WHO Technical Report Series
924

WHO EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION

Fifty-second Report

This report presents the recommendations of a WHO Expert Committee commissioned to coordinate activities leading to the adoption of international requirements for the production and control of vaccines and other biologicals and the establishment of international biological reference materials. The report starts with a discussion of general issues brought to the Committee's attention and provides information on the status and development of reference materials for various antibodies, antigens, blood products and related substances, cytokines, growth factors, and endocrinological substances. The second part of the report, of particular relevance to manufacturers and national regulatory authorities, contains recommendations for the production and quality control of meningococcal group C conjugate vaccines, guidelines for regulatory expectations for clinical evaluation of vaccines, guidelines for the production and quality control of inactivated oral cholera vaccines and guidelines on viral inactivation and removal procedures intended to assure the viral safety of human blood plasma products.

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World Health Organization
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Introduction

The WHO Expert Committee on Biological Standardization met in Geneva from 26 to 30 November 2001. The meeting was opened on behalf of the Director-General by Dr Yasuhiro Suzuki, Executive Director of the Health Technology and Pharmaceuticals cluster.

Dr Suzuki noted that the past 2–3 years had been a time of considerable change and strengthening for the work on biologicals at WHO. This had been in response both to the general restructuring of WHO under the leadership of the Director-General, and to the resolution on the quality of biological products moving in international commerce (WHA 50.20), unanimously adopted by the World Health Assembly in 1997, and the subsequent report of the independent review of the remit and activities of WHO in the biologicals field and of the biologicals unit, which was submitted to WHO in November 1998. As a consequence, substantially more resources had been secured for work on the standardization and control of biological medicines during 2000 and 2001. Staffing levels had risen significantly, and had included a high-level secondment for 2 years from the Korea Food and Drug Administration, for which the Organization expressed gratitude to the Government of the Republic of South Korea, and significantly more funding was available for biological standardization activities. Nevertheless, more effort was needed to reach an appropriate level of funding and staffing for WHO's work in the biologicals field, which is undertaken by the Quality Assurance and Safety of Biologicals (QSB) and Quality Assurance and Safety of Plasma Derivatives and Related Substances (QSD) units working together even though managerially they were placed in different departments. There was also increasing interaction between the biologicals team and other groups in WHO.

Once again, the Expert Committee meeting had a heavy agenda, reflecting the ever-increasing complexity and sophistication of biological products, as well as their increasing numbers. Major challenges include the development of a coordinated international approach to the standardization and control of both traditional biologicals and of those developed by novel biotechnologies, as well as efforts at capacity-building in developing countries to help national regulatory authorities and manufacturers provide safe and effective biological medicines.

General

Developments in biological standardization

The work of QSB and QSD during 2000 and 2001 was reviewed. Critical indicators of performance included the number of guidelines or recommendations produced and adopted during these 2 years, as well as those on which work was well advanced, and the number of international standards and reference materials developed and established. Achievements included the increasing use of molecular methods for the characterization of vaccines (e.g. the mutant analysis by PCR and restriction enzyme cleavage (MAPREC) assay for testing oral polio vaccines), the development of guidelines on procedures for viral inactivation and removal for use during the production of human plasma products, the updating of the WHO catalogue of international standards and reference materials and its translation into an electronic form available on the Internet, and work on the coordination of regulatory research, including investigations of the contamination of cell substrates with viruses such as SV40. There had also been several valuable workshops and seminar sessions dealing with blood products held in Latin America and Japan, as part of the outreach and capacity building programme of QSD. Plans for 2002–2003, included an increased emphasis in QSB on products and assays arising from new biotechnologies, coverage of wider regulatory issues, such as clinical evaluation and batch release of vaccines, and the promotion of the use of international and working standards, particularly in developing countries. Priority was to be given to issues related to the safety of biologicals, and of vaccines in particular. Also, the early identification of gaps in the available regulatory guidance and the development of appropriate guidelines was recognized as an essential element in facilitating the licensing of much-needed vaccines and biologicals. Promotion of a scientific consensus as the basis for regulatory decisions would remain a main focus, together with the improvement and consolidation of changes in the standard-setting process to render it more transparent and interactive. The major challenges were related to the scientific issues of standardization and to assuring the safety of biologicals; the early harmonization of approaches to quality control testing, and access to adequate funding from flexible sources to meet unexpected needs and to respond to urgent issues of global consequence.

The outline of the QSB workplan for the next biennium included development or updating of several guidelines and recommendations for specific products, the preparation of new or replacement international standards and reference materials, the monitoring of the per-

formance of international standards in the field and the development of selected WHO working standards. Another area was coordination of regulatory research that would include the standardization of assays to measure immune responses to vaccine antigens, the improved characterization and quality control of vaccines including BCG and yellow fever and a task force on cell substrate safety. The proposed outreach and capacity-building activities of QSB include work on the control of polio vaccines and workshops on new developments in the regulation and quality control of vaccines and other biologicals.

The activities planned in QSD for 2002–2003 focus on guidelines on quality assurance, biological reference preparations and technical advice to regulatory authorities in the area of blood products and related biologicals. A Working Group on Plasma Issues had recently been formed under the auspices of the Global Consortium on Blood Safety and this would operate within the QSD structure. The objective of this group is to examine areas of concern related to the provision of plasma-derived medicinal products appropriate to the needs of a particular country. This would include identifying factors that could influence the continuity of the supply of plasma for fractionation. The group will follow the WHO Requirements for the Collection of Blood, Blood Components and Plasma Derivatives adopted by the Expert Committee in 1992 (WHO Technical Report Series 840, 1994), complemented by using further guidance documents as well as appropriate aide memoires/fact sheets on particular topics. The Expert Committee noted that two such documents were in an advanced stage of preparation, namely one on ensuring the quality and safety of human blood plasma products and another on plasma contract fractionation. The development of guidelines on plasma contract fractionation, on a plasma master file and on good manufacturing practices for blood and plasma collection establishments were considered a high priority, as were those for biological measurements. In addition, a number of reference materials were in development including standards for nucleic acid amplification technology and serological tests for bloodborne viruses and standards relating to transmissible spongiform encephalopathies (TSEs). Future activities for QSD would also include promotion in developing countries of the use of international standards and reference reagents both to validate assays and help in selection of diagnostic kits, both for the diagnosis of HIV in clinical practice and in the testing of donated blood for bloodborne viruses. These activities, which significantly contribute to the safety of blood products worldwide complement those undertaken by other parts of the Organization in the area of blood transfusion safety.

Further development of the Biologicals Internet web site (www.who.int/biologicals) was reported to be under way. The web site was initially set up in 1999 as an electronic version of the WHO catalogue of international standards and reference materials. It is intended to improve the transparency of the standard-setting process and to accelerate the dissemination of information on the availability of guidance documents and recommendations, to present a diary of events and meetings, and list calls for research proposals and details of contacts. In addition, links to other relevant organizations are being incorporated. These developments were warmly welcomed by the Committee.

Although the Committee was grateful for the increased resources both in personnel and funding that had been made available to QSB, and to a much lesser extent to QSD, it nonetheless considered that the global importance of its work justified still further investment. It recommended that an action plan be developed to attract funds from potential donors, and suggested that donor representatives might be invited to attend meetings of the Committee.

A major and recurrent issue raised by the Committee was the unacceptable delay in publishing the full proceedings of its meetings. The delay in making the recommendations and guidelines adopted by the Committee officially available has recently been of the order of 2 years, which is unacceptable in an area moving as rapidly as biological medicines. Although the use of the web site may make access to documents quicker prior to official publication, the delays are unacceptable and not all countries will have easy access to the web site. The delay has significant implications for public health, for example in the case of guidance dealing with the viral safety of blood or blood products. The Committee recommended that the Secretariat seek approval for the final version of all Guidelines and Recommendations adopted at its meetings to be made available on the Biologicals web site in the form of pre-publication drafts pending their final publication in the WHO Technical Report Series.

Bioterrorism

A consultation on bioterrorism involving experts from the region of the Americas was called by the Pan American Health Organization (PAHO)/WHO Region of the Americas (AMRO) on 24 October 2001, after the terrorist attacks of 11 September in New York, USA, and the subsequent anthrax-related incidents. The nature of the global economy and modern travel mean that an outbreak of anthrax in one country could affect the entire world. Although anthrax is not

spread by person-to-person contact, and is treatable early after infection with appropriate antibiotics, several vaccines exist that are reported to be effective if correctly administered. WHO has requirements for attenuated spore vaccines for veterinary use, but not for anthrax vaccine for human use. This highlights a gap in the guidance available from WHO and the Expert Committee recommended that the data on existing vaccines for human use, and new initiatives to develop novel vaccines, should be urgently reviewed.

The risk from smallpox is thought to be greater because it is highly infectious and global immunity is currently very low because the disease was eradicated almost 25 years ago. It was reported that current vaccine production capacity was limited and that additional production would be needed to meet any major demand for supplies of this vaccine. It was further reported that PAHO/AMRO planned to convene a meeting on 19 and 20 December 2001, to discuss the feasibility of re-establishing production of vaccines in the WHO Region of the Americas. It was also reported that the International Association of Biological Standardization had convened a meeting on orphan vaccines in Cairns, Australia, in August 2001 where many general issues concerning the development of vaccines against biological agents, including licensing, were discussed.

In 1999, the Expert Committee had recommended that the WHO requirements for the smallpox vaccine should be kept in place (WHO Technical Report Series, 904, 2002), and proposals for an update were already included in the workplan for QSB. The issues to be addressed include whether traditional production methods using scarification of animals are still acceptable in the twenty-first century and, if so, whether good manufacturing practice could be implemented; what guidance is required or available for smallpox vaccine production in cells or eggs, and what seed viruses should be used. The Expert Committee was informed that a seed lot based on the Lister strain is held by the National Institute of Public Health and Environmental Protection, Bilthoven, the Netherlands, on behalf of WHO, but that guidance was needed on the management of this stock and on its release to potential vaccine manufacturers and national regulatory authorities. The Committee recommended that further information on this seed be urgently sought and that WHO policy on its use be clarified and implemented. Another strain widely used in the production of vaccine for use during the smallpox eradication era was the New York City Board of Health strain and its use for vaccine production is much favoured by some countries.

The Committee was informed of the status of smallpox vaccine production in a number of countries. The Russian Federation had contin-

ued production of smallpox vaccine on calves in Siberia. The strain used, designated LIVP, was considered to be a more attenuated derivative of the Lister strain. The use of a combined inactivated/live vaccination schedule, including a live oral preparation, had also been explored in the former Soviet Union.

In Germany in the 1970s, the MVA strain had been used in a clinical trial involving 120000 people, and appeared to have had fewer side-effects than other smallpox vaccines available at the time. In 1975, a trial was conducted in 50000 people in Japan with a cell-grown vaccine and a low incidence of side-effects was reported.

More recently, clinical trials had been conducted in the United States to establish whether the existing vaccine stocks could be diluted without losing effect. A tenfold dilution gave 70% vaccine take and a one hundred-fold dilution 20–30%. The study is being repeated to see if a fivefold dilution would be satisfactory. However, it was agreed that more stocks of vaccine are urgently required and that several important issues needed clarification, including the selection of strains, methods of production and the availability of standards. It was recommended that WHO convene a meeting to discuss these and other issues in depth. However, because WHO is potentially concerned with production for use anywhere in the world, and the development of new production methods would require clinical trials of vaccine candidates, which was likely to be a lengthy process, it was concluded that it was unwise to close off any option. Thus, while cell-culture or egg-grown vaccines were to be strongly encouraged, the Expert Committee concluded that vaccines produced in the traditional way on animal skins were not to be ruled out. Experience in veterinary vaccines seemed potentially useful in the context of establishing good manufacturing practices for production in animals. Finally, the Committee also noted the importance of considering the production of stocks of vaccinia immune globulin, as well as vaccine production.

International nonproprietary names for biotechnology-derived medicinal products

The International Nonproprietary Names (INN) Committee of WHO has the responsibility for providing international non-proprietary names (INNs) for medicinal products. So far the INN Committee has assigned about 8000 names, some of which are for biological or biotechnology products. Contacts between the INN Committee and the Expert Committee on Biological Standardization with respect to the nomenclature of biological products have so far been informal.

However, with the increasing number and complexity of biological and biotechnology products it was felt that more formalized liaisons should be established. The Expert Committee welcomed this development. An informal consultation was planned for early 2002 and representatives of the Expert Committee on Biological Standardization were to attend. The issues to be addressed included a review of current policies on nomenclature of biological and biotechnological products and the provision of advice on the nomenclature of products derived from transgenic animals, gene therapy products, new vaccines and blood products and their recombinant equivalents, before requests for INNs in these areas were submitted.

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

Guidelines on clinical evaluation of vaccines: regulatory expectations

Draft guidelines (BS/01.1940) had been developed in response to requests from national regulatory authorities for assistance in evaluating clinical trials as a part of the regulatory overview. The intention was to provide guidance on regulatory expectations for the clinical evaluation of vaccines, rather than a manual of how to perform clinical trials. The draft document had been widely circulated for comments and much welcomed. It had also been discussed in detail at a consultation held in Geneva in October 2001, at which time amendments had been proposed for consideration by the Expert Committee.

The preclinical section of the document provided general principles, but did not define in detail the regulatory expectations in this area. As suggested by the Committee at its meeting in 2000, a separate guideline will be produced to cover this topic and the Committee recommended that it should be developed as soon as possible. Certain aspects of the evaluation of clinical trials were considered in some detail including adverse events, study population, size of trials, bridging studies, issues of consistency and combination vaccines. After making modifications to the text, the Committee adopted the document and agreed that it should be annexed to its report (Annex 1). The need for further guidance on specific issues such as clinical trials in which a particular disease (e.g. HIV) is endemic was identified and would be considered on a case-by-case basis.

Group C meningococcal conjugate vaccines

Recommendations (formerly known as requirements) for meningococcal polysaccharide vaccines were adopted by the Expert Committee in 1975 (WHO Technical Report Series 594, 1976) and amended in 1978 and 1981 (WHO Technical Report Series 626, 1978 and 658, 1981). They deal exclusively with unconjugated polysaccharide preparations used for control of epidemics and in groups of adults at high risk. These vaccines have several shortcomings that have precluded their incorporation into routine immunization programmes. They are poorly immunogenic in young infants (<2 years), are unable to induce immunological memory, and repeated vaccination with group C polysaccharide induces immune depression.

Following the successful introduction of *Haemophilus influenzae* type b conjugate vaccine, considerable progress had been made in the development of similar conjugate vaccines based on group C meningococcal capsular polysaccharide. Controlled trials have shown these vaccines to be highly immunogenic in all age groups and, as T-cell-dependent antigens, to induce immunological memory and affinity maturation of anti-capsular antibodies. In 1999, group C meningococcal conjugate vaccines were licensed and introduced into the routine immunization programme in the UK where they have been found to be safe and highly effective in decreasing the incidence of disease. Following their success in the UK, other European countries have also licensed and introduced group C meningococcal conjugate vaccines into their routine vaccination programmes.

The Committee noted a draft of proposed recommendations for the production and control of group C meningococcal conjugate vaccines (BS/01.1939) that had been widely circulated for comment, and reviewed in detail at a consultation held in Geneva in October 2001. The issues identified for consideration during the consultative process included the effect of polysaccharide size distribution and its *O*-deacetylation, the nature of the carrier protein and the extent of conjugation, and the possible effects of concomitant administration of other vaccines or combination vaccines. The Committee also considered the question of stability and the demonstration of lot consistency in clinical trials. The Committee agreed with a proposal to draft, at a later date, an addendum on serological assays and correlates of protection and to review the current recommendations in the light of data emerging from the UK following the introduction of the vaccine, especially those data related to the demonstration of immunological memory. It was reported that an immunogenicity test in mice may be a useful animal model for monitoring manufacturing consistency. The

Expert Committee recommended that the Secretariat evaluate the correlation between this assay and immunogenicity in humans and, if appropriate, incorporate the test in the proposed amendment on serological assays. After making some minor modifications, the Committee adopted the text as Recommendations for the production and control of group C meningococcal conjugate vaccine and agreed that it should be annexed to its report (Annex 2).

These recommendations would now serve as the basis for recommendations on other new meningococcal conjugate vaccines containing group A, Y or W135 antigens. The Committee was informed that a new public/private partnership programme had been set up with funding from the Bill and Melinda Gates Foundation to develop a group A meningococcal conjugate vaccine for use in Africa.

