorbed) and for Tetanus vaccine (adsorbed) into two subsections to clearly distinguish the recommendations for licensing from those for routine batch release;

3. Simplification of the routine testing for batch release and use of fewer animals than used for licensing;

4. Amendment of the recommendations for diphtheria and tetanus potency testing in the diphtheria, tetanus, pertussis combined vaccine section to bring them in line with the changes outlined (in 2 and 3) above.

No changes have yet been made to the pertussis section of the Requirements for pertussis vaccines published in Technical Report Series, No 800, 1990 (annex 2).

Requirements for diphtheria vaccine (adsorbed)

Part A. Manufacturing recommendations

Replace section A.1.3, *International reference materials,* by the following:

A.1.3 *International reference materials*

The first International Reference Reagent of Diphtheria Toxoid for Flocculation Tests was established in 1988 (*7*).

The Third International Standard of Diphtheria Toxoid Adsorbed was established in 1999 (*8*) for determining the potency of vaccines containing diphtheria toxoid. The assigned activity of 160 IU/ampoule is based on its calibration in guinea-pig challenge assays. Potencies calculated by other methods should not be assumed to be transferable without validation. When potency tests are carried out in mice instead of guinea-pigs, transferability should be demonstrated.

The International Standard for Diphtheria Antitoxin[1] was established in 1934. It is made from horse hyperimmune serum for use in toxin neutralization potency assays, in vivo.

The above-mentioned reference materials are in the custody of the National Institute for Biological Standards and Control, Potters Bar, Herts., England (web site: http://www.nibsc.ac.uk). The WHO catalogue of international biological standards should be consulted for the latest list of appropriate international standards and reference materials (http://www.who.int/biologicals). International reference materials are intended for the calibration of national reference materials for use in the manufacture and laboratory control of diphtheria antitoxin and vaccines.

[1] The original standard is a freeze-dried preparation and new standard is a liquid fill of 10 IU/ml, made every 2 years.

Replace section A.3.5.6, *Potency,* by the following:

A.3.5.6 ***Potency***

a) Potency test for licensing

The potency of the final bulk is determined by comparison with an appropriate reference material[1] calibrated against the International Standard for Diphtheria Toxoid, Adsorbed. A three-dilution assay should be used to evaluate consistency of production of the vaccine in question. Three-dilution assays should also be used to test product stability for the purpose of establishing shelf-life as well as to calibrate reference preparations.

Potency should be determined by the inoculation of guinea-pigs with appropriate doses or dilutions of both the tested product and the reference material. After immunization, guinea-pigs may be challenged either by the subcutaneous or the intradermal route, or bled to obtain sera for measurement of the antitoxin or antibody response. When guinea-pigs are bled, the antibody levels of the individual animals may be titrated by means of toxin neutralization tests in vivo or in vitro, such as the Vero cell assay.

> The ELISA assay (*9*) or another suitable in vitro method may be used to measure the antibody response to diphtheria toxoid provided these assays have been validated against the challenge assay or the toxin neutralization test, using the particular product in question. A minimum of three assays with a suitable dose–response range is likely to be required for validation.

Appropriate statistical methods should be used to calculate the potency of the final bulk (*9*). The national regulatory authority should approve the method and the interpretation of the results.

If mice are used for the potency assay, they should be bled and antibody levels of the individual animals titrated by means of toxin neutralization tests in vivo in guinea-pigs, or in vitro using the Vero cell assay. Because mice are not sensitive to diphtheria toxin, challenge with diphtheria toxin is not possible.

> The ELISA or toxoid-binding inhibition (ToBI) assay (*9*) or another suitable method may be used to measure the antibody response to diphtheria toxoid, provided these assays have been validated against the toxin neutralization test, using the particular product in question. A minimum of three assays with a suitable dose–response range is likely to be required for validation.

The potency of diphtheria vaccine used for the immunization of children should not be less than 30 IU per single human dose. The results

[1] Such material could be monocomponent or multicomponent.

of all statistically valid tests should be combined in a geometric mean estimate and the confidence limits calculated. If the lower limit of the 95% confidence interval of the estimated potency is less than 30 IU per single human dose, then the limits of the 95% confidence interval should be within 50–200% of the estimated potency.