Inactivated oral cholera vaccines

A new generation of inactivated oral vaccines against cholera have been developed and licensed in some countries. These are now being considered for wider public health application, and WHO is establishing a vaccine stockpile for emergency use. Inactivated oral cholera vaccine is a new type of vaccine and a WHO Working Group met in 1999 to consider issues of standardization and control. The Working Group considered that the WHO Requirements for the parenteral inactivated cholera vaccine, first adopted by the Expert Committee in 1959, and discontinued at the fiftieth meeting of the Committee in 1999 (WHO Technical Report Series 904, 2002) were inappropriate. New guidelines were recommended to reflect the production and control of the new generation of oral cholera vaccines; further regulatory research would, however, be necessary on some aspects. Draft Guidelines for the production and control of inactivated cholera vaccines for oral administration (BS/01.1938) had therefore been prepared and were considered by the Committee. These had been circulated for comment in the usual way and had also been reviewed in detail at a consultation held at the International Vaccine Institute, Seoul, the Republic of Korea, in October 2001.

The vaccines consist of inactivated *Vibrio cholerae* alone or in combination with the B-subunit of cholera toxin expressed by rDNA methods. A number of issues were identified at the consultation in Seoul and amendments to the draft text were proposed for consideration by the Expert Committee. There was no precedent for controlling this type of vaccine (i.e. an inactivated oral vaccine) and, in particular, there was no animal model available that could meaningfully measure or predict the potency of such vaccines in humans. Some manufactur-

ers used a parenteral rabbit immunogenicity assay to assign potency. However, in this assay there was no dose-response relationship and it was not known whether this assay was a reliable indicator of the protective effect of the vaccines when administered orally in the target human population. In the light of these difficulties, it was suggested that emphasis should be placed on the characterization and quantification in vitro of the critical antigens and components. Key antigens are thought to include the lipopolysaccharide (LPS), toxin co-regulated pili and cholera toxin.

The draft guidelines reflected these concerns and the Committee agreed that no potency test could be recommended at present. Furthermore, it considered that the general safety test in animals should not be applied to this oral presentation. The Expert Committee also agreed that in view of the urgent need for guidance, the text should be published as guidelines instead of recommendations which would allow greater flexibility with respect to further developments in the field. The need for a reference serum was also agreed by the Committee. After making some modifications, the Committee adopted the text as Guidelines for the production and control of inactivated oral cholera vaccines and agreed that it should be annexed to its report (Annex 3).

Guidelines on viral inactivation and removal procedures intended to assure the viral safety of human blood plasma products

The Committee reviewed proposed guidelines that were intended to complement existing Recommendations on the collection, processing and quality control of blood, blood components and plasma derivatives (WHO Technical Report Series, 840, 1994). The draft document had been considered at a WHO consultation held in Geneva from 25–26 June 2001, and at various other seminars and meetings. Although human blood plasma products have, in the past, transmitted serious bloodborne infections, certain countries have had no major incidents for over 15 years. This has been the result of the implementation of a range of procedures, including the selection of donors, the screening of donations and the modification and evaluation of production processes so as to inactivate or remove infective viruses. Many regulatory documents deal with this subject, but the proposed guidelines are intended to cover general scientific principles and the processes generally regarded as effective in improving the safety of human plasma-derived products. A review of the infectious load and the incidence of contaminated plasma pools is followed by a consideration of those process steps considered to inactivate viruses, including dry heat, pasteurization, vapour heat, solvent/detergent treatment

and exposure to low pH. Process steps that remove viruses include ethanol fractionation, column chromatography and nanofiltration. The proposed guidelines review the advantages and uncertainties of each procedure, and considers the effect of various procedures on the product, the implementation of different process steps in a production setting, and possible new methodologies, as well as the treatment of fresh frozen plasma. The proposed guidelines were discussed at some length, and were welcomed by the Committee as a useful contribution to the viral safety of blood-derived products. After making some modifications, the Committee adopted the text as Guidelines on viral inactivation and removal procedures intended to assure the viral safety of human blood plasma products and agreed that it should be annexed to its report (Annex 4).

Pneumococcal conjugate vaccines

The Committee was informed of progress in the standardization and validation of IgG enzyme-linked immunosorbent assay (ELISA) to measure immune responses to pneumococcal conjugate vaccines. Because serological assays will play a pivotal role in the licensing of future pneumococcal conjugate vaccines, their standardization is vital for assuring the comparability of data from different clinical studies. Following the recommendations of a WHO Working Group established to promote standardization in this field, two WHO collaborating laboratories had been established to assist in this work. These laboratories are the Institute of Child Health, London, England, and the University of Birmingham, Alabama, USA. The Committee agreed with the specific tasks of these laboratories, namely:

— to train third-party laboratories to run pneumococcal serotype-specific IgG ELISAs to a standard that enables them to achieve appropriate and consistent quality of results;
— to accept visiting scientists in the laboratory for the above-mentioned training;
— to dispatch reagents to third-party laboratories for use in qualifying their assays, accept samples from third-party laboratories and run these in their qualified assay to help validate third-party assays and accept data from laboratories for analysis; and
— to produce a range of written training materials (manuals and standard operating procedures) intended to assist the third-party laboratories which will be made available to WHO for a wider distribution.

The Committee encouraged the efforts in this field and awaits with interest the opportunity to review their progress.

Potency assays for diphtheria and tetanus vaccines

The Committee was informed of the current situation concerning the development of a proposed simplified potency assay for the routine lot release of vaccines containing diphtheria and tetanus toxoids. It was reminded of the fundamental problems that still exist in standardizing and controlling the potencies of these toxoids and of the different approaches taken by different countries which leads to problems with the international exchange of vaccines and their licensing. At its fiftieth meeting in 1999, the Committee had welcomed the report of a WHO working group on the harmonization of antigen content and potency measurement of diphtheria and tetanus vaccines and endorsed the recommendation to develop a simple, robust and standardized assay that could be used worldwide to demonstrate the consistency of immunological characteristics of vaccine lots once clinical efficacy and safety of the vaccine, and consistency of production had been firmly established. There had been agreement in principle to move forward on this basis but the details still needed to be resolved. Because of the urgent need to give clear guidance on this problem, the Expert Committee recommended that the working group meet as soon as possible to review progress and to seek agreement on outstanding issues. The Committee also acknowledged the necessity to provide guidance specifically on future specifications for reference material in a new simplified lot release assay. It therefore recommended that those manufacturers currently conducting clinical trials retain sufficient quantities of the clinical trial lots in case they are needed for this purpose in the future.

Pertussis vaccines

The Committee noted the report of a workshop on the standardization and control of pertussis vaccines that had been held at the Center for Biologics Evaluation and Research Food and Drug Administration, Bethesda, MA, USA, in November 2000. The workshop participants had considered current issues concerning the testing of whole-cell and acellular pertussis vaccines and the need for new reagents. Reference materials were regarded as being essential in some cases and useful in others, although the priorities had not yet been firmly established. Various assays for the toxicity of vaccine preparations were also considered at the workshop: these included the mouse weight gain test, the Chinese hamster ovary cell test for pertussis toxin, the histamine sensitization factor test and a newly-developed chromogenic assay for active pertussis toxin. The implications of these tests for the WHO requirements for pertussis vaccines would continue to be evaluated and proposals for updating its current

recommendations would be made to the Expert Committee for its consideration as appropriate.

It was reported at the workshop that the modified Kendrick test, used in some countries for potency testing of acellular pertussis vaccines, had been assessed in a collaborative study. The study was undertaken in eight laboratories using acellular vaccines free of active pertussis toxin; two laboratories also performed the respiratory challenge test. The results showed that both methods could distinguish animals receiving vaccine from unvaccinated controls, and both gave a dose–response curve. Thus, the modified Kendrick test is able to measure protective responses to pertussis antigens in the absence of active pertussis toxin. However, the inclusion of non-immunogenic quantities of active pertussis toxin greatly enhanced the response, and there were some differences according to the strain of mouse used in the study. The Committee welcomed these developments and encouraged the Working Group on Pertussis Vaccines to continue its evaluation of methods to improve the quality and safety of pertussis vaccines.

Preventive human immunodeficiency virus vaccines

The Committee was informed that a WHO–Joint United Nations Programme on HIV/AIDS (UNAIDS) consultation on preventive human immunodeficiency virus (HIV) vaccines had been held in March 2001. The objectives of the meeting were to discuss the evaluation of potential vaccines, to consider the steps to be taken should a vaccine show promise and to consider the regulatory framework within which HIV vaccines should be assessed. The types of vaccine under consideration include inactivated viruses, recombinant derived proteins, DNA vaccines, vaccines based on virus or bacterial vectors carrying antigens, and live attenuated vaccines. Many vaccine candidates are in phase I and phase II clinical trials whereas one is in phase III trials in two countries. The issues addressed included standardization of assays for HIV viral load, genetic variation of HIV, assays for potency of vaccines and the use of animal models. Possible clinical end-points were also discussed and include prevention of infection, reduction of virus load and effects on cell-mediated immunity. It is not known if a reduction in virus load leads to a reduction in virus transmission. The effect of concurrent infections on the progression of the disease and the effect of antiretroviral therapy were identified as confounding variables in the assessment of the outcomes of clinical trials. The choice of the target population for vaccination was an issue raised by those countries most affected by the AIDS epidemic and should include adults, adolescents and pregnant women. The vaccina-

tion of pregnant women raises specific safety concerns. It was agreed that only countries with a framework for ethical and legal oversight should be considered for clinical trials, although, where such expertise was lacking, it might be sought from other countries in the area. Additional international and national funding should be directed towards developing countries for the specific purpose of strengthening regulatory expertise and building the infrastructure ready to facilitate swift approval of safe, effective vaccines particularly those against life-threatening diseases. The Committee concluded that there was a need to develop guidelines for vectored vaccines, to review cell substrate use for HIV vaccines, especially packaging cell lines and to review current guidelines to identify any further gaps in the existing regulatory guidance. Research on assays for vaccine potency and the development of a consensus on what constitutes a clinically significant response to antiretroviral treatment was to be encouraged. Further development of a network of interested parties and a forum of experts was required and a specific meeting on regulatory issues had been proposed.

Cell substrate safety

The Committee recalled previous discussions on cell substrate safety and the need to coordinate research and quality control work in this area. The importance of continued vigilance and sound science is illustrated by two specific issues that were brought to the attention of the Committee.

The presence of sequences of the monkey virus SV40 in certain human tumours had been reported to the Committee at previous meetings since some scientists postulated the sequences derived from the presence of live SV40 in early preparations of polio vaccine. Soon after the virus was discovered as a potential contaminant of monkey kidney cells, precautions were put in place by the Committee to prevent contamination of vaccine batches. Data previously presented had indicated that SV40 was not present in vaccine batches manufactured in Europe and the US after these precautions had been put in place. In further studies, a number of current vaccines and working seeds from manufacturers outside the Europe and the US were tested for SV40 sequences by sensitive molecular methods and shown to be negative, confirming that the precautions were also effective elsewhere. More recently, one research group had claimed that the SV40 cell-culture detection systems specified in one country would not detect naturally occurring SV40 viruses that contain a single copy of the enhancer region, but would detect culture-adapted viruses which usually have two or three copies of this region. Studies of a range of

viruses with various numbers of copies in a cell culture system used for detection of SV40 showed that there was no relationship between rate of growth of SV40 and numbers of copies of the enhancer region. This gives confidence in the specified cell culture detection tests. In view of these developments the potential need for virus standards for assays of SV40 was recognized by the Committee.

Simian cytomegalovirus (SCMV) is found in the kidneys of infected animals. Recently, genomic sequences of SCMV have been detected in a high proportion of the polio vaccines manufactured on cells from African green monkeys. Such sequences have not been detected in vaccines made on other cell substrates, including primary cell cultures from cynomolgous and erythrocebus monkeys, Vero cells (continuous cells derived originally from African green monkeys), or the human diploid MRC5 cells. MRC5 cells have been shown to be very sensitive to infection with SCMV. Investigations in at least three laboratories have failed to detect infectious SCMV in any vaccine preparation, and it is considered that the genomic sequences of SCMV represent inactivated virus. The Committee reiterated that the proposed Task Force on Cell Substrates would be a good forum for a review of detailed scientific data on these and other cell substrate issues and asked to be kept informed of developments.

Cell banks

The Committee was advised that a cell banking activity has been set up at the National Institute for Biological Standards and Control, Potters Bar, England, to establish cell banks for specific uses. They include cells used in diagnosis, such as Hep2c, RD and L20B cells used in the polio eradication initiative for virus detection, and cells used in assays, such as the HL-60 cells used in the opsonophagocytic assay of antibodies to pneumococcal conjugate vaccines, and a particular variety of Vero cell used in the assay of live dengue virus vaccines. Cells from the cell bank for these uses are subjected to a level of quality control which they would not otherwise receive, including the establishment of adequate master and working cell banks of cells known to be free of detectable viruses and mycoplasma, and of defined passage level.

The canine cell line MDCK has been proposed for the isolation of influenza viruses from clinical specimens, and a cell bank is to be established. MDCK cells have also been developed by some manufacturers for the production of influenza vaccine. WHO previously established a cell bank of Vero cells that was extensively characterized. The data on this cell bank are being collated for publication.

The Committee expressed interest in the activities described, and recommended that a working group be established to review WHO cell banking activities, including the possible extension of WHO cell banks to include cells for both diagnostic and production purposes. Collated information on the cell banks, including their characterization and intended use should be submitted to the Expert Committee for review.

Yellow fever

The WHO requirements for yellow fever vaccine were first formulated by a WHO Study Group in 1958 and were last revised by the Expert Committee in 1995. Live attenuated yellow fever vaccines that comply with WHO production and quality control specifications have been administered to millions of vaccinees, yet serious adverse events have been extremely rare and mainly neurological in nature. However, the Committee was informed that between 1996 and 2001, seven serious adverse events were reported, of which six were fatal. This was still an extremely low level, given the number of doses administered during this time. Of particular note were the clinical presentations of the events. The one case reported from Australia and the two from Brazil, that were fatal, appeared to be classical yellow fever. The four cases in the United States, three of which were fatal, involved multisystem disease. These adverse events had been reviewed by expert groups and were considered to be idiosyncratic host reactions rather than the results of quality defects in the vaccine.

Independently of these developments, a WHO collaborative study was under way to evaluate potency determinations for yellow fever vaccines. The specifications are currently expressed in terms of mouse infectious units, although in practice vaccines are formulated by their in vitro infectivity. The objectives of the study are to evaluate a candidate international standard and to eliminate the need for an animal potency test.

In view of the range of activities under way, including the development of possible tests of molecular consistency of vaccine production and further standardization of the neurovirulence test performed in primates, it was proposed that a working group on the quality control of yellow fever vaccines be formed, and the Committee welcomed this proposal.

Poliomyelitis vaccine, oral

The Committee was informed of three areas of activity concerning poliomyelitis vaccine, oral, all related to neurovirulence. Concerns

had recently arisen related to several batches of type 1 polio vaccine bulk from one manufacturer that had failed the monkey neurovirulence test. The manufacturer had a long history of satisfactory production of poliovirus vaccine bulks of all three types and the bulks in question appeared to be satisfactory in terms of production conditions and process controls. A variety of retest procedures including repeat testing in primates failed to reveal a credible problem or cause, and the manufacturer considered that the failure was caused by changes in the performance of the test. At the request of the national regulatory authority involved, WHO had convened a group of experts to investigate the matter. Their investigations revealed a need for clarification of the WHO recommendations for poliomyelitis vaccine, specifically the criteria for concluding that the mean lesion score of the reference is compatible with previous experience, the number of positive animals per test that should be included in the calculation of the statistical "c" values and the impact of the inoculator and histopathologist on the "c" value.

The use of transgenic mice in the evaluation of polio vaccine neurovirulence was introduced in 2000 into the WHO Recommendations for poliomyelitis vaccine, oral. An implementation process was agreed, but there was a need to clarify which laboratories should undertake the process. Independent assessment by a national regulatory authority of tests in transgenic mice performed by a manufacturer was also an issue. The monkey neurovirulence test uses histological criteria that allow an independent assessment to be made by the national regulatory authority of slides prepared by the manufacturer. The scoring of the test in transgenic mice is based on clinical criteria and discussion is needed on the most appropriate way to provide for independent assessment of the test other than by a retest, which may be difficult to justify in some countries.

The Committee was reminded that the MAPREC assay has been incorporated into the WHO Recommendations for poliovirus type 3. A collaborative study of candidate reference materials had been completed for poliovirus type 2 and a study was in progress for the application of MAPREC to poliovirus type 1. The Committee anticipated review of these studies at a future meeting.

The Committee was informed that WHO and the European Department for the Quality of Medicines were organizing a joint meeting in 2002 to discuss the above issues. The Committee supported the concept of the meeting and welcomed more extensive discussions in this complex area.

Transmissible spongiform encephalitis and the safety of biologicals

The safety of vaccines, blood products and other biologicals with respect to transmissible spongiform encephalopathies (TSEs) was discussed by the Expert Committee at its fifty-first meeting and considered to be assured by a combination of ensuring that materials used in manufacture came from safe sources (i.e. country, herd and animal), and by employing only tissues that have no demonstrable infectivity. At its fifty-first meeting the Committee had also requested that the recommendations made by WHO in 1997 on minimizing the risk of transmission of TSAs by biological medicines (WHO/BLG/97.2) be updated. However, in view of the rapid developments in this area it was decided to defer the updating until after a consultation sponsored by WHO, the Food and Agriculture Organization of the United Nations and the International Office of Epizootics (OIE) had been held in June 2001 and the process of classifying countries with respect to their freedom from bovine spongiform encephalopathy (BSE) had been reconsidered. There are currently several schemes operating worldwide, including those of the European Union, the United States Department of Agriculture and the OIE itself. As a result of the Paris meeting, the OIE was to consider using the scheme developed by the European Union.[a]

Views on acceptability of blood donors and specifications for cell banks for use in the production of biologicals are changing rapidly and the Expert Committee therefore recommended a full re-evaluation of current guidance. For this purpose a consultation would be required and the timely dissemination of any conclusions reached would be essential.[b] As a general point, the Committee agreed that replacement of animal materials used in the production of biologicals should be more strongly encouraged to speed up the process.

Antivenoms

The Expert Committee was informed of a WHO Workshop on the Standardization and Control of Antivenoms that had been held at the National Institute for Biological Standards and Control, Potters Bar, England, in 2001.[c] This was the first WHO meeting to deal with this

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[a] *Joint WHO/FAO/OIE/Technical Consultation on BSE: public health, animal health and trade. Paris, 10–14 June 2001* Geneva, World Health Organization, 2001 (WHO/CDS/CSR/APH/2001/8/EN).

[b] These revised Guidelines were published in February 2003: *WHO Guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical Products*. Geneva, World Health Organization, 2003 (WHO/BCT/QSD/03.01).

[c] Theakston RDG, Warrell DA, Griffiths E. Report of a WHO workshop on the standardization and control of antivenoms. *Toxicon*, 2003, 41:541–557.

subject since 1979 and it had attracted considerable interest worldwide. Participants included manufacturers (mainly small-scale national organizations), and national control authorities from 16 countries. The importance of snake and scorpion stings as public health issues was re-emphasized and this was reflected by the fact that most antivenoms were against snake or scorpion venoms. Most of the antivenoms were produced by traditional methods in horses, although some antisera were produced commercially in sheep and rabbits. Current production methods and quality control measures were reviewed in some detail. The methods used for plasma fractionation included salt and heat coagulation, caprylic acid stabilization or ion exchange chromatography, immunoglobulin digestion with pepsin to produce $F(ab')_2$ or with papain to produce Fab fragments. The participants at the meeting had agreed that there was much room for improving the production, quality control and safety profile of these products and that lessons could be learnt from the experience gained with the production of human immunoglobulins. Very little attention had been paid to the potential for the transmission of microbial agents from the immunized animals to patients and it had been agreed that this aspect needed attention. The possibility of introducing a validated viral inactivation step based on the use of caprylic acid and low pH, which is already used in some production processes, had been recommended for investigation.

The Expert Committee noted the workshop recommendation on the need to update current WHO guidelines on the production and control of antivenoms. It noted in particular that the WHO Requirements for Snake Antivenins (1971) and the WHO Requirements for Immune Sera of Animal Origin (1969) were very old and needed updating. The Committee recommended the development of a new guidance document on the production and control of antivenoms that took account of the recent progress made in the production and quality control of biologicals, and discontinued both existing documents (see page 20).

The Expert Committee also noted the workshop conclusion that international standards and reference materials were inappropriate in the antivenom field because the venoms from similar species of snake vary between regions. Instead, the workshop had recommended that national or regional standards for venoms and antivenoms be developed and used. The Expert Committee therefore disestablished the First International Standard for *Naja* (*Naja* and *Hemachatus* species) antivenin, equine (1964), (WHO Technical Report Series, No. 293, page 19) which is the only International Standard for Antivenom ever established.

Gene therapy

The Committee heard that the field of gene therapy was now very active worldwide and that many products were already at various stages of clinical evaluation in a number of countries. These included products for treating a range of genetic diseases as well as for treating cancer, diabetes, rheumatoid arthritis and high blood pressure. A variety of vectors have been used in gene therapy, including those based on retroviruses, adenoviruses, pox virus and plasmid vectors. An informal Working Group on Biological Standardization in Gene Therapy, convened by the National Institute for Biological Standards and Control, Potters Bar, England, had proposed the development of reference materials for replication-competent retroviruses and the validation of assays. The Committee recommended that the Secretariat monitor progress in this rapidly changing field and consider developing guidelines for gene therapy products. A document along the lines of the existing guidelines for assuring the quality of DNA vaccines could be used as a model. The possibility of WHO acting as co-sponsor of the Working Group on Biological Standardization in Gene Therapy was also raised and supported by the Expert Committee.

Discontinuation of requirements and guidelines

On the recommendation of an international workshop on antivenoms (see page 19), and bearing in mind the age of the documents concerned and the progress that has been made in the quality control of biological products in recent years, the Expert Committee discontinued the 1969 Requirements for Immune Sera of Animal Origin (WHO Technical Report Series, No. 413) as well as the 1971 Requirements for Snake Antivenins (WHO Technical Report Series, No. 463). These will be replaced in due course by a new document on the production and control of antivenoms.

The Committee was reminded of its decision at its forty-ninth meeting in 1998 (WHO Technical Report Series, No. 897, 2000, p. 5) no longer to be responsible for reference preparations or documents concerning agents that pose no threat to human health. Bearing this decision in mind, the Secretariat proposed that a few documents that dealt specifically with the production and control of veterinary vaccines, most of which had not been reviewed or updated for many years, should now be discontinued. These were:

- Requirements for Anthrax Spore Vaccine (Live, for Veterinary use) (WHO Technical Report Series, No. 361, 1967).

- Requirements for Rinderpest Cell Culture Vaccine (Live) and Rinderpest Vaccine (Live) (WHO Technical Report Series, No. 444, 1970).
- Requirements for *Brucella abortus* Strain 19 Vaccine (Live, for Veterinary Use) (WHO Technical Report Series, No. 444, 1970, addendum WHO Technical Report Series, No. 594, 1976).
- Requirements for *Brucella melitensis* Strain Rev. 1 Vaccine (Live, for Veterinary Use) (WHO Technical Report Series, No. 610, 1977).
- Requirements for Rabies Vaccine for Veterinary Use (WHO Technical Report Series, No. 840, 1981, Amendment WHO Technical Report Series, No. 840, 1994).
- Requirements for Rift Valley Fever Vaccine (Live Attenuated) for Veterinary Use (WHO Technical Report Series, No. 700, 1984).

The Committee agreed to the discontinuation of the above requirements provided there was no objection from the OIE, which has international responsibility for animal health. The Secretariat agreed to seek such agreement before implementing the discontinuation[a].

International reference materials

Biological substances: international standards and reference reagents

The Committee was informed of the continued demand for international reference materials distributed by the two International Laboratories for Biological Standards. It endorsed the programmes of work in progress and work planned by the two International Laboratories and the Collaborating Centre at the Centre for Biologics Evaluation and Research, Rockville, MD, USA.

The Committee reviewed proposals for the disestablishment of International Biological Reference Preparations arising from the annual review of usage and stocks. It was informed that no response had been received to the enquiry about the continued need for the International Reference Preparations or International Standards for amikacin, capreomycin, chlortetracycline, lymecycline, methacycline, novobiocin and paromomycin. It was also informed that no response had been received to the enquiry about the continued need for standards for porcine calcitonin for bioassay, porcine kininogenase, ovine prolactin for bioassay and bovine thyrotrophin for bioassay, in view of

[a] The Committee was informed that OIE had not raised objections and therefore discontinued the Requirements and Guidelines at its fifty-third meeting.

the availability of the corresponding human materials. All of these preparations were therefore disestablished. On the recommendation of an international consultation on antivenoms (see page 19), the Committee also disestablished the First International Standard for *Naja* (*Naja* and *Hemachatus* species) antivenin, equine (1964). Because of depleted stocks of the First International Standard for the tetanus toxoid flocculation test, the First International Standards for Hepatitis B DNA and Hepatitis C RNA, and the WHO reference reagent for anti-human platelet antigen 1A, the Committee requested the National Institute for Biological Standards and Control, Potters Bar, England, to take steps to replace them. The International Standard for erythromycin was not to be replaced because it was anticipated that the microbiological assay for erythromycin would shortly no longer be used. The need for a replacement for the International Reference Reagent for Hepatitis B vaccine should be reviewed.

International biological standards for in vitro diagnostic procedures

The Committee was reminded of a consultation held in September 2000, to consider the scientific issues arising from the establishment of reference preparations for in vitro diagnostic procedures. The outcome of that meeting was a decision to continue to collaborate with the International Standards Organization (ISO) to develop appropriate standards for in vitro diagnostic procedures. The differences between WHO and the ISO in the establishment of biological reference preparations were evident in approaches to the traceability of standards to previous standards, the uncertainty of measurement and unitage assignment and the commutability of results obtained with standards (and standard methods) to samples. Draft ISO document 17511 related to the development of standards for in vitro diagnostic procedures, and was discussed at a meeting at ISO European Committee for Standardization (CEN) in November 2001. Minor amendments suggested by WHO had been accepted, but the concept that the highest metrological order was represented by the expression of analyte content in SI units using a reference method was retained. The Expert Committee was requested to consider the principles embodied in ISO 17511 when establishing International Standards for diagnostics use, particularly with respect to uncertainties of measurement for replacement standards. During discussion, the difficulties of expressing the activity of most biological reference materials in SI units was again emphazised by the Committee. Furthermore, it was pointed out that ISO 34 specifically excluded assignment of uncertainty to reference materials in the pharmacopoeial field.

However, the Expert Committee heard that issues relating to the validation and calibration of diagnostic tests using nucleic acid based techniques (NAT) not only for testing the virological safety of blood and blood products, but also in clinical diagnostic testing, including the possible use of synthetic nucleic acid standards expressed in SI units, were to be discussed at a WHO Consultation planned for early 2002. Participants in the Consultation would be drawn from WHO International Laboratories for Biological Standards, regulatory bodies, clinicians with relevant expertise and experts in the manufacture of in vitro diagnostics employing nucleic acid based technologies, including the Industrial Liaison Committee of the in vitro diagnostic industry.

International reference materials for the diagnosis and study of transmissible spongiform encephalitis

The possibility of transmitting Creutzfeld–Jakob disease (CJD) through blood or blood products and other biologicals, or through surgical or other medical procedures, has caused considerable concern throughout the world in the wake of the appearance of a CJD variant (vCJD) in France and the United Kingdom, following the epidemic of BSE in cattle. Although there is no evidence to date of transmission of either CJD or vCJD in blood products, national regulatory authorities have given serious consideration to precautionary measures in the pharmaceutical and biologicals field. Reliable diagnostic procedures to detect asymptomatic carriers during the long incubation periods of CJD and vCJD would be very useful. The relative sensitivity of assays is an important subject of debate in the development of such diagnostic procedures which would be used to exclude potentially infectious donated blood, organs and tissues, as well as in the validation of the ability of pharmaceutical manufacturing processes to remove the abnormal forms of the prion proteins (PrP^{sc}).

The Expert Committee was informed that the Working Group established in 1999 to develop international reference materials for diagnostic procedures for TSEs had met in March 2001 to review progress. The characterization of four human-brain-derived materials, donated by the CJD Surveillance Unit in the United Kingdom, was being carried out in a WHO collaborative study, dealing in the first instance with in vitro assays of PrP^{sc} content, following a protocol agreed by the Working Group. This study was now near completion. Homogenates of four human-brain-derived materials, one from a normal individual, two cases of sporadic CJD and one case of variant CJD (uninfected, sp1CJD, sp2CJD and vCJD), had been prepared

and distributed in 2000 vials of each of the four homogenates. Thirteen international laboratories had participated in the study. The same materials would be studied in a variety of conventional and transgenic mouse infectivity assays so that the sensitivities of different assays and assay types can be compared. The WHO reference materials would be available as calibrants to laboratories attempting to optimize in vitro and in vivo diagnostic procedures for TSEs.

As a result of the study, it became important to clarify the nature of the two sporadic CJD strains proposed as candidate reference preparations as they appeared to be of mixed types according to their physicochemical properties. The brain materials were checked by two types of DNA fingerprinting and shown to be from unique human individuals, and not a mixture. A physicochemical typing collaborative study had been initiated to investigate this issue further and the results were expected to be discussed at the next meeting of the group in February 2002.

Antibodies

Human antibody against human platelet antigen 5b

The Committee noted the report (BS/01. 1945) of four collaborative studies describing the calibration of a freeze-dried preparation of pooled human plasma, coded 99/666, containing IgG antibodies against the human platelet antigen 5b (HPA-5b). The material was a pool from two donors that was obtained by plasmapheresis. Because it is intended as a minimum sensitivity reagent, and not a standard, it was proposed to be established as a Reference Reagent.

The Committee noted that no report had been received on this proposal from the International Society on Thrombosis and Haemostasis (ISTH) and the International Society of Blood Transfusion. It therefore deferred decision until further discussion had taken place.

Blood products and related substances

International reference panels for the validation of serological and nucleic acid based tests for the detection of hepatitis B, hepatitis C and human immunodeficiency virus in blood screening

The Committee was informed of progress in developing international reference preparations for the validation of diagnostic tests applied to the detection of nucleic acid and antibody viral markers for hepatitis B, hepatitis C and HIV. The preparations that have been developed

in this programme are designed to assist in defining the analytical sensitivity of tests and will provide a means of ensuring comparability of data between laboratories worldwide and of helping to guide surveillance and quality control programmes.

A meeting of a core group of experts from the WHO Working Group on Reference Preparations for Evaluating Diagnostic tests used for detection of hepatitis B surface antigen (HBsAg), anti-hepatitis C antibody (HCV) and anti-HIV antibodies for blood screening was held at the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, in February 2001. The purpose of the meeting was to advance the preparation of the proposed antigen and antibody subtype reference panels.

The proposed WHO HBsAg reference panel, subtype adw, encompasses a broad range of antigen concentrations so that it can be used to determine the levels of sensitivity of all the HBsAg tests kits available worldwide. The stock reagent was purified and heat-inactivated at CLB and diluted in serum and freeze-dried at the National Institute for Biological Standards and Control, England. The panel will be calibrated against a primary reference material defined in micrograms of HBsAg using physicochemical methods[a] to assure traceability. The proposed anti-HCV reference material will include anti-core, anti-NS3, anti-NS4 and anti-NS5 antibodies and the Working Group is currently seeking to identify and obtain the monospecific raw materials. The subtype reference panel for HIV under development will be composed of antibodies to HIV-1 types A, B, C, E and O, and HIV-2. Each panel component will be diluted in normal recalcified human plasma, inactivated by solvent/detergent and freeze-dried. The international collaborative study being planned to calibrate these materials will include representative laboratories from all WHO Regions. Further progress will be discussed at a meeting of the full WHO Working Group scheduled for the second half of 2002.

The Expert Committee was also informed about the composition of a WHO subtype reference panel for NAT that is also being developed; 10 HIV-1 subtypes, at approximately 5000 copies/ml, and two controls are included. None of the preparations are inactivated or freeze-dried and there will be 500 sets of this panel prepared, of which approximately one hundred will be used during the collaborative studies currently in progress. The Committee considered it important that

[a] Gerlich W, Thomssen R. Standardized detection of hepatitis B surface antigen: determination of its serum concentration in weight units per volume. *Developments in Biological Standardization*, 1975, 30:78–87.

indications for the appropriate use of such a panel should be clearly defined and published. The Committee also expressed an interest in studying possible approaches to inactivating NAT reference materials.

The Expert Committee requested a review of developments in both of these areas at its next meeting.

Factors II, VII, IX, X, Plasma

Low stocks of the second International Standard for Factors II, VII, IX, X, Plasma, Human, coded 94/746, had necessitated the calibration of a replacement preparation. The Committee noted the report of a WHO Collaborative Study (BS/01.1946) describing the calibration of the proposed third International Standard plasma (99/826) by assaying the material against the second International Standard and against pooled fresh human plasma from normal subjects. Nineteen laboratories participated in the study. Inter-laboratory variability for all four factors was low for assays of the proposed candidate replacement measured against the second International Standard (geometric coefficient of variation <4%), but was somewhat higher when measured against the fresh normal plasma pools (geometric coefficient of variation 6–10%). No major differences related to the use of different reagents and methods were observed, except for estimates of factor VII for which the chromogenic assays gave different results from the clotting assays. Potency estimates calculated relative to the fresh normal plasma pools were 3%, 10%, 8% and 6% lower for factors II, VII, IX and X, respectively, when compared to potencies calculated relative to the second International Standard.