The potency values mentioned above do not apply to diphtheria vaccine for use in adolescents or adults.

b) Potency test for routine lot release

Following licensing, and once consistency in production and quality control of the vaccine has been further confirmed on a continuous basis, then the determination of potency in routine lot release may, with the approval of the national regulatory authority, be based on the results of serological assays, or on a challenge assay, both involving a reduced number of animals and/or doses.

To further confirm consistency on a continuous basis, the potency of about ten recent batches of vaccine should be tested using the full three-dilution assay. If potency expressed in International Units is relatively uniform and if the expectations of linearity and parallelism are consistently satisfied, then fewer doses may be used and the assumptions of linearity and parallelism need not be tested in each assay. When vaccine lots consistently give a lower limit of the 95% confidence intervals for the estimated potency well in excess of 30 IU per single human dose, one-dilution tests may offer advantages. If one-dilution assays are not advantageous, a reduction in animal usage may, nevertheless, be achieved by use of two-dilution assays or another suitable design modification.

> A one-dilution assay is based on the same principles for evaluating the response as the three-dilution assays. The assay involves the selection of a dose of the reference vaccine, expressed as a fraction of 30 IU (i.e. of the minimum potency of a single human dose), that elicits a minimum protective effect in guinea-pigs, and comparing its effect with the response elicited by the same fraction of a human dose of the test vaccine. If the response to the test vaccine is significantly greater than the response to the reference vaccine ($P \leq 0.05$), the potency of the test vaccine is satisfactory.

One-dilution assays provide assurance that the lower limit of the estimated potency is in excess of the minimum requirement. A disadvantage of such an approach is that strictly quantitative estimates of vaccine potency will not be possible.

If in vitro serological assays are used, they should show that the product induces an appropriate antibody response in animals in comparison with a reference material calibrated against the International Standard for Diphtheria Toxoid, Adsorbed.

The ELISA assay (*9*) or another suitable in vitro method may be used to measure the antibody response to diphtheria toxoid, provided these assays have been validated against the challenge assay or the toxin neutralization test, using the particular product in question. A minimum of three assays with a suitable dose–response range is likely to be required for validation of a particular product in a particular laboratory. These methods will require precise definition of the characteristics of reagents critical for successful performance of the testing method which may include positive and negative control sera, antigen and others.

There is a need to support the data generated by a simplified potency assay with physicochemical methods to ensure overall consistency of production.

Lot release based on a simplified approach will require periodic review to ensure that the validity of all procedures is maintained. The timing of the review should be decided on a case-by-case basis depending on the number of batches of vaccine produced annually and/or by time (at least every 2 years), as agreed by the national regulatory authority.

Recommendations for tetanus vaccine (adsorbed)

Part A. Manufacturing recommendations

Replace section A.1.3, *International reference materials*, by the following:

A.1.3 *International reference materials*

The first International Reference Reagent of Tetanus Toxoid for Flocculation Tests was established in 1988 (*7*).

The third International Standard of Tetanus Toxoid, Adsorbed, was established in 2000 (*10*) for determining the potency of vaccines containing tetanus toxoid. The assigned value of 469 IU/ampoule is based on its calibration in guinea-pig challenge assays. Potencies calculated by other methods should not be assumed to be transferable without validation. When potency tests are carried out in mice instead of guinea-pigs, transferability should be demonstrated.

The first International Standard for Tetanus Immunoglobulin, human was established in 1992 (*11*) for use in toxin neutralization potency tests.

The above-mentioned international standards are in the custody of the National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, EN6 3QG. England (web site: http://www.nibsc.ac.uk). The WHO catalogue of international biological standards should be consulted for the latest list of appropriate international standards and reference materials (http://www.who.int/

biologicals). The international reference materials are intended for the calibration of national reference materials for use in the manufacture and laboratory control of tetanus antitoxin and vaccines.

Replace section A.3.5.6, *Potency,* by the following:

A.3.5.6 *Potency*

a) Potency test for licensing

The potency of the final bulk should be determined by comparison with an appropriate reference material[1] calibrated against the International Standard for Tetanus Toxoid, Adsorbed. A three-dilution assay should be used to evaluate consistency of production of the vaccine in question. Three-dilution assays should also be used to test product stability for the purpose of establishing shelf-life as well as to calibrate reference preparations.

The potency should be determined by the inoculation of guinea-pigs or mice with appropriate doses or dilutions of both the tested product and the reference material. After immunization, animals may be challenged by the subcutaneous route, or bled to obtain sera for measurement of the antitoxin response. When animals are bled, the antibody levels of the individual animals may be titrated by means of toxin neutralization tests in vivo.