On the basis of the data from the collaborative study, the Committee established the preparation, coded 99/826, as the third International Standard for Factors II, VII, IX, X Plasma, Human, and assigned to the preparation the mean values of the potencies calculated against the second International Standard and the fresh normal plasma pools for each factor. These are as follows:

— factor II: 0.91 IU/ampoule;
— factor VII: 1.00 IU/ampoule;
— factor IX: 0.86 IU/ampoule; and
— factor X: 0.93 IU/ampoule.

Agreement on these assignments had been obtained from the laboratories participating in the study as well as from the Scientific and Standardization Committee of the International Society of Thrombosis and Haemostasis at its meeting in Paris in July 2001.

It was noted that the predicted degradation rates for the four factors, particularly at temperatures likely to be encountered during shipment by post, were higher than for the estimates obtained for the previous International Standard, coded 94/746. The Committee recommended that additional stability data should be obtained before distribution of the International Standard and full information provided with the "Instructions for use".

Von Willebrand factor

Von Willebrand disease is a haemorrhagic disorder caused by a deficiency and/or abnormality of plasma von Willebrand Factor (vWF). Purified concentrates containing vWF used in replacement therapy for von Willebrand disease must carry labels that state the concentration of vWF. Characterization of the vWF in therapeutic concentrates has been based on measurement of antigen (vWF:Ag), ristocetin cofactor activity (vWF:RCO), multimer composition and more recently collagen binding activity (vWF:CB). The vWF in therapeutic concentrates has been found to have a lower ratio of function/antigen than vWF in normal plasma and to lack the highest-molecular-weight multimers, probably as a result of degradation during purification. These properties, together with the obvious differences in purity between concentrates and plasma suggest that the currently available primary standard for vWF (the fourth International Standard for Factor VIII and von Willebrand Factor, Plasma, Human, coded 97/586), may not be the most appropriate reference material for the quantitation of vWF in concentrates.

The Committee noted the report of a WHO collaborative study (BS/01.1947) to calibrate two candidate vWF concentrate preparations, coded 00/514 and 00/482, as prospective WHO International Standards for use in the estimation of the biological activity of therapeutic concentrates. The study involved 26 laboratories in nine countries. Calibration for vWF:Ag, vWF:RCO and vWF:CB was carried out by assay in which they were compared to the fourth International Standard for Factor VIII and von Willebrand Factor, Plasma, Human, coded 97/586). Since this International Standard had no assigned value for vWF:CB, it was first necessary to calibrate the fourth International Standard plasma for vWF:CB by assay relative to locally collected pools of frozen plasma from normal humans. The study also allowed the effect of using either a plasma or concentrate standard on the inter-laboratory variability of vWF estimates to be compared.

All but four estimates of vWF:Ag were carried out using ELISA techniques and of the remainder, only two estimates were obtained

using immuno-turbidimetric and two using Laurell electro-immunoassay techniques. There was good agreement between the mean estimates obtained using all three methods, for both candidates, and the overall combined means were 11.01 IU/ampoule for preparation 00/514 and 14.01 IU/ampoule for candidate 00/482.

Most laboratories used aggregometric techniques to measure the vWF:RCO of preparations although some used visual agglutination. No significant difference was found between mean estimates from the two methods for either candidate. The overall means corresponded to 9.38 IU/ampoule for candidate 00/514 and 10.19 IU/ampoule for candidate 00/482.

All laboratories measured wVF:CB using ELISA-type methods and various collagen reagents. Before candidates 00/514 and 00/482 could be calibrated, it was necessary to assign a value for vWF:CB to the fourth International Standard, plasma, by an assay in which it was compared to locally collected frozen normal plasma pools. Estimates for the fourth International Standard, plasma, showed good agreement between the results obtained using different collagen reagents and an overall combined mean of 0.83 units per ampoule. Estimates of vWF:CB in candidates 00/514 and 00/482, relative to the fourth International Standard plasma, showed poor agreement both between laboratories using the same type of collagen reagent and between laboratories using different collagen reagents.

Preliminary accelerated degradation studies on ampoules of both candidates stored at 20,37 and 45 °C for up to 7.5 months showed no detectable loss of vWF:Ag or vWF:CB when compared to ampoules stored at –70 °C. Studies on the fourth International Standard plasma were unable to detect degradation even after 35 months of storage at elevated temperatures. These results are consistent with the known stability of vWF in plasma and concentrates.

The Committee accepted the report of the WHO collaborative study. It was agreed that a value of 0.83 IU/ampoule for the collagen binding activity (vWF:CB) be assigned to the current fourth International Standard for Factor VIII and von Willebrand Factor, Plasma, Human, coded 97/586. However, the Committee accepted the recommendation of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis that no values for vWF:CB should be assigned to the candidate preparations because of the wide variability of results within and between different collagen reagents when assayed against the fourth International Standard for Factor VIII and von Willebrand Factor, Plasma, Human. Candidate preparation 00/514 was selected and established as the first Interna-

tional Standard for von Willebrand Factor, concentrate, with potencies of 11.0 IU/ampoule for vWF:Ag and 9.4 IU/ampoule for vWF:RCO.

The Expert Committee agreed that the fourth International Standard for VIII and von Willebrand Factor, Plasma, Human, coded 97/586, should be relabelled with the newly assigned value of 0.83 IU/ampoule for the collagen-binding activity (vWF:CB).

Streptokinase

The Committee noted the report of the WHO collaborative study (BS/01.1948) organized to calibrate a candidate material as a replacement for the current second International Standard for Streptokinase, 88/826, stocks of which are almost exhausted. Two candidate preparations were assayed against the second International Standard in a study involving 16 laboratories from 12 countries representing four WHO Regions: preparation 88/824, coded B, and preparation 00/464, coded C and D (coded duplicates). Preparation 88/824 had been included in the previous WHO collaborative study, in which the second International Standard had been established and preparation 00/464 was a material from a different manufacturer newly distributed into ampoules. The assays used in the participating laboratories were either a fibrin clot lysis assay, a chromogenic method provided with the protocol of the study, or one of a variety of in-house fibrin and non-fibrin-based methods.

With the exception of one laboratory in which outlying results were reported for preparation 00/464, there was good agreement between laboratories and no significant differences between potencies as measured by the different methods. Inter-laboratory variability, expressed as the geometric coefficient of variation was 6.4% for preparation 88/824 and 4.8% for preparation 00/464. The potency of 88/824 reported in this study was very similar to that obtained in the previous collaborative study in 1988, indicating its good long-term stability and the good continuity of methodology.

Although preparations 88/824 and 00/464 were both suitable, in terms of comparability and assay variability, as candidate replacements for the second International Standard, preparation 00/464 was considered to be more representative of currently manufactured materials and more ampoules of this preparation are available (4000). Accelerated degradation studies on ampoules of preparation 00/464, stored for 12 months at temperatures of up to 45 °C have found no loss of biological activity, indicating that its stability is acceptable.

The Committee therefore established preparation 00/464 as the third International Standard for Streptokinase with a potency of 1030 IU/ampoule. This proposal had been agreed to unanimously by the participants of the collaborative study and by the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis.

Unfractionated heparin and low-molecular-weight heparin

The Committee was informed of efforts to develop an internationally harmonized method to resolve differences in potency of about 10% between the International Standard and the United States Pharmacopeia (USP) standard for Low-Molecular-Weight Heparin. A working group had met in Paris in July 2001, and proposed that a study using the chromogenic method for measuring potency should be carried out which would also include the methods of USP, European Pharmacopoeia and WHO. The current International Standard, the European Pharmacopoeia standard and the current and proposed new USP standards were to be included. Filling of the material was to take place shortly.

The Committee was reminded that the first International Standard for low-molecular-weight heparin was established in 1986 as a result of the identification of non-parallelism when low-molecular-weight heparin was assayed against unfractionated heparin. More products are now marketed and several of those used in the original study are no longer manufactured. Moreover, methods for the assay of low-molecular-weight heparin are increasingly harmonized, specifically in the use of assays based on anti-thrombin (2A) or anti-thrombin (10A) activity. The need for a specific standard for low-molecular-weight heparin and its suitability for current products were therefore examined in a collaborative study. Eight products were examined in preliminary studies by the two assays. The variability in potency was much greater when expressed against the unfractionated heparin. Two of the preparations showed statistically significant deviations from parallelism when the eight preparations were standardized each against the other. Two preparations were chosen as potential standards, and large amounts obtained for distribution into ampoules. The physicochemical properties of the proposed low-molecular-weight heparin standards and their method of production are to be provided at submission to assist traceability, and the *European Pharmacopoeia* standard will be included in the study to ensure continuity. Concern was expressed about the two products found to deviate from parallelism in the assays, and it was requested that the manufacturers be approached to establish their views on the suitability of either of

the proposed standards for assaying their materials before the main collaborative study was undertaken.

Cytokines, growth factors and endocrinological substances

Human chorionic gonadotrophin

The Committee noted the report of the collaborative study of six molecular forms of human chorionic gonadotrophin (hCG) proposed as candidate WHO Reference Reagents (BS/01.1944). These preparations had been extensively characterized by physicochemical and immunological methods and calibrated in nanomols by amino acid analysis. The Committee agreed that the design of the collaborative study was appropriate and considered that the preparations would be valuable reagents for characterizing the specificity of hCG immunoassays.

However, the Committee requested that the report be amended in the following ways:

— to reflect the status and proposed use (immunoasay) of the preparations in the title of the document;
— to include a revised estimate for each of the extinction coefficients in the results section; and
— to clarify the intended use of the preparations in the text.

In view of the results of the study, but subject to the above modifications, the Committee established:

- Preparation coded 99/688 as the first WHO Reference Reagent for immunoassay of intact hCG, with a content of 1.88 nmol/ampoule
- Preparation coded 99/642 as the first WHO Reference Reagent for immunoassay of nicked hCG (hCG-n), with a content of 0.78 nmol/ampoule
- Preparation coded 99/650 as the first WHO Reference Reagent for immunoassay of hCG beta subunit (hCG-β), with a content of 0.88 nmol/ampoule
- Preparation coded 99/720 as the first WHO Reference Reagent for immunoassay of hCG alpha subunit, (hCG-α), with a content of 0.84 nmol/ampoule
- Preparation coded 99/708 as the first WHO Reference Reagent for immunoassay of hCG beta core fragment) hCG-βcf, with a content of 1.02 nmol/ampoule

- Preparation coded 99/692 as the first WHO Reference Reagent for immunoassay of nicked hCG beta subunit (hCG-βn), with a content of 0.33 nmol/ampoule.

The Committee further noted and endorsed the recommendation that the existing International Standard for human chorionic gonadotrophin should not be disestablished, but should remain the primary standard for the calibration of diagnostic immunoassays for hCG. The use of the above Reference Reagents should, in the first instance, be in investigating and characterizing the specificity of existing hCG assays.

Ciliary neurotrophic factor

The Committee noted the report of a collaborative study (BS/01.1943) that showed the suitability of a lyophilized recombinant human ciliary neurotrophic factor, coded 94/684, to serve as a Reference Reagent for the bioassay of ciliary neurotrophic factor (CNTF). The Committee requested that, in accordance with established guidelines for the preparation of WHO Reference Reagents, a certificate of analysis of the bulk material should be appended to the copy of the report lodged with WHO.

Subject to this amendment of the report lodged with WHO, the Committee established preparation 94/684 as the as the first WHO Reference Reagent for the bioassay of Human Ciliary Neurotrophic Factor with an assigned potency of 8000 units per ampoule.

In making this recommendation, the Committee further noted that:

— in the event of the development of bioassays of different specificity, the suitability of the Reference Reagent may need to be re-evaluated; and
— for the purposes of immunoassay calibration, a nominal content of 6.5 µg CNTF per ampoule should be assumed.

Prolactin and its glycosylated and non-glycosylated components

A preparation of recombinant DNA-derived human prolactin and two of its purified components, a glycosylated form and a non-glycosylated form, were examined and compared with the current International Standard for Prolactin, coded 84/500, in bioassays and immunoassays in a collaborative study in 15 laboratories in eight countries to assess their suitability as Reference Reagents. The Committee noted the report of this collaborative study (BS/01.1942), and agreed that the candidate preparations of rDNA prolactin and its

glycosylated and non-glycosylated components were suitable to serve as Reference Reagents for immunoassays of these analytes.

The Committee requested that the report of the collaborative study be amended to:

— reflect in the official titles of the three preparations their primary function as immunoassay standards;
— reflect the primary function of the preparations as immunoassay standards in the title of the document; and
— include appropriate details of the characteristics of the bulk preparations.

Subject to these modifications the Committee agreed that:

- The preparation coded 97/714 be established as the first WHO Reference Reagent for Prolactin, recombinant human, with a nominal content of 24.5 µg/ampoule
- The preparation coded 98/580 be established as the first WHO Reference Reagent for Prolactin, recombinant human, glycosylated, with a nominal content of 5.5 µg/ampoule
- The preparation coded 98/582 be established as the first WHO Reference Reagent for Prolactin, recombinant human, non-glycosylated, with a nominal content of 10.5 µg/ampoule.

The Committee further agreed that, for the purposes of bioassay calibration, the preparation coded 97/714 may be assumed to contain 1400 mU/ampoule; the preparation coded 98/580 may be assumed to contain 88 mU/ampoule; and the preparation coded 98/582 may be assumed to contain 670 mU/ampoule.

The Committee further noted and endorsed the recommendation that the existing International Standard for Prolactin should not be disestablished, but should remain the primary standard for the calibration of diagnostic immunoassays for prolactin. The use of the above reference reagents should, in the first instance, be in investigating and characterizing the specificity of existing prolactin assays and determination of the suitability of using recombinant DNA-derived prolactin as an immunoassay standard should be a priority.

Miscellaneous

Pertussis toxin standard

A preparation designated JNIH-5 has been used as an unofficial standard in pertussis toxin assays for many years. It appears to have functioned adequately with all types of preparation and a study was

undertaken to regularize the status of JNIH-5, specifically with regard to its use in the quality control of pertussis vaccines with respect to the histamine sensitizing and Chinese hamster ovary cell assay for pertussis toxin. The collaborative study involved six participants in six countries who assayed JNIH-5, the in-house standard from the National Institute for Biological Standards and Control (NIBSC standard) and any in-house reference available.

The Committee noted a report of this study (BS/01.1949) and the proposal that JNIH-5 be established as the International Standard for histamine-sensitizing and Chinese hamster ovary cell assays of pertussis toxin. In all laboratories the potency of the NIBSC standard relative to JNIH-5 was comparable although the absolute values varied widely. However, the Committee questioned the designation of JNIH-5 as an International Standard with an activity expressed in International Units. The need for a standardized method for assay of pertussis toxin was stated. It was pointed out that there were many different in-house pertussis toxin reference materials that appeared to differ in specific pertussis toxin activity, but that it was difficult to differentiate the impact of variations in methodology from intrinsic differences in toxin activity. The Committee recommended that the issue be referred to the WHO Working Group on pertussis vaccines and that a decision be taken on the status of the material at a future meeting of the Expert Committee.

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Annex 1
Guidelines on clinical evaluation of vaccines: regulatory expectations

This document provides guidance for national regulatory authorities and vaccine manufacturers on the clinical evaluation of vaccines by outlining the international regulatory expectations applicable to the different stages of vaccine development and for marketing approval. For this reason, the guidance in this document could also be useful for clinical researchers and investigators.

The text is presented in the form of guidelines rather than recommendations because vaccines are a heterogeneous class of agents, and the preclinical and clinical testing programmes will need to be adapted for each individual product. Guidelines allow greater flexibility than recommendations with respect to specific issues related to particular vaccines.

A separate WHO document intended to provide more detailed guidance on preclinical and laboratory evaluation of vaccines is in preparation. This was subsequently established by the 54th meeting, November 2003, of the WHO Expert Committee or Biological Standardization and is to be published in the WHO Technical Report Series. The section of this document that discusses preclinical and laboratory evaluation consequently provides general guidance, but does not define international regulatory expectations in this area.

Introduction

This document provides guidance to national regulatory authorities (NRAs), manufacturers, clinical researchers and investigators on the clinical evaluation of vaccines by outlining the data that should be obtained during the different stages of vaccine development to support an application for marketing approval. This document has been prepared in response to requests from NRAs for assistance in the evaluation of clinical trials, both during the clinical development of a new vaccine and during the regulatory review of dossiers submitted in support of applications for marketing authorization. The NRAs

should have a mandate to review protocols, and when this is necessary to protect the safety of subjects, to require revision of the protocol and/or termination of the trial. This document is intended to provide basic guidance to NRAs on how to achieve these objectives. Because it is common practice for the clinical development programmes and the individual clinical trials to take place in different countries, each NRA should, as far as possible, collaborate with the other regulatory authorities involved to benefit from shared experiences and to align regulatory considerations (*1*).