> The ELISA or ToBI assay (*9*) or another suitable method may be used to measure the antibody response to tetanus toxoid, provided these assays have been validated against the challenge assay or the toxin neutralization test, using the particular product in question. A minimum of three assays with a suitable dose-response range is likely to be required for validation.

Appropriate statistical methods should be used to calculate the potency of the final bulk (*9*). The national regulatory authority should approve the method and the interpretation of the results.

The potency of tetanus vaccine used for the immunization of children should not be less than 40 IU per single human dose. The results of all statistically valid tests must be combined in a geometric mean estimate and its confidence limits should be calculated. If the lower limit of the 95% confidence interval of the estimated potency is less than 40 IU per single human dose, then the limits of the 95% confidence interval should be within 50–200% of the estimated potency.

> In some countries these potency values may not apply to tetanus vaccine for adolescent or adult use.[2]

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[1] Such material could be monocomponent or multicomponent.

[2] Further guidance will be developed.

b) Potency test for routine lot release

Following licensing, and once consistency in production and quality control of the vaccine has been further confirmed on a continuous basis, then the determination of potency in routine lot release may, with the approval of the national regulatory authority, be based on the results of serological assays, or on a challenge assay, both involving a reduced number of animals and/or doses.

To further confirm consistency on a continuous basis, the potency of about ten recent batches of vaccine should be tested using the full three-dilution assay. If potency expressed in International Units is relatively uniform and if the expectations of linearity and parallelism are consistently satisfied, then fewer doses may be used and the assumptions of linearity and parallelism need not be tested in each assay. When vaccine potencies consistently give a lower limit of the 95% confidence intervals for the estimated potency in excess of 40 IU per single human dose, one-dilution tests may offer advantages. If one-dilution assays are not advantageous, a reduction in animal usage may, nevertheless, be achieved by use of two-dilution assays or another suitable design modification.

> A one-dilution assay is based on the same principles for evaluating the response as the three-dilution assays. The assay involves the selection of a dose of the reference vaccine, expressed as a fraction of 40 IU (i.e. of the minimum potency of a single human dose), that elicits a minimal protective effect, and comparing its effect with the response elicited by the same fraction of a human dose of the test vaccine. If the response to the test vaccine is significantly greater than the response to the reference vaccine ($P \leq 0.05$), the potency of the test vaccine is satisfactory.

One-dilution assays provide assurance that the lower limit of the estimated potency is in excess of the minimum requirement. A disadvantage of such an approach is that strictly quantitative estimates of vaccine potency cannot be obtained.

In vitro serological assays should show that the product induces an appropriate antibody response in animals in comparison with a reference material calibrated against the International Standard for Tetanus Toxoid, Adsorbed.

> The ELISA or ToBI assay (*9*) or another suitable method may be used to measure the antibody response to tetanus toxoid, provided these assays have been validated against the challenge assay or the toxin neutralization test, using the particular product in question. A minimum of three assays with a suitable dose–response range is likely to be required for validation for a particular product in a particular laboratory. These methods will require precise definition of the characteristics of reagents critical for successful performance of the testing method which may include positive and negative control sera, antigen and others.

There is a need to support the data generated by a simplified potency assay with physicochemical methods to ensure overall consistency of production.

Lot release based on a simplified approach will require periodic review to ensure that validity of all procedures is maintained. The timing of the review should be decided on a case-by-case basis depending on the number of batches of vaccine produced annually, or by time (e.g. every 2 years), as agreed by the national regulatory authority.

Recommendations for combined vaccines (adsorbed)

Part A. Manufacturing recommendations

A.2 *Special tests for DTP vaccines*

A.2.1 *Final bulk*

Replace section A.2.1.1, *Potency test*, by the following:

The following tests should be carried out on the final bulk vaccine.

A.2.1.1 *Potency test*

For the Diphtheria component, the recommendations for the licensing and routine lot release of Diphtheria vaccine (adsorbed) should apply (section A.3.5.6).

For the Tetanus component, the potency of which is tested in guinea-pigs, the recommendations for licensing and for routine lot release of Tetanus vaccine (adsorbed) should apply (section A.3.5.6). However, when tetanus toxoid is in combination with whole-cell pertussis vaccine and when the potency test for licensing is performed in mice, the estimated potency of tetanus vaccine used for immunization of children should be not less than 60 IU per single human dose. The same potency criteria should also apply when carrying out the routine lot release test.