The World Health Organization (WHO) has made available the following guidelines and requirements that are relevant to the evaluation of vaccines: good clinical practice for trials on pharmaceutical products (*2*), good manufacturing practice for pharmaceutical preparations (*3*, *4*), good manufacturing practice for biological products (*5*), regulation and licensing of biological products in countries with newly developing regulatory authorities (*1*) and guidelines for national authorities on quality assurance for biological products (*6*). Guidelines and recommendations for the production and control of specific vaccines have been reviewed in detail in a series of WHO technical reports (*7*), which should be consulted where applicable but will not be discussed further here. However, there is no existing WHO document that gives guidance on the planning, performance and assessment of clinical studies on vaccines with a regulatory perspective. Specific WHO guidelines that complement this document are available for malaria (*8*) and dengue (*9*) or are in preparation in the case of certain candidate vaccines, such as for human immunodeficiency virus (HIV). Basic standards of care, including details about the cold chain required for transport and storage of vaccines, proper injection techniques for delivery of vaccines and safety of injections have already been described in the WHO manual *Immunization in practice* (*10*).

Guidance on various aspects of clinical trials of vaccines is also available from several other bodies such as the International Conference on Harmonization (ICH), the European Agency for the Evaluation of Medicinal Products (EMEA), the United States Food and Drug Administration (FDA) and the United Kingdom Medical Research Council (MRC). These WHO guidelines are not intended to conflict with, but rather to complement, these other documents (*11–16*, *18–39*).

Regulation of vaccines

Regulatory issues related to a particular candidate vaccine should be considered early in the development process, since compliance with regulatory requirements is the basis for eventual approval. It is strongly recommended that dialogue with the appropriate national regulatory authority be established early on. The national regulatory authority should review the plans for development of the candidate vaccine and clarify requirements for carrying out clinical trials, as well as for marketing approval.

The regulation of vaccines can be divided into three stages: developmental, licensure and postlicensure (*40*). The developmental stage consists of two parts, preclinical research and development, and clinical research and development.

Preclinical testing

Preclinical research and development are carried out in the laboratory using in vitro techniques or, when necessary, in vivo techniques in animals. The data from preclinical and laboratory research include details of the development and production of a vaccine together with reports of control testing, which should be adequate to justify subsequent clinical studies in humans.

Phases of clinical development (I–III)

Clinical trials in humans are classified into three phases: phase I, phase II and phase III and in certain countries formal regulatory approval is required to undertake any of these studies. This approval takes different forms in different countries (e.g. Investigational New Drug Application (IND) in the United States and Clinical Trial Certificate or Clinical Trial Exemption (CTX) in the United Kingdom). This is in addition to ethical clearance which is required for clinical trials in all countries. All studies of human subjects require proper ethical review, in accordance with the Declaration of Helsinki (see http://www.wma.net/e/).

The phase I clinical studies carry out initial testing of a vaccine in small numbers (e.g. 20) of healthy adults, to test the properties of a vaccine, its tolerability, and, if appropriate, clinical laboratory and pharmacological parameters. Phase I studies are primarily concerned with safety. Phase II studies involve larger numbers of subjects and are intended to provide preliminary information about a vaccine's ability to produce its desired effect (usually immunogenicity) in the target population and its general safety. To fully assess the protective

efficacy and safety of a vaccine, extensive phase III trials are required. The phase III clinical trial is the pivotal study on which the decision on whether to grant the licence is based and sufficient data have to be obtained to demonstrate that a new product is safe and effective for the purpose intended.

By the beginning of the phase III stage of development, a vaccine should have been fully characterized and the final manufacturing process, specifications and batch release testing procedures should have been established. An application for market authorization may be submitted to an NRA on the basis of the data from phase III testing and if approved, the vaccine then becomes commercially available in that particular country. If a product contains or consists of genetically modified organisms an environmental risk assessment should also be undertaken and approved by the appropriate agency.

The structure of the clinical development programme must be tailored to the type of vaccine and the antigenic content. For example, the clinical evaluation of a vaccine that contains only novel antigen(s) may of necessity be very different from that of a vaccine that contains one or more previously evaluated antigens. Such factors also influence whether clinical protection trials will be required, whether or not they are feasible, or whether an approval may reasonably be based on immunogenicity data. In all instances, it is the obligation of the applicant to justify the content and structure of the clinical development programme. Pre-submission meetings with regulatory authorities may assist in ensuring that the content of the final data package is likely to be acceptable.

Issues to be considered after the initial licensure

In addition to phase I, II and III studies that may be performed before or after the first licensure of a new vaccine, which are described under other relevant trials as outlined above, the postmarketing period is critical for the collection of data on the safety and effectiveness of a vaccine in large numbers of recipients; these data may come from both active and passive modes of surveillance. Following licensing, there is continued surveillance of vaccinees for adverse events, especially for those rare events that can be detected only in very large numbers of subjects.

Any change in production methods or scale-up following licensing will necessitate further product characterizations to demonstrate equivalence, although the extent of re-characterization required depends on the nature of the changes implemented. Further characterizations should be documented and the NRA should be notified of all

changes. Regulatory authorities should clearly define and implement in their regulations which changes require only a notification and which changes require a formal approval before they can be introduced. This will be decided on a case-by-case basis and, in all instances, regulatory approval for a change must be obtained before the vaccine is used.

Glossary

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

Adverse event
Any untoward medical occurrence in a clinical trial subject to whom a vaccine has been administered; it does not necessarily have a causal relationship with the vaccine/vaccination.

Adverse reaction
A response to a vaccine that is noxious and unintended and that occurs at doses tested in humans for prophylaxis, or during subsequent clinical use, following licensure. The term adverse reaction is usually reserved for a true causal association with a drug or a vaccine.

Attack rate
The proportion of the population exposed to an infectious agent who become (clinically) ill.

Audit
A systematic examination, carried out independently by persons not directly involved in the clinical trial, to determine whether the conduct of a trial complies with the agreed protocol and whether the data reported are consistent with the records on site, e.g. whether data reported or recorded in the case report forms are consonant with those found in hospital files and other original records.

Blinding
A procedure in which one ore more parties to the trial are kept unaware of the treatment assignment(s). Single blinding usually refers to the subject(s) being unaware of the treatment assigned to them, and double blinding usually refers to the subject(s), investigator(s) and, in some cases, data analyst(s) being unaware of the treatment assignment.

Booster vaccination
Vaccination given at a certain time interval (at least 6 months) after primary vaccination in order to induce long-term protection.

Bridging studies
Studies intended to support the extrapolation of efficacy, safety and immunogenicity data from one formulation, population or dose regimen to another.

Case–control study
An observational study in which the exposure to a particular risk factor (the vaccine in the case of vaccine studies) is determined retrospectively, and the effect of this exposure is compared between individuals (the cases) who experience an event (the disease, in vaccine studies) and individuals who do not (the controls).

Case definition
A set of diagnostic criteria that must be fulfilled to confirm a case of a particular disease. Case definitions can be based on clinical criteria, laboratory criteria or combinations of the two.

Case report form
A document used to record data on a subject participating in a clinical trial during the course of the trial, as defined by the protocol. The data should be collected by procedures that guarantee preservation, retention and retrieval of information and allow easy access for verification, audit and inspection.

Cluster
Aggregation of relatively uncommon events or diseases in space and/or time in amounts that are believed or perceived to be greater than could be expected by chance.

Cohort study
A retrospective or prospective study in which the development of a disease or infection, or any other relevant event, is observed over time in a defined group of subjects.

Colonization
The asymptomatic, often transient, presence of a microbe as a part of the normal microflora of a host (e.g. pneumococci on the mucosae of the upper respiratory tract).

Community investigation
A population-based trial in large predefined segments of the population to investigate the impact of a treatment on a preventable infectious disease.

Comparator product
A pharmaceutical or other product (which may be a placebo) used as a reference in a clinical trial.

Contact
An individual who has had contact with an infected person (case) in a way that is considered as having caused significant exposure and therefore a risk of infection.

Control
Any comparator suitable for validation of the trial. The comparator may be either an active treatment or a placebo control.

Equivalence trial
A trial having the primary objective of showing that the response to two or more treatments differs by an amount that is clinically unimportant. Showing that the true treatment difference is likely to lie between a lower and an upper equivalence margin of clinically acceptable differences usually demonstrates this.

Experimental study
A study in which the conditions are under the direct control of the investigator. Such studies may include random allocation of subjects to treatment or control groups and blinding of subject and investigator to the placement status (i.e. whether in the treatment or control group).

Exposure
Having contact with an infectious agent in a way that experience has shown may cause disease.

Foreign clinical data
Clinical data generated outside the target region (i.e. in a foreign region).

Geometric mean titre
Calculation of the average titre for a group of subjects by multiplying all values and taking the *n*th root of this number, where *n* is the number of subjects.

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

Good manufacturing practice
That part of the pharmaceutical quality assurance process which ensures that products are consistently produced and to meet to the quality standards appropriate to their intended use and as required by the marketing authorization. In these guidelines, good manufacturing practice refers to the current good manufacturing practice guidelines published by WHO.

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

Incidence
The number of persons who fall ill with a certain disease during a defined time period.

Informed consent
A subject's voluntary confirmation of his or her willingness to participate in a particular trial, and the documentation thereof. This consent should be sought after giving the subject appropriate information about the trial, including an explanation of its status as research, its objectives, potential benefits, risks and inconveniences, alternative treatment that may be available, and of the subject's rights and responsibilities in accordance with the current revision of the Declaration of Helsinki.

Inspection
An officially conducted examination (i.e. review of the conduct of the clinical trial, including quality assurance, personnel involved, any delegation of authority and audit) by relevant authorities at the site of the trial and/or the site of the sponsor in order to verify adherence to good clinical practice as set out in these guidelines.

Internal control
An additional control arm in a vaccine trial, usually a placebo, which may be required when the efficacy of the active comparator is not adequately established or is known to give inconsistent results.

Investigator
A person responsible for the clinical trial and for the rights, health and welfare of the subjects in the trial. The investigator should have qualifications and competence in accordance with the local laws and regulations as evidenced by an up-to-date curriculum vitae and other relevant credentials. Decisions relating to medical or dental care, and their provision must always be the responsibility of a clinically competent person legally allowed to practice medicine or dentistry.

Minimal risk
A level of risk similar to the risk encountered during an individual's usual daily activities. Minimal risk would apply to activities such as physical examination, venipuncture or urine sample collection.

Non-inferiority trial
A trial with the primary objective of showing that the response to the product under investigation is not clinically inferior to the control vaccine (active or placebo).

Observational studies
Observational studies focus on events, exposures and diseases occurring in the population during their everyday life, not subject to experimental interventions.

Outbreak
The occurrence of two or more linked cases of a communicable disease.

Placebo control
A comparator in a vaccine trial that does not include the antigen under study. In studies of monovalent vaccines this may be an inert placebo (e.g. saline solution or the vehicle of the vaccine), or an antigenically different vaccine. In combined vaccines, this may be a control arm in which the component of the vaccine being studied is lacking.

Post-marketing surveillance
A system for monitoring adverse events following licensure. Postmarketing surveillance can be passive or active and its objectives include, but are not limited to, the following:

— the identification of rare adverse reactions not detected during pre-licensure studies; and
— the identification of risk factors or pre-existing conditions that may promote reactions.

Potency
The quantitative measure of the specific ability or capacity of the product to achieve a defined biological effect.

Pre-exposure trial
A prospective trial in a population expected to be exposed to the pathogen under study within a predefined, relatively short, period.

Prevalence
The number of persons who have a particular disease at a specific time.

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.

Protocol
A document that states the background, rationale and objectives of the clinical trial and describes its designs, methodology and organization, including statistical considerations, and the conditions under which it is to be performed and managed. The protocol should be signed and dated by the investigator, the institution involved and the sponsor. It can also serve as a contract.

Randomization
In its simplest form, randomization is a process by which n individuals are assigned to a test (n_T) or control (n_C) treatment so that all possible groups of size $n = n_T + n_C$ have equal probability of occurring. Thus randomization avoids systematic bias in the assignment of treatment. It also promotes balance with respect to known and unknown prognostic factors that could affect the outcome of interest. While it does not guarantee that treatment groups will be exactly equal with respect to these factors, it does guarantee that any imbalance that occurs arose purely by chance. The process of randomization guarantees the validity of statistical analyses of treatment effect, and (with adequate sample size) allows the detection, or ruling out, of small or moderate treatment differences.

Reactogenicity
Reactions, either local or systemic, that are considered to have a causal relationship to the vaccination.

Reproductive rate
The average number of secondary cases of an infection arising from a single primary case. The measure is inherent to the potential (infectiousness, susceptibility, measures of protection) of a microorganism to spread from person to person in a population.

Secondary attack-rate study
An outbreak investigation in a defined susceptible population. The population to be studied is either a cluster (in an urban or semi-urban setting) or a household (or family). Outbreak investigations may be either observational or experimental. The unit of randomization may be the individual, a household or a cluster.

Sensitivity (statistical)
The probability that a test will detect a disease/condition when it is used on an individual who truly has the disease/condition. It is estimated in a study as the proportion of individuals with positive test results out of all individuals classified by a gold standard as having the disease/condition.

Serious adverse event
An event occurring in connection with the clinical trial that results in death, admission to hospital, prolongation of a hospital stay, persistent disability or incapacity, or is otherwise life-threatening.

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 preexisting antibodies, seroconversion is defined by a transition from a predefined low level to a significantly higher defined level such as a fourfold increase in geometric mean antibody concentration.

Serological surrogate
Predefined antibody concentration correlating with clinical protection.

Serosurveillance
The surveillance of an infectious disease by measuring disease-specific antibodies in a population or subpopulation.

Specificity (statistical)
The probability of a negative test result when a test is used on an individual who truly does not have the disease/condition. It is esti-

mated in a study as the proportion of individuals with negative test results out of all individuals classified by a gold standard as not having the disease/condition.

Sponsor
An individual, a company, an institution or an organization that takes responsibility for the initiation, management and/or financing of a clinical trial. When an investigator initiates and takes full responsibility for a trial, the investigator has then also assumed the role of the sponsor.

Standard deviation
The measure of the variability of a sample of observations around the mean.

Superiority trial
A trial with the primary objective of showing that the response to the product under investigation is superior to the control vaccine (active or placebo).

Surveillance
The systematic collection, collation and analysis of data and the dissemination of information to those who need to know in order that appropriate action may be taken.

Survey
An investigation in which information is systematically collected. It is usually carried out in a sample of a predefined population group for a defined time period. A survey is not a continuous investigation and may be repeated after a period of time. If repeated regularly, surveys can form the basis of a surveillance system.

Vaccine (protective) efficacy
The reduction in the chance or odds of developing clinical disease after vaccination relative to the chance or odds when unvaccinated. Vaccine efficacy measures direct protection (i.e. protection induced by vaccination in the vaccinated population sample). Vaccine efficacy is calculated according to the following formula:

$$\mathrm{VE} = \left(\frac{Iu - Iv}{Iu}\right) \times 100\% = \left(1 - \frac{Iv}{Iu}\right) \times 100\% = (1 - \mathrm{RR}) \times 100\%$$

where Iu = incidence in unvaccinated population; Iv = incidence in vaccinated population; RR = relative risk

Vaccine effectiveness
The protection rate conferred by vaccination in a specified population. Vaccine effectiveness measures both direct and indirect protection (i.e. protection of non-vaccinated persons by the vaccinated population). Vaccine effectiveness is also determined by vaccination coverage, correlation of vaccine strains with circulating strains and incidence of disease due to strains not included in the vaccine following introduction of the vaccine in that population.

Vaccine failure
The onset of infection or disease, biologically confirmed, in a subject who is supposed to be protected, following completion of age-appropriate immunization as recommended by the manufacturer.

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

Vector
A carrier, most often an animal or arthropod that transfers a pathogen from an infected person(s) or animal to a susceptible individual.

Scope of the document

Vaccines are a heterogeneous class of prophylactic medicinal products containing antigenic substances capable of inducing specific, active and protective host immunity against an infective agent or toxin, or against other important antigenic substances produced by infective agents. Vaccines for human use contain one of the following: microorganisms inactivated by chemical and/or physical means that retain adequate immunogenic properties; living microorganisms that are avirulent to humans or have been selected for their attenuation whilst retaining immunogenic properties; or antigens extracted from organisms, secreted by them, or produced by recombinant DNA technology. The antigens may be in their native state, detoxified by chemical or physical means and/or aggregated, polymerized or conjugated to a carrier to increase immunogenicity.

This document also covers novel products such as DNA vaccines and live genetically engineered microorganisms used themselves as vaccines or used as carriers for other antigens. However, therapeutic

vaccines (e.g. viral-vector-based gene therapy, tumour vaccines and anti-idiotypic vaccines such as monoclonal antibodies used as immunogens) are *not* considered here.

Part A. Preclinical and laboratory evaluation of vaccines

A.1 General remarks

The preclinical evaluation of a vaccine is a prerequisite for the initiation of clinical trials. Laboratory evaluation should however be continued throughout both the preclinical and clinical phases of vaccine development. This section on preclinical and laboratory testing discusses the general principles for the nonclinical evaluation of vaccines which should be taken into consideration both before and during clinical trials. (A document which deals with the nonclinical and laboratory evaluation of vaccines in more detail has also been prepared by WHO. Established by the 54th meeting, November 2003, of the WHO Expert Committee on Biological Standardization and to be published in the WHO Technical Report Series.)

The primary goal of preclinical testing of a new vaccine product, or a new combination vaccine comprised of previously licensed antigen(s), or vaccines presented in new formulations or new delivery systems, should be to demonstrate that the vaccine is suitable for testing in humans.

Preclinical and laboratory studies are aimed at defining the characteristics (physical, chemical and biological) of a product, including the indicators of safety and immunogenicity in an appropriate animal model. When preclinical testing is performed in animals, there should always be a clear rationale for doing so, and the study should be performed in compliance with Good laboratory practice guidelines (*11*) and with national guidelines on animal experimentation. In addition to establishing the characteristics of the candidate vaccine, preclinical and laboratory studies may also identify possible risks to the vaccinees, and can be used to plan protocols for subsequent clinical studies in human subjects in which safety and efficacy of the candidate vaccine are evaluated.

Close collaboration between the preclinical and the clinical investigators is particularly important in assessing the first results of the administration of vaccines in humans. The clinician, in consultation with the appropriate advisers, has, however, the responsibility of ensuring that the preclinical experiments are adequate in scope and for requesting a full account of all relevant data.

A.2 Production, characterization and quality assurance of candidate vaccines

The basic principles for the production and control of vaccines are set out in the relevant publications in WHO Technical Report Series which cover general requirements (*41–46*). Specific guidelines and recommendations for particular vaccines are also available (*7*) and should be consulted as appropriate. The WHO guidelines and recommendations are often adopted by national regulatory authorities as definitive national requirements. Other useful guidance may be obtained from the documents produced by other bodies (*47*). The characterization, standardization and control of the components, safety and potency of vaccine preparations are key issues during development. The amount of data collected to support clinical studies should increase throughout phases I and II, and product characterization should be completed by the beginning of the phase III stage of development. In-process testing should be performed to ensure adequate control over the manufacturing process and manufacturing consistency. Analytical criteria should be established during product development and used subsequently to evaluate new batches and to establish batch-to-batch consistency. The tests adopted for routine batch release should be a selection of those tests used for the initial characterization of the vaccine. A batch release protocol providing an outline of production and a summary of the test results and establishment specifications should be available for each batch.

Candidate vaccines for clinical trials should be prepared according to good manufacturing practices. The general manufacturing recommendations contained in good manufacturing practices for pharmaceutical and biological products (*3–5*) should be applied by all establishments involved in producing candidate vaccine for clinical studies. Standard operating procedures covering all aspects of production, quality control, storage and distribution should be documented.

Any proposed change in the formulation of a vaccine should be considered carefully both by the manufacturers and NRAs. Some changes in formulation may have a serious effect on the quality, safety and efficacy of vaccines and will subsequently require clinical trials.

Sufficient stability data should be generated to support clinical trials. Accelerated stability data could be used to support preliminary data generated at the normal storage temperature. Further data on stability to support the expiry date of the product for licence should be based on long-term, real-time, stability studies under the real conditions of use. All relevant documentation should be made available to the regulatory authorities.

In accordance with good clinical practice, sufficient samples of each batch of candidate vaccine, together with a record of analyses and characteristics, must be kept for future reference by the manufacturer and ideally a national control laboratory (NCL) for possible subsequent re-testing and investigation. The product should be stored under safe and stable conditions for at least the duration of its anticipated or approved shelf-life and preferably longer.

A.3 Toxicity and safety testing

Toxicity studies in animals may be considered for the assessment of the potential toxic effects of a vaccine in target organs, including the haematopoietic and immune systems as well as to assess systemic toxicity. Such studies may help to identify potential toxicity problems requiring further clinical monitoring. Detailed guidance on toxicological and pharmacological testing may be found in the EMEA *Note for guidance on preclinical pharmacological and toxicological testing of vaccines* (*12*). However, it should be recognized that a suitable animal model may not be available for undertaking toxicological evaluation of candidate vaccines, and such models are not necessarily predictive of human responses the interpretation of the results may be difficult. Furthermore, a classical repeated dose toxicity test as applied to medicines may or may not be applicable for vaccines. Applicability of repeated dose toxicity tests depends on the vaccine dose regimen and the composition of the vaccine. Usually there is no chronic exposure of the subject to a vaccine through repeated administration.

The design and value of repeated-dose toxicity tests should therefore be considered on a case-by-case basis, as should the selection of the animal species used for these investigations. If a vaccine is intended to be clinically tested in women of childbearing age, the need for reproductive toxicity studies and studies of embryo/fetal and perinatal toxicity should be considered on a case-by-case basis. Reproductive toxicity studies, where appropriate, will need to be undertaken before licensing.

Toxicity tests should include:

— an evaluation of the initial safe dose and of subsequent dose escalation schemes relevant to the clinical dose;
— an evaluation of single and repeated doses as appropriate;
— a determination of a set of relevant safety parameters for clinical monitoring;
— a demonstration of potential reversibility of virulence of attenuated vaccine strains;

— a demonstration of the completeness of inactivation for inactivated vaccine strains;
— a demonstration of the completeness of inactivation as well as reversibility to toxicity of toxoids;
— local tolerability studies; and
— an evaluation of the potential of the vaccine antigen(s) to induce antibodies that cross-react with human tissues, where appropriate (e.g. streptococcal vaccine).

Where different routes of administration are proposed, multiple safety and toxicity studies in a suitable animal model should be considered. These should address the specific safety concerns associated with administration of the vaccine by each of the proposed routes. Caution is recommended when extrapolating safety data obtained using one route of administration to other routes.

A.4 Potency and immunogenicity

A.4.1 *Potency*

Where relevant, potency tests should be established during vaccine development and used for routine batch release. Examples of potency assays are challenge models such as the intracerebral mouse test for pertussis and rabies vaccines, and evaluations of infectious units of live attenuated organisms for viral vaccines and bacille calmette-Guèrin (BCG). Ideally, the potency assay should mimic the clinically expected function of the vaccine in humans (as for rabies vaccine). However, in many cases, this is not possible and the assay is based on artificial challenge procedures that assess clinical protection (e.g. potency test for whole cell pertussis vaccine). For polysaccharide vaccines chemical characterization may be sufficient. For products for which little is known about the pathogenic mechanism and or the protective factors, animal testing with subsequent serological evaluation or challenge testing is informative. However, as understanding of the mechanism of protection and immunity to vaccine increases, every effort should be made to replace in vivo potency assays with validated in vitro alternatives based on the biological activity of the product, test systems and novel laboratory methods as they become available.

A.4.2 *Immunogenicity*

Data obtained from the immunization of animals with candidate vaccine preparations will provide valuable information to support a clinical indication. Such studies may include testing in non-human primates, but only if an appropriate disease model is available. Immu-

nogenicity data derived from animal models can help in the selection of the doses, schedules and routes of administration to be evaluated in clinical trials. Preclinical studies should be designed to assess the relevant immune responses, e.g. seroconversion rates, geometric mean antibody titres, or cell-mediated immunity in vaccinated animals. Such studies may also address interference between antigens and/or live viruses. If a vaccine consists of more than one antigen (e.g. acellular pertussis vaccine) the response to each antigen should be evaluated. Immunogenicity studies may include the characterization of antibody class, avidity, affinity, half-life, memory, and potential induction of cell-mediated immunity as well as release of soluble mediators affecting the immune system, as appropriate.

Of primary concern in interpreting the data obtained from such studies should be how closely the animal models resemble the human disease and human immune responses. For example, the demonstration of humoral antibody responses in an animal model to a vaccine delivered mucosally (i.e. oral or nasal) may be irrelevant to the evaluation of the clinically expected secretory and cell-mediated immune response.

Although immunogenicity testing in animals may be necessary during the development of a vaccine to demonstrate its ability to induce an appropriate immune response, an animal immunogenicity test may not always be needed for routine lot release (e.g. *Haemophilus influenzae* type b conjugate vaccine) (*48*).

A.5 Special considerations

A.5.1 *Adjuvants*

Adjuvants may be included in new vaccines to promote appropriate immune responses to particular antigens, 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 reactogenicity.

Compatibility of the adjuvant(s) with all the antigenic components of the vaccine should be demonstrated. Where relevant, adsorption of all the antigenic components present in the vaccine, should be shown to be consistent on a lot-to-lot basis. Possible desorption of antigen during the shelf-life of the product should be evaluated, reported and specifications set. If a new adjuvant is proposed for use in a vaccine formulation, appropriate preclinical studies are necessary (*12*, *49*). It should be noted that no adjuvant is licensed in its own right, but only as a component of a particular vaccine. If no toxicological data exist for a new adjuvant, toxicity studies of the adjuvant alone should first

be performed. Preclinical animal studies to determine the safety profile of the combination of adjuvant and vaccine should also be undertaken.

Preclinical studies should evaluate the combination of adjuvant and antigen as formulated for clinical use. In the case of new adjuvants prepared to replace the well-established aluminium adsorbants in a vaccine already in use, the inclusion of appropriate control groups of animals is important. These groups may include one group receiving the antigen alone, and a group receiving the antigen adsorbed to an aluminium compound.

A.5.2 *Additives (excipients and preservatives)*

If a new additive such as a preservative or excipient is to be used, its safety should be investigated and documented. If a new preservative is used, its safety as well as efficacy or appropriateness for use in a particular product must be documented. The safety of new additives can be evaluated using vaccine formulations without antigen. However, the compatibility of a new additive with all vaccine antigens should be documented as well as the toxicological profile of the particular combination of antigen(s) and additive in animal models.

A.5.3 *Other types of product requiring special considerations*

Some types of data and testing are specific for certain types of product, such as genetic stability for recombinant vaccines, data concerning the inactivation and attenuation methods, demonstration of comparability of combination vaccines, contribution of adjuvants and safety/toxicity studies for particular vaccines.

A.5.3.1 Combination vaccines

New combinations of antigens or serotypes should be studied for appropriate immunogenicity in an animal model, if available, before initiation of clinical trials in humans (*13*, *14*). The response and the quality of response to each of the antigens in the vaccine should be assessed. 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. Interference between live vaccine strains may also be studied in animal immunogenicity tests.

A.5.3.2 DNA vaccines

Special considerations concerning the production and control of DNA vaccines as well as their preclinical evaluation are covered in WHO guidelines for assuring the quality of DNA vaccines (*42*).

A.5.3.3 Recombinant vaccines

WHO guidelines for assuring the quality of pharmaceutical and biological products prepared by recombinant DNA technology should be consulted (*43*).

A.5.3.4 Synthetic peptide vaccines

Detailed information concerning the production and control of synthetic peptide vaccines, including preclinical safety evaluation is available in guidelines for the production and quality control of synthetic peptide vaccines (*15*, *44*).

A.5.3.5 Live attenuated vaccines

The major concern related to live attenuated vaccines is potential reversion to virulence and the possible transmissibility and exchange of genetic information with wild type or other microorganisms. Every effort should be made to identify markers of attenuation (genetic sequences) which should be used in clinical trials to monitor the results of excretion studies and during clinical evaluation, phase by phase. A specific example of a live attenuated vaccine is the poliomyelitis vaccine, oral (*50*).

Part B. Clinical evaluation of vaccines

B.1 General remarks

Before the start of clinical trials (particularly phase III trials), a sound understanding of the epidemiology of the pathogen or disease of interest in the intended study population is needed. This requires population-based or outbreak evaluations of individuals exposed to, at high risk of, or suffering from, the disease in question. Such studies define disease incidence, the proportion of infected persons who develop clinical disease and the risk of transmission. The understanding of the full clinical spectrum of illness and the optimization of diagnostic criteria as well as definition of the high-risk groups frequently defined by age, gender, ethnic or population group membership, social characteristics as well as geography and seasonality of exposure, is essential for accurate vaccine evaluation. Consideration should also be given to defining laboratory values (e.g. for platelet counts and leukocyte counts) in the intended study population. The use of inappropriate laboratory values often results in too many people failing to meet the "criteria for inclusion". The laboratory values in the protocol should therefore reflect "normal" values in the population in question. In some developing countries, these may differ consider-

ably from those accepted as normal in industrialized countries due to widespread concurrent infections (e.g. with helminths). Seroprevalence studies should also be undertaken, where appropriate, to assess at-risk populations and to evaluate potential protective mechanisms, such as persistence of maternal antibodies. This is particularly important for the evaluation of live attenuated vaccines in infants because pre-existing maternal antibodies can prevent infection with attenuated vaccine strains. The determination of sample size of study population as well as the duration of the trial necessary to achieve a statistically meaningful result with respect to efficacy and safety requires a clear understanding of the incidence of the disease in question. An understanding of the background incidence of various adverse reactions, including those that are specific to the wild type pathogen is essential.

All clinical trials should adhere to the standards described for good clinical practice. The general principles of the WHO guidelines for good clinical practice already in place for trials of pharmaceutical products, also apply to vaccine studies. However, vaccines demand special consideration because:

- Vaccines are given to healthy individuals, mostly children and infants.
- Vaccines are given to prevent disease; this limits tolerability of adverse events.
- Vaccines are biological products which are highly complex substances derived from living materials, and sometimes comprising living organisms. They require specialized assays and testing to assure their quality and safety on a lot-to-lot basis.

Consistency of manufacturing for the vaccine lots used in clinical trials should be demonstrated and well documented. These lots should be adequately representative of the formulation intended for marketing. Clinical data may be required to help to demonstrate manufacturing consistency.

B.2 Methodological considerations

This section describes some methodological considerations common to the different phases of vaccine evaluation. Methodological considerations are vital to the outcome of all clinical studies and they should be given careful attention during the trial design stage. The methods used in all trial protocols should be clearly delineated. Existing effective preventive measures (e.g. bednets for malaria, counselling for HIV) should be continued for trial participants (*2*, *51*).

B.2.1 *Study population*

The initial phase I study is usually conducted in healthy, immunocompetent adults who are at low risk of the infection or complication against which the vaccine protects. Generally, the trial population for phases II and III should be chosen to represent the group that will be the target for the vaccination in an immunization programme. Care should be taken to identify the target population correctly. If a vaccine is intended for children or other vulnerable populations, it should be tested in a small number of subjects from the intended population, usually after at least one phase I study has been completed in healthy adults, but before proceeding to studies in a larger number of subjects from the intended population. Definitive criteria for inclusion or exclusion of subjects in the clinical trial should be established in advance.

B.2.1.1 Inclusion and exclusion criteria for enrolment in the trial

Specific inclusion and exclusion criteria should be defined for each phase of a trial. The subjects enrolled in the trial should be in the required age group, resident within the defined study area(s) during selection, examined by the study physician and able to give their signed informed consent (in the case of children, the consent of the parent(s) or guardian is required). Previous exposure to vaccines and antigens should be recorded for all participants.

Subjects should be excluded from the trial if they do not meet the medical or other eligibility criteria, for example, if they suffer a chronic illness with signs of cardiac or renal failure, suspected progressive neurological disease, uncontrolled epilepsy or infantile spasms, have received other vaccinations within 1 or 2 weeks of administration of the test vaccine, or are receiving long-term treatment with antibiotics. Immune status should also be considered when deciding whether or not an individual may participate in the study (e.g. immunodeficiency, immunosuppression and/or prematurity). Other criteria for exclusion of participants from a study might include a planned move from the study area within the period of follow-up, social and/or language difficulties or other circumstances that interfere with communication and follow up. However, the number of potential participants excluded should be kept to minimum.

Criteria should also be established for contraindications to the administration of a subsequent (second or third) dose of vaccine, if applicable. These might include serious reaction after the first or second dose (e.g. neurological reaction), fever greater than or equal to 40 °C within 48 hours of administration or a generalized allergic reaction within 48 hours of administration.

B.2.2 *Outcome measurement*

The primary end-point should be the most relevant for the disease in the target population.

B.2.3 *Safety*

When safety is the primary end-point in a clinical trial, the adverse event or reactogenicity (local or systemic) considered to be of primary importance should be the major focus in trial design. The safety profile should be representative of, and predictive for, the target population for which the vaccine is to be used in practice (see also B.2.7, monitoring and reporting adverse events).

B.2.4 *Immunogenicity*

In phases I, II and III, immunogenicity data are recorded as an outcome, and in certain circumstances may be used to demonstrate clinical efficacy (see below).

B.2.4 *Efficacy*

In phases II and III, clinical protection outcomes may be measured. Studies in which the end-point is clinical efficacy should be performed in areas where an appropriate impact of active immunization can be expected, and where a controlled trial is feasible. Pre-exposure studies should thus preferably be performed in an area with low endemicity, or in an area with few individuals who have natural long-term protection.