Authors

The first draft of this amendment was prepared at a WHO Informal Consultation (meeting of drafting group) held in Geneva, 30 June–2 July 2003 and attended by the following participants: Dr J. Arciniega, Office of Vaccines Review and Research, Center for Biologics Evaluation and Research, Bethesda, MD, USA; Dr M. Corbel, Division of Bacteriology, National Institute for Biological Standards and Control, Potters Bar, Herts., England; Dr R. Gaines Das, Statistics Department, National Institute for Biological Standards and Control, Potters Bar, Herts.,

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The final draft was prepared by the drafting group, taking into account comments made by the reviewers of the document.

References

1. Assay of diphtheria vaccine adsorbed (2.7.6); Method of analysis. *European Pharmacopoeia*, 4th ed. Strasbourg, Council of Europe, 2003: Supplement **7**:2630–2632.
2. Assay of tetanus vaccine adsorbed (2.7.8), Method of analysis. *European Pharmacopoeia*, 4th ed. Council of Europe, Strasbourg, 2003: Supplement **7**:4257–4260.
3. US Department of Health, Education and Welfare Public Health Service, National Institute of Health. *Minimum requirements: tetanus toxoid.* Fourth revision. Bethesda, MD, NIH, 1952.
4. US Department of Health, Education and Welfare Public Health Service, National Institute of Health. *Minimum Requirements*: *Diphtheria Toxoid.* Fourth revision. Bethesda, MD, NIH, 1947.
5. US Department of Health, Education and Welfare Public Health Service, National Institute of Health. *Minimum Requirements: Tetanus and Diphtheria Toxoids Combined Precipitated Adsorbed (For Adult Use).* Bethesda, MD, NIH, 1953.
6. US Department of Health, Education and Welfare Public Health Service, National Institute of Health. *Minimum Requirements: Tetanus and Diphtheria Toxoids Combined Precipitated Adsorbed (For Adult Use).* Amendment 1. Bethesda, MD, NIH, 1956.
7. *WHO Expert Committee on Biological Standardization. Thirty-ninth report.* Geneva, World Health Organization, 1989 (WHO Technical Report Series, No. 786).
8. *WHO Expert Committee on Biological Standardization. Fiftieth report.* Geneva, World Health Organization, 2002 (Technical Report Series, No. 904), Annex 5.
9. *WHO Manual of laboratory methods for testing of vaccines used in the WHO Expanded Programme on Immunization.* Geneva, World Health Organization (WHO/VSQ/97.04).
10. *WHO Expert Committee on Biological Standardization. Fifty-first report.* Geneva, World Health Organization, 2002 (WHO Technical Report Series, No. 910).
11. *WHO Expert Committee on Biological Standardization. Fiftieth report.* Geneva, World Health Organization, 1994 (WHO Technical Report Series, No. 840).

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Annex 6
Biological substances: international standards and reference reagents

A list of International Biological Reference Preparations was issued in WHO Technical Report Series, No. 897, 2000 (Annex 4) and is available on the Internet at http://www.who.int/biologicals. Copies of the list 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-toxoplasma IgG, human	20 IU/ampoule	First International Standard 2003

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.

Antigens and related substances

Yellow fever vaccine	$10^{4.5}$ IU/ampoule	First International Standard 2003
Pertussis toxin	10 000 IU/ampoule	First International Standard 2003

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.

Blood products and related substances

Factor VIII, concentrate, plasma, human	11.0 IU/ampoule	Seventh International Standard 2003
Factor VIII and von	0.68 IU/ampoule factor	Fifth International

Willebrand factor, plasma, human	VIII:C 0.94 IU/ampoule factor VIII:antigen 0.91 IU/ampoule von Willebrand factor: antigen 0.78 IU/ampoule von Willebrand factor:ristocetin cofactor 0.94 IU/ampoule von Willebrand factor:collagen binding	Standard 2003
Prekallikrein activator, human	29 IU/ampoule	Second International Standard 2003
Low-molecular-weight heparin	1097 IU/ampoule Anti-Xa 326 IU/ampoule Anti-Iia	Second International Standard 2003

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.