The outcome of a trial is measured as vaccine efficacy and/or vaccine effectiveness. Immunogenicity studies may be sufficient to demonstrate clinical efficacy for vaccines containing a known antigen for which the level of protective antibody is well established (see Correlates of protection, B.7.2.3). If protection cannot be measured as an end-point alternative parameters to be measured should be justified.

B.2.5 *Factors influencing the choice of outcome measurement*

The choice of outcome measurement in a specific trial may be constrained by scientific, logistical, economic or ethical considerations. When a randomized-controlled trial using clinical end-points is not feasible, alternative strategies need to be considered (*52*). The feasibility and validity of such alternative strategies should be considered in the protocol. Evaluation of the feasibility of a serological correlate of protection should address the relationship between the surrogate end-point and the clinical end-point, bearing in mind that this relationship may not necessarily be linear or direct.

B.2.5.1 Vaccine efficacy

Vaccine efficacy could be measured as an outcome of clinical protection and/or as an immunological surrogate end-point based on immunological response. The definition of clinical cases should be given in the protocol (see Case definition and case ascertainment, B.2.6). The inclusion of cases for whom confirmation (e.g. microbiological) was not possible should be justified in the protocol. When relevant, both clinical and serological end-points should be studied and the data presented in the report. The formula by which vaccine efficacy is calculated should be defined and validated (see Glossary) (*53*, *54*).

B.2.5.2 Vaccine effectiveness

The effects of vaccination at the population level depend on the coverage and distribution of the vaccine, as well as on its efficacy in preventing disease and preventing colonization (*54*). In addition to the intrinsic efficacy of the vaccine, its effectiveness depends on the heterogeneity in susceptibility, rates of exposure to infectious agents and protection conferred by the vaccination (*55*). Vaccine effectiveness may also be influenced by time-related changes in protection caused by intrinsic properties of the vaccine (waning of efficacy and boosting) (*54*, *56*, *57*), changes in vaccination coverage, and population characteristics (such as age distribution).

B.2.6 *Case detection, case ascertainment and case definition*

The outcome of trials of clinical protection by a vaccine will depend critically on case definition, as well as on the sensitivity and specificity of case detection and case ascertainment. Sensitivity determines the power of the study, specificity of the predictive value and safety estimate (*54*).

It is essential that the case definitions for the trial end-points be clearly defined at the outset. Case definitions and methods of case detection should be justified and described in the study protocol. The protocol should substantiate and provide a full discussion of the consequences of the anticipated sensitivity and specificity of the case definition. Defined and validated methods should be applied consistently for the duration of the study, at all study sites.

B.2.6.1 Case detection

The methods used for detecting cases should be the same in both vaccinated and unvaccinated populations.

- If attack rates are high, the number of cases in the population of interest may be sufficient to estimate vaccine efficacy accurately in a relatively small population and a relatively short time.

- If attack rates are low, enrolment (sample size) and/or duration of follow-up may need to be increased to detect sufficient cases to enable a precise estimation of efficacy. If this is not possible, other surveillance data may be used to detect other potential cases and subsequently increase the precision of the estimate.

In cohort studies all cases from both the vaccinated and non-vaccinated groups should be included in the analysis. This practice is consistent with the philosophy of "intent-to-treat" (*58*).

In secondary attack rates trials all cases in the target group found in the surveyed household or cluster during the predefined time period should be included, as well as the case which led to the cluster being studied.

Case–control studies use the same case-detection methods as other study designs, but not all cases need be detected.

B.2.6.2 Case ascertainment and case definition

The case definitions should be developed, defined and clearly documented in the study protocol before any efficacy study commences. This ordinarily involves using the efficacy definition(s) in an earlier phase of clinical development. The validity of the diagnosis is most important for an adequate evaluation of the efficacy or safety of a vaccine. When the diagnosis is based on defined clinical criteria, justification and validation of these criteria should be provided. Confirmation of cases using laboratory methods, antigen detection and the clinical signs is necessary to support a clinical case definition.

Specific and sensitive methods properly validated for case ascertainment and consistent use of a reliable and valid case definition are vital to the useful outcome of a study (*59*). Highly specific methods may be needed in certain cases, but are not always available.

Consideration should also be given to defining in the study protocol when and how, in the event of a vaccine failure, the immunological evaluation of study subjects and typing of the infecting microorganism will be performed after unblinding, or as part of planned interim analysis, including where possible:

— evaluation of clustering of cases of the disease in the population with serological and/or microbiological confirmation; and
— information on the antigenic match between vaccine strains or serotypes and circulating strains or serotypes, to provide insight into the possibility of strain or serotype selection.

B.2.7 *Monitoring and reporting adverse events*

An adverse event in a vaccine trial is any untoward medical occurrence in a clinical trial subject administered the vaccine; it does not necessarily have a causal relationship with the vaccine or vaccination. It is critically important, especially in vaccine trials, that adverse events are actively monitored and reported swiftly. The NRA may require the sponsor and/or the investigator to report certain types of adverse events or reactions (e.g. serious or previously unknown events) to itself and to the Independent Ethics Committee. Investigators should report all serious adverse events to the sponsor immediately unless they are identified by the protocol as not needing to be reported immediately. Investigators should also comply with the applicable regulatory requirements related to the reporting of unexpected serious adverse reactions to the NRA and the independent ethics committee. Investigators should be trained adequately for this purpose. After the trial has been completed or terminated, all recorded adverse events should be listed, evaluated and discussed in the final report. Reporting of adverse events should be part of the protocol design.

Standardized methods should be used for investigating and reporting local and systemic adverse events following vaccination. All safety information should be recorded and the procedure for reporting adverse events should be described in the protocols (see guidelines for good clinical practices (2)). The instructions should include details of:

— who is going to make the report (e.g. study investigators or nurses subjects, parents or guardians);
— how the reporting is planned (e.g. using questionnaires or diary cards);
— duration of follow-up; and
— the intervals of reporting (e.g. daily, weekly).

Adverse events following vaccination should be well documented.[a] The report should include evaluation of injection-site reactions (pain, induration, erythema) and systemic events (fever, nausea, malaise, headache, anaphylaxis), at baseline, at pre-specified vaccination times and following vaccination. Any difference in safety profile related to injection site or route of administration should be recorded. For vaccines administered to children and infants, reactions should be recorded both by the parents and by the study investigator or nurse in a structured manner. Parents should be contacted by the study inves-

[a] A useful set of recently established definitions is available at: http://www.brightoncollaboration.org.

tigator or nurse at defined intervals after vaccination to check for any reactions. Before the second and/or third doses (if applicable) parents of infants and children, or the vaccine recipients themselves, should be asked by the study investigator or nurse about reactions to the previous dose. Also, the investigator or nurse should consult the previous vaccination records of the individual in question.

The procedure for recording adverse events should be defined and carried out at appropriate intervals and for a sufficient duration. Every effort should be made to improve the quality of the reporting of adverse events, for example by the use of standardized forms (e.g. case report forms, subject diaries). Furthermore, such forms should include questions about specific adverse events or findings including qualitative and quantitative parameters, as appropriate. For example, temperature should be measured by pre-specified methods. The forms should also allow for the recording of unsolicited events. Prior instructions for the use of diary cards and follow-up visits or contacts by clinical study staff should be given. All model forms to be used for monitoring should be provided with each protocol.

For some trials, such as large-scale phase II and phase III trials and post-marketing surveillance studies, data safety monitoring boards (DSMBs) need to be in place, to ensure adequate safety monitoring. In special cases DSMBs may also be required for phase I studies (*51*). DSMBs must be independent and preferably linked to the independent ethics committee (see guidelines for good clinical practices). If necessary, a DSMB may initiate a new study to further investigate the nature of the adverse events following vaccination seen in the original trial. In the case of serious adverse events an Institutional Review Board should unblind a study and, if necessary, stop a trial and report its findings to the appropriate NRA. Safety monitoring of trial participants should continue for a defined period after the trial has ended.

Consistency in safety reporting may be improved by increased reporting in the published literature. Issues that pertain to the publication of study data should be considered in the design of study protocols.

B.3 Statistical considerations

B.3.1 *General principles*

Statistical analysis should be based on the recommendations made in relevant WHO documents, where available, and or other suitable guidelines. Early phase trials are often exploratory and may lack the statistical power for definitive inferences. However, if the aim of a study is to provide conclusive information, e.g. the final determination

of the optimal dose for use in a pivotal, phase III trial, then the study should be rigorously designed, powered and statistically analysed, regardless of the phase of investigation. Otherwise, the issues discussed below pertain primarily to phase III trials. Essentially, the recommendations are as follows:

- The procedures for randomization and blinding should be described in the study protocol.
- The primary and secondary objectives of the study should be clearly stated.
- The protocol should state explicitly the outcome variables to be analysed, the null and alternative hypothesis to be tested, the significance level the anticipated power and the statistical methods to be used for assessing each end-point.
- For the evaluation of efficacy, intent-to-treat estimates should accompany traditional per-protocol estimation. Intent-to-treat estimates will include all protocol-defined cases of disease, without regard to completion of vaccine series or compliance with protocol, and will include follow-up from the time of randomization (*58*). The reasons for removal of any subject from the efficacy or safety analysis should be described in detail in the study reports.
- If interim analyses for efficacy are planned, this information should be included in the protocol together with appropriate significance level adjustments to be implemented.
- Statistical estimates should include confidence intervals (*60*).

B.3.2 *Trial objectives: efficacy and safety*

B.3.2.1 Establishing efficacy

The efficacy of a new vaccine can most convincingly be demonstrated in a randomized, double-blind, placebo-controlled trial based on a clinical disease end-point. The placebo may be an inactive product or a vaccine for a different disease, believed to be ineffective in preventing the disease of interest. This type of trial is called a superiority trial, because the vaccine must be sufficiently superior in efficacy to the placebo to be acceptable (see section B.3.3.1). High specificity of case definition is desired because it is well known that low specificity has a deleterious effect on the ability of a study to estimate vaccine efficacy accurately (*59*). The aim of these trials is not to test a hypothesis regarding efficacy, but rather to estimate efficacy with both a point estimate and the corresponding confidence interval (usually 95%). The size of sample chosen for these trials depends on disease incidence rates in the study population, as well as on the anticipated level of efficacy of the vaccine that is considered to be clinically relevant.

There are, however, situations in which vaccine efficacy cannot be determined from cases of disease. For example incidence of a disease in a population may have been reduced to very low levels by widespread immunization with a previously licensed vaccine. When the serological parameters are known to correlate with clinical protection, evaluation of a new vaccine for the same disease is based on measures of the vaccine's immunogenicity. One or more immune response outcome variables thus serve as "surrogates" for determining efficacy. Since the comparator in this setting is typically the already-licensed vaccine, evaluation of the new vaccine is based on establishing its "non-inferiority" to the licensed vaccine (see section B.3.3.2). Statistical inference of non-inferiority is based on the appropriate confidence interval excluding a pre-specified difference in immune response believed to be clinically meaningful. The size of sample required for establishing non-inferiority of immune response depends upon the variability in the immunogenicity measurements and on the level of efficacy of the comparator vaccine.

B.3.2.2 Evaluating safety

Most vaccine trials are not aimed at testing specific hypotheses regarding adverse events. Consequently, safety assessment is generally characterized by exploratory data analysis. Descriptive statistics are presented and confidence intervals are often informative. *P*-values may be useful for detecting signals of possible vaccine-associated adverse events for further evaluation.

If the detection of a few serious adverse events that have been specified prospectively is the primary focus of a large pre-licensure safety trial, it is advisable to consider a multiplicity adjustment for testing the corresponding small number of hypotheses. This multiplicity adjustment should be accounted for in the determination of the sample size. Otherwise, if there are no a priori hypotheses regarding specific adverse events, meaning that an undetermined number of safety analyses will be performed, adjustment for multiplicity is not generally performed during initial evaluations of the clinical trial data. Signals in the data suggesting possible vaccine-related adverse events may be investigated further for the determination of a potential causal association. However, the effect of multiple testing should be considered before the final decisions are made regarding any safety signals detected. If a serious, unexpected event occurs, prospective monitoring for additional events might be added to the protocol, and formal statistical testing could be implemented. Further general guidance on the statistical evaluation of safety has been published by the International Conference on Harmonization (*39*).

B.3.3 *Study designs (superiority, non-inferiority and two-sided equivalence trials)*

B.3.3.1 Superiority trials

Superiority trials of vaccines are generally based on cases of disease. The control is either a placebo or a vaccine that has no effect on the disease of interest. The purpose of these trials is to estimate the percentage reduction in the incidence rate of disease due to use of the vaccine. The point estimate of this percentage reduction may be obtained by various methods: as a ratio of risks, incidence rates, or hazards (see definition of vaccine efficacy in the glossary). There are also a number of statistical methods for obtaining the confidence interval on vaccine efficacy (*60*).

B.3.3.2 Non-inferiority (one-sided equivalence) trials

A non-inferiority trial of vaccine efficacy is generally designed to show that the use of a new vaccine gives a relative risk, relative incidence rate or relative hazard rate of a disease, infection, etc., when compared to the control, is not greater than a pre-specified clinically relevant quantity. In a non-inferiority trial based on immune response, the relative effect of interest may be a difference in proportions of subjects responding in a pre-specified manner, or a ratio of geometric mean titres or concentrations. For the former, the trial is designed to show that the proportion of subjects responding to the new vaccine is not less than the proportion of subjects responding in the control group by as much as a pre-specified quantity (often 0.10). For the evaluation of titres, the trial may be designed to demonstrate that the ratio of the geometric mean titre (or concentration) of the new vaccine relative to the control is not less than some pre-specified ratio (e.g. 0.50 or 0.67).

The comparative outcome measure for a non-inferiority trial for an adverse event can be either a difference or a ratio of risks. If a ratio is to be obtained, the trial is designed to show that the relative risk of the adverse event occurring with the new vaccine relative to the occurrence in the control is not greater than a pre-specified ratio (e.g. 1.5). If the difference in rates of adverse events, is required, the trial is designed to show that the risk of the adverse event occurring with the new vaccine is not greater than the risk with the control by as much as a pre-specified quantity.

Because non-inferiority evaluations are one-sided, statistical inference is based only on the upper or lower confidence limit, whichever is appropriate for the aim of the study. The null hypothesis (to be rejected) is that the difference between vaccinated and control subjects is greater than the lower or upper equivalence margin. Alterna-

tively, inference may be based on the corresponding one-sided confidence limit.

B.3.3.3 Two-sided equivalence trials

A two-sided equivalence trial, such as might be used to compare two vaccine lots, is designed to show that the outcome measure for one group is similar in both directions to that for another group. The reason that the evaluation of lot consistency is inherently two-sided is that there would be concern if an outcome measure for one lot were either too high or too low when compared to another lot. Such a finding might suggest that the two lots are not similar enough to be considered to be consistently manufactured. The lots are considered equivalent, or consistently manufactured, when a two-sided confidence interval for the appropriate relative effect (e.g. ratio of geometric mean antibody concentrations or relative risk of adverse event) falls entirely within pre-specified limits. The choice of the equivalence margins should be scientifically justified. Thus, statistical inference is based upon both upper and lower confidence limits.

B.3.3.4 Accepted difference or ratio in equivalence and non-inferiority trials

The quantity to be ruled out as the criterion for non-inferiority or equivalence should be based on clinical, laboratory and statistical judgement. It may be based on evidence from previous trials and/or laboratory assay data. In a trial of relative efficacy, the equivalence or non-inferiority criterion should be sufficiently achievable so that, if the new vaccine meets the criterion, it is clear that it will provide an acceptable level of protection from disease. The feasibility of attaining a sample of the appropriate size may also be a factor in the choice of the criterion; the calculated sample size can be very large when the criterion is easily achievable or the variability of the outcome measure is large.

B.3.4 ***Sample size***

The number of subjects participating in a clinical trial must be sufficient to provide a reliable answer to the questions posed. The sample size in a trial of vaccine efficacy should be large enough to allow precise interval estimation of efficacy. Sample size is usually determined by the primary end-point chosen. Generally, the sample size should be large enough to ensure that the lower confidence limit for efficacy will be considerably greater than zero. A sufficiently high lower confidence limit is desirable to ensure a minimal level of vaccine efficacy.

The protocol should clearly explain calculations of sample size required for each primary end-point (immunogenicity, safety and efficacy) and the largest estimate should determine the number of subjects to be enrolled. The amount of information requested prior to licensing and the feasibility of obtaining it need to be carefully balanced.

B.3.4.1 Sample size in non-inferiority/equivalence trials

The sample size should be such that, if a new vaccine is truly non-inferior, there is a high probability that the appropriate confidence interval for the relative effect of interest will not exceed the predefined non-inferiority criterion. Alternatively, for equivalence trials, there should be a high probability that both the upper and lower confidence intervals will fall within the predefined upper and lower equivalence margins. Methods of sample size calculation specially designed for non-inferiority/equivalence trials should be used. Non-inferiority trials of vaccine efficacy based on clinical outcomes usually require much larger samples than placebo-controlled superiority trials or non-inferiority trials based on immunogenicity measurements (*61*).