Cytokines, growth factors and endocrinological substances

Interferon, beta, human, recombinant, glycosylated	40 000 IU/ampoule	Third International Standard 2003
Tumour necrosis factor, alpha, human, recombinant	46 500 IU/ampoule	Second International Standard 2003
Luteinizing hormone, human recombinant	189 IU/ampoule	First International Standard 2003
Thyroid-stimulating hormone, human, for immunoassay	11.5×10^{-3} IU/ampoule	Third International Standard 2003

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.

Diagnostic reagents

Hepatitis B surface antigen	33 IU/vial	Second International Standard 2003
Hepatitis B surface antigen panel (set of 4 dilutions and control)	No assignment	First International Reference Panel 2003

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.

Lipoprotein (a) for immunoassay	0.107 nanomoles/vial	First Reference Reagent 2003

This substance is held and distributed by Northwest Lipid Research Laboratories, University of Washington School of Medicine, 2121 North 35th Street, Seattle, WA 98103, USA.

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Annex 7
Recommendations and guidelines for biological substances used in medicine and other documents

The recommendations (previously called requirements) and guidelines published by the World Health Organization are scientific and advisory in nature but may be adopted by a national regulatory authority as national requirements or used as the basis of such requirements.

These international recommendations are intended to provide guidance to those responsible for the production of biologicals as well as to others who may have to decide upon appropriate methods of assay and control in order to ensure that these products are safe, reliable and potent.

Recommendations concerned with biological substances used in medicine are formulated by international groups of experts and are published in the Technical Report Series of the World Health Organization,[1] as listed here. A historical list of requirements and other sets of recommendations is available on request from the World Health Organization, 1211 Geneva 27, Switzerland.

Reports of the Expert Committee on Biological Standardization published in the WHO Technical Report Series can be purchased from:

Marketing and Dissemination
World Health Organization
1211 Geneva 27
Switzerland
Telephone: +41 22 79 12 476
Fax: +41 22 79 14 857
email: publications@who.int

Individual recommendations and guidelines may be obtained free of charge as offprints by writing to:

Quality Assurance and Safety of Biologicals
Department of Immunization, Vaccines and Biologicals
World Health Organization
1211 Geneva 27

Switzerland

[1] Abbreviated in the following pages as TRS.

Recommendations, guidelines and other documents

Recommendations and Guidelines	Reference
Acellular pertussis component of monovalent or combined vaccines	Adopted 1996, TRS 878 (1998)
Animal Cells, use of, as in vitro Substrates for the Production of Biologicals	Revised 1996, TRS 878 (1998); Addendum 2003, TRS 927 (2005)
BCG Vaccine, dried	Revised 1985, TRS 745 (1987); Amendment 1987, TRS 771 (1988)
Biological products prepared by recombinant DNA technology	Adopted 1990, TRS 814 (1991)
Blood, Blood Components and Plasma Derivatives: collection, processing and quality control	Revised 1992, TRS 840 (1994)
Blood plasma products, human: viral inactivation and removal procedures	Adopted 2001, TRS 924 (2002)
Cholera Vaccine (Inactivated, oral)	Adopted 2001, TRS 924 (2002)
Diphtheria, Tetanus, Pertussis and Combined Vaccines	Revised 1989, TRS 800 (1990); Ammendment 2003, TRS 927 (2005)
DNA Vaccines	Adopted 1996, TRS 878 (1998)
Haemophilus influenzae Type b Conjugate Vaccines	Revised 1998, TRS 897 (2000)
Haemorrhagic Fever with Renal Syndrome (HFRS) Vaccine (Inactivated)	Adopted 1993, TRS 848 (1994)
Hepatitis A vaccine (inactivated)	Adopted 1994, TRS 858 (1995)
Hepatitis B Vaccine prepared from Plasma	Revised 1987, TRS 771 (1988)
Hepatitis B Vaccines made by Recombinant DNA Techniques	Adopted 1988, TRS 786 (1989); Amendment 1997, TRS 889 (1999)
Human Interferons made by Recombinant DNA Techniques	Adopted 1987, TRS 771 (1988)
Human Interferons prepared from Lymphoblastoid Cells	Adopted 1988, TRS 786 (1989)
Influenza Vaccine (Inactivated)	Revised 2003, TRS 927 (2005)
Influenza Vaccine (Live)	Adopted 1978, TRS 638 (1979)
Japanese Encephalitis Vaccine (Inactivated) for Human Use	Adopted 1987, TRS 771 (1988)
Japanese Encephalitis Vaccine (Live) for Human Use	Adopted 2000, TRS 910 (2002)
Louse-borne Human Typhus Vaccine (Live)	Adopted 1982, TRS 687 (1983)

Recommendations and Guidelines	Reference
Measles, Mumps and Rubella Vaccines and Combined Vaccine (Live)	Adopted 1992 TRS 848 (1994); Note TRS 848 (1994)
Meningococcal Polysaccharide Vaccine	Adopted 1975, TRS 594 (1976); Addendum 1980, TRS 658 (1981); Amendment 1999, TRS 904 (2002)
Meningococcal C conjugate vaccines	Adopted 2001, TRS 924 (2002); Addendum 2003, TRS, 926 (2004)
Monoclonal Antibodies	Adopted 1991, TRS 822 (1992)
Pneumococcal conjugate vaccines	Adopted 2003, 927 (2005)
Poliomyelitis Vaccine (Inactivated)	Revised 2000, TRS 910 (2002); Amendment 2003, TRS 926 (2004)
Poliomyelitis Vaccine, Oral	Revised 1999, TRS 904 (2002); Addendum 2000, TRS 910 (2002)
Rabies Vaccine (inactivated) for Human Use, Produced in Continuous Cell Lines	Adopted 1986, TRS 760 (1987); Amendment 1992, TRS 840 (1994)
Rabies Vaccine for Human Use	Revised 1980, TRS 658 (1981); Amendment 1992, TRS 840 (1994)
Rift Valley Fever Vaccine	Adopted 1981, TRS 673 (1982)
Smallpox Vaccine	Revised 2003, TRS 926 (2004)
Sterility of Biological Substances	Revised 1973, TRS 530 (1973); Amendment 1995, TRS 872 (1998)
Synthetic Peptide Vaccines	Adopted 1997, TRS 889 (1999)
Thiomersal for vaccines: regulatory expectations for elimination, reduction or removal	Adopted 2003, TRS 926 (2004)
Thromboplastins and Plasma Used to Control Oral Anticoagulant Therapy	Revised 1997, TRS 889 (1999)
Tick-borne Encephalitis Vaccine (Inactivated)	Adopted 1997, TRS 889 (1999)
Tuberculins	Revised 1985, TRS 745 (1987)
Typhoid Vaccine	Adopted 1966, TRS 361 (1967)
Vaccines, Clinical Evaluation: regulatory expectations	Adopted 2001, TRS 924 (2004)
Vaccines, nonclinical evaluation	Adopted 2003, TRS 927 (2005)
Varicella Vaccine (Live)	Revised 1993, TRS 848 (1994)
Vi Polysaccharide Typhoid Vaccine	Adopted 1992, TRS 840 (1994)
Yellow Fever Vaccine	Revised 1995, TRS 872 (1998)

Other documents	Reference
Biological standardization and control: a scientific review commissioned by the UK National Biological Standards Board (1997)	Unpublished document WHO/BLG/97.1
Development of national assay services for hormones and other substances in community health care	TRS 565 (1975)
Good manufacturing practices for biological products	TRS 822 (1992)
Guidelines for national authorities on quality assurance for biological products	TRS 822 (1992)
Guidelines for the preparation, characterization and establishment of international and other standards andreference reagents for biological substances	TRS 800 (1990)
Guidelines for quality assessment of antitumour antibiotics	TRS 658 (1981)
Guidelines for the safe production and quality control of inactivated poliovirus manufactured from wildpolioviruses	Adopted 2003, TRS 926 (2004)
Guidelines on Transmissible Spongiform Encaphalopathies in relation to biological and pharmaceutical products	Unpublished document WHO/BCT/QSD/03.01
Laboratories approved by WHO for the production of yellow fever vaccine, revised 1995	TRS 872 (1998)
Production and testing of WHO yellow fever virus primary seed lot 213-77 and reference batch 168-73	TRS 745 (1987)
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)
Regulation and licensing of biological products in countries with newly developing regulatory authorities	TRS 858 (1987)
Report on the standardization and calibration of cytokine immunoassays	TRS 889 (1997)
Standardization of interferons (reports of WHO Informal Consultations)	TRS 687 (1983) TRS 725 (1985) TRS 771 (1988)
Summary protocol for the batch release of virus vaccines	TRS 822 (1992)