Undersized superiority trials that give non-significant results will not generally allow any conclusions to be made regarding non-inferiority or equivalence.

Useful information on statistical principles for clinical trials is published by the International Conference on Harmonization (*39*).

B.3.4.2 Considerations underlying sample size determination in efficacy evaluations

The criteria underlying the determination of sample size are based on methodological and statistical considerations, as well as on epidemiological and scientific judgement. Factors to be taken into account include the expected incidence of the disease and its prevalence (endemic spread, epidemic spread, or low-incidence disease). These factors may vary from product to product and from one setting to another.

B.3.4.3 Sample size considerations in immunogenicity evaluations

The evaluation of immunogenicity, when part of an efficacy trial with a clinical end-point, should ideally be conducted in a randomly selected subsample from the population initially enrolled. When immunogenicity is the only primary end-point, it should be studied in individuals representative of the target population. Sample size will depend upon the aim and design of the study, as well as the variability

of the immune response measurements. In certain situations (e.g. when too few subjects are available for immunogenicity testing) additional methodologies could be used in order to increase the number of study subjects. Aspects such as the appropriate choice of control and expected protection rates should always be taken into account.

B.3.4.4 Sample size considerations in safety evaluations

Prior to licensure, comparative studies of common adverse events (e.g. injection site reactions with diphtheria, tetanus, pertussis, whole cell DTPw) require large numbers of subjects to give them sufficient power to detect small differences. The same is true for cohort studies intended to detect serious uncommon adverse events. For evaluation of common local reactogenicity, approximately 300 subjects are needed for each comparison group. However, depending on the type of vaccine, the disease indication, and the target population, enrolment of more than 5000 subjects may be appropriate to provide reasonable assurance of safety pre-licensure in randomized, controlled settings. These numbers are based on a one-sided confidence interval when no adverse events are observed. They increase if one adverse event is observed.

The investigation of uncommon or rare events already occurring in the study population requires long-term prospective population-based surveillance studies. These are often not feasible in pre-marketing trials and such data are obtained from postmarketing surveillance studies. In practice, such events are studied either in retrospective closed cohorts and/or in case–control studies. Valuable sources of information for such purposes are large databases with records of vaccinees. These databases may include several hundreds of thousands of subjects for evaluation.

B.3.5 ***Duration of study***

The impact of a particular vaccination schedule is evaluated by the primary outcome measure of the clinical trial. In principle, all vaccines under development need a long-term evaluation plan. In most confirmatory clinical trials this implies a follow-up period of at least 6 months subsequent to the last vaccination. However, this will depend upon the outcome measurement chosen (i.e. clinical end-point, immunogenicity or safety), the vaccination strategy and the novelty and/or type of the vaccine. Long-term follow-up may be undertaken for the whole study population or in a relevant subset.

For vaccines intended for use in immunization programmes, subjects should be followed up for at least 1 year following the last vaccination to obtain serological and clinical information on the persistence of

protection and the possible need for a booster vaccination. In situations where safety evaluation is a primary outcome, different follow-up periods may be appropriate and should be considered on a case-by-case basis. Fully documented information on follow-up should be obtained for as many individuals enrolled in the trial as possible until all final outcomes are recorded.

B.4 Ethical considerations

For information on the clinical standards and ethical issues to be considered in the design and conduct of vaccine trials, WHO guidelines for good clinical practices should be followed (*2*). Compliance with these standards provides assurance that the rights, safety and well-being of trial subjects are protected, in accordance with the principles that have their origin in the Declaration of Helsinki (*16*). For any study, a review by an independent ethics committee, functioning in accordance with good clinical practice standards, is mandatory (*17*).

To assure protection of the rights of research subjects, the approval of the appropriate independent ethics committee must be obtained before the start of the trial. No subject may be included in a clinical trial without proper informed consent in writing. Informed consent for children should be obtained from their parent or guardian.

The specific roles and responsibilities of the ethical review boards and regulatory authorities are country-specific.

Special attention should be given to the ethical considerations underlying testing of vaccines in healthy infants, children, pregnant women and the elderly. The use and nature of a placebo should be carefully considered as should the use of human challenge studies. Human challenge studies are appropriate only for selected diseases that have no serious complications or long-term sequelae and for which successful treatment is available. Such studies can provide valuable information on the pathophysiology, clinical manifestations, diagnosis, immunology, treatment response and most importantly protective efficacy of vaccines.

Subjects participating in vaccine trials should not be exposed to unreasonable or serious risks of illness or injury and measures should be in place to ensure that all subjects receive the full benefits of scientific innovation. An adjustment may be needed to an existing national vaccination programme after careful consideration of the possible benefits of innovations. It is important to ensure that economically and socially deprived communities, which are often those at the great-

est risk of disease, are not exploited in conducting research that will be of no benefit to them. Detailed information is available in the ethical guidance documents issued by WHO, Council for International Organizations of Medical Sciences (CIOMS), UNAIDS and other bodies (*17–20*) and these should be consulted as appropriate. Other relevant national or international requirements must also be considered (such as from the US Office for Human Research Protections (OHRP)).

B.5 Phase I studies

If appropriate animal challenge models for the evaluation of immunogenicity or efficacy parameters are available, data from such studies should be provided before starting the clinical trial programme. However, if such models are not available, relevant data from alternative approaches and/or from in vitro testing may need to be considered to provide proof of concept in support of a proposed clinical development plan.

Phase I studies should be undertaken to define acceptable safety and reactogenicity of a vaccine candidate as well as to obtain preliminary information on its immunogenicity (*62*). The dose and method of administration should also be assessed with respect to these parameters. Generally phase I studies are small-scale studies of which the primary focus is the determination of clinical tolerance and safety.

All phase I studies should be conducted in research environments with adequate laboratory support and very carefully monitored. Phase I studies are usually-open label studies and are not randomized with placebo control groups. However, there is a recognized need for controlled trials, even in phase I, to allow at least some comparison of intercurrent common non-vaccine induced events. When possible, the concomitant use of other vaccines or therapeutic agents should be avoided to optimize the safety evaluations. Phase I studies might be conducted in several different age or population groups because of differences in, for example, dose, safety, vaccine schedule, route of administration or disease risk. Where appropriate, laboratory testing (e.g. complete blood count and liver function tests) should be undertaken to establish a baseline database. A short period of evaluation in a clinical research centre or extended observation in a clinic, day-care centre or home environment is recommended for close monitoring of vaccinees. Less intensive phase I trials might involve daily visits by a research nurse to the home or day-care centre or daily return visits by the subject to the clinic.

Live attenuated vaccines (viral or bacterial) are potential causes of clinically significant infections in the recipient or in contacts. Major concerns in the evaluation of a live attenuated vaccine include the possible shedding of the agent, transmission to contacts, potential genetic variability and reversion to a more virulent state. Therefore, such vaccines require intensive investigations in closely monitored clinical settings. Initial studies of candidate attenuated vaccines should be undertaken to make preliminary evaluations of dose ranges, immune responses, clinical signs of infection and reactogenicity (immediate, early and late). Phase I studies may provide preliminary information on shedding, reversion characteristics, transmission to contacts and genetic stability.

Phase I studies may provide data that are useful in the design of further clinical phase studies.

B.6 Phase II studies

Once phase I studies have been successfully completed with a satisfactory outcome, a candidate vaccine should then undergo phase II clinical evaluation. The main distinction between phase I and phase II studies is that phase II studies involve larger numbers of subjects, and are often randomized and well controlled. The outcome measures, however, are often similar. Phase II vaccine trials are intended to demonstrate the immunogenicity of the relevant active component(s) and the safety profile of a candidate vaccine in the target population. Ultimately, the phase II studies should define the optimal dose, initial schedule and safety profile of a candidate vaccine before the phase III trials can begin.

Phase II studies should be undertaken to evaluate multiple variables associated with the host immune response such as age, ethnicity, gender and presence of maternal or pre-existing antibodies. In future trials, genotype may also need to be considered. Other factors to be investigated to determine their influence on immune response include:

— dose of vaccine;
— sequence or interval between vaccine doses;
— number of doses of vaccine; and
— route of vaccine administration.

The duration of immunity, potential need for booster immunizations and qualitative aspects of the immune response may also be investigated. A single study can address several questions, although several

studies are often required to obtain definitive evaluations. If the answer to the scientific question under study will be final, e.g. the determination of the optimal dose to be used in a large phase III efficacy trial, then the phase II trial should be rigorously designed, adequately powered and appropriately analysed to provide conclusive information.

For a live attenuated vaccine, continued active monitoring of specific parameters into the second and third week, or more, post-vaccination is recommended. The duration of follow-up may be determined by a number of factors that may have been identified in the phase I studies including the degree of shedding, transmission and potential reversion characteristics.

The immune responses to vaccine antigen(s) should be carefully evaluated and are a critical part of phase II clinical studies. Such studies are intended to further characterize immune responses elicited by a particular immunogen thought to be relevant to protection, such as level, class, subclass and function of the specific antibodies produced, as well as appearance and duration of adequate antibody titres. Other relevant information such as presence of neutralizing antibodies or cross-reactive antibodies, formation of immune complexes, cell mediated immunity and any interaction that might affect the immune system (e.g. preexisting antibodies, concomitant administration of another vaccine or drugs) should be recorded.

The percentage of responders should be defined and described based on predefined criteria for assessing the immune response (e.g. antibodies and/or cell-mediated immunity). For vaccines for which the immunological correlates of protection are not known, the immunological profile should be studied in detail. Subjects who fulfil immunogenicity criteria (often seroconversion) are regarded as responders (having seroconverted) and the result of an immunogenicity study includes the proportion of responders. For the validation of an immune response, sera should be collected from all participants at regular, predefined intervals throughout the study period. For certain vaccines (e.g. nasally administered vaccines) the investigators should consider whether samples from other body fluids should also be collected. Immunological data from phase II trials should be documented, including geometic mean titre, median, standard deviation, and the range of antibodies in pre and post-vaccination sera (*63*). In the case of vaccines for which the end-point is the induction of antibodies, the immunological data should be presented by dividing the pre- and post-vaccination titres, or antibody concentrations according to arbitrary (or, if known, protective) antibody levels (e.g. 0.01, 0.1

and 1 IU/ml for diphtheria and tetanus antibodies). Presenting reverse cumulative distribution curves may provide additional insight (*64*, *65*). When available, standardized assay methodologies should be used, and details may be found in WHO recommendations, European Pharmacopoeia monographs or US Food and Drug Administration documents. Each assay should be fully documented and consistent use of a validated assay is essential.

B.7 Phase III studies

The phase III studies are large-scale clinical trials designed to provide data on vaccine efficacy and safety. These studies are usually performed in large populations to evaluate efficacy and safety of formulation(s) of the immunologically active component(s). In large-scale efficacy studies of this type, that may enroll many thousands of subjects, serological data are usually collected from at least a subset of the immunized population at pre-defined intervals. It is also important to collect serological data from all persons classified as vaccine failures.

When vaccines containing the same antigens are already in common use and/or the incidence of disease is very low, it may not be feasible to perform a formal study of protective efficacy. In such instances, the phase III trials, although involving larger numbers of persons than previous phases, will be confined to the evaluation of immune responses and comparison with any recognized correlates of protection. However, sometimes there are no established and unequivocal immunological correlates of protection. In such cases, it is important that some attempt should be made to estimate the effectiveness of the vaccine after its licensure and widespread introduction. Phase III trials involve a larger number of subjects than were included in the earlier phases of development and, thus, provide expanded safety assessments.

The duration of follow-up should be determined taking into account the type of vaccine and other relevant factors (e.g. disease incidence, characteristics of immune response to vaccine, and anticipated and safety profile of the vaccine.)

Whether or not a prophylactic vaccine is ultimately accepted as a general public health measure depends upon the availability of clear and definitive evidence that the vaccine is safe and actually able to prevent the infectious disease in question or to significantly reduce the adverse consequences of the disease.

B.7.1 *Considerations for formal trials of protective efficacy*

Vaccine efficacy is the percentage reduction in the incidence rate of a specific disease in vaccinated individuals as compared to that in unvaccinated individuals. Vaccine efficacy measures direct protection (i.e. protection induced by vaccination in the vaccinated population sample).

B.7.1.1 Trial design

Two general approaches can be applied to efficacy studies; they can be either experimental studies or observational studies. The gold standard for assessing the prevention of disease or infection in a phase III trial is the prospective randomized double-blind controlled trial of protective efficacy. This design will control for other variables that might affect disease risk and avoids potential bias in the assessment of end-points. Thus this design maximizes the chance that a difference in disease incidence between two equivalent groups is due to a true effect of the vaccine being evaluated. However, in certain circumstances other approaches may be necessary. Great care should be taken when designing a vaccine trial to maximize efficiency and to eliminate bias. Observational studies of efficacy or effectiveness are usually part of phase IV post licensure studies.

B.7.1.2 Randomized double-blind controlled trials

The most effective efficacy trials are double-blind, randomized and controlled. This design controls for other variables that might affect disease risk by prospectively randomizing groups being studied. Double-blinding is necessary to avoid bias in the assessment of endpoints. The choice and feasibility of blinded, randomized-controlled trials depends on the vaccination strategy and on the demographic and epidemiological characteristics of the study population. The following approaches may be used:

— prospective cohort studies for population-based vaccination strategies; and
— pre-exposure cohort studies in-groups at risk of the target infection (e.g. vaccination for travellers).

A double-blinded evaluation of disease outcomes minimizes potential ascertainment bias and, therefore maximizes the chance that a difference in disease incidence observed between two equivalent groups is due to a true effect of the vaccine being evaluated.

Randomization is necessary to avoid bias in the assignment of the participants to one of the study groups and it permits statistically valid comparisons to be made between different arms of a study. It allows

the detection of small differences between vaccines and comparators; this is particularly important when an active control is used. Non-randomized study designs such as the use of historical controls or case–control studies allow only larger differences to be detected. If possible, these non-randomized approaches should be avoided in phase III trials.

The unit of randomization is usually the individual included in the trial and this is ideally the unit of statistical analysis. In some situations, however, it may be necessary to randomize on the basis of clusters or groups, e.g. school, geographical or political region (*66*). It is important to specify the randomization procedures and to adhere to them. Failure to do so may lead to biased results. Every effort should be made to use randomized well-controlled designs for phase III trials. However, such studies can be technically difficult and the decision to undertake them should be made on a case-by-case basis.

B.7.1.3 Other approaches for obtaining efficacy data

Several alternative types of study may be considered, depending upon the incidence and epidemiology of the disease of interest, the characteristics of the population and the expected efficacy of the vaccine or prophylactic agent. However, the use of designs other than double-blind randomized-well controlled trials to provide efficacy data is allowed only when fully justified. The possible alternative approaches include:

— secondary attack rate study, or household contact study (which can be randomized);
— uncontrolled, open studies (used only to collect additional information on serological responsiveness and tolerance);
— observational cohort studies; and
— case–control studies.

Secondary attack rate study

A secondary attack rate study is a specific type of pre-exposure cohort trial that usually requires smaller sample sizes than other randomized controlled trials. This may be the method of choice in studies of infections with a relatively high secondary attack rate in closed communities and/or susceptible populations (*53*, *67*). The unit to which the intervention is applied may be the individual, family (household) or community (environment) and the unit of randomization will correspond with this. Randomization of groups or clusters rather than of individuals may be preferred in the following situations:

— when a vaccination programme is to be conducted in a geographical area or community
— when it is logistically easier to administer the vaccine to groups than to individuals; and
— when the purpose of the vaccination is to reduce transmission of the infection, where the unit is the "transmission zone" (the area in which humans, vectors and intermediate hosts interact and share a common pool of pathogens).

Groups of subjects (or clusters), the population and the geographical area under investigation should all be defined in the protocol. Data regarding the presence of infecting pathogens and their attack rates are essential. The follow-up period for subjects after contact with the index case may be short; as a minimum it should cover the assumed incubation period and infectious period of the index cases and secondary contacts. The inclusion period for new cases and controls and their contacts should be set at a maximum of 6 months following the detection of the first case. Inclusion over a longer period may introduce bias in favour of vaccine efficacy, because the exposure to the infecting pathogen and thus the risk of infection will be reduced in the vaccinated groups or clusters compared with that in unvaccinated groups or clusters (*54*).

Observational cohort studies

Supportive evidence may be obtained from observational cohort studies if randomized-controlled trials or secondary attack rate trials are not ethically justified, or are not feasible due to low incidence of the disease or there is a requirement for long-term follow-up for the calculation of efficacy. Such studies provide an estimate of the value of a vaccine for operational purposes.

Observational cohort studies in a clinical programme for marketing approval may be considered in those unusual situations in which a double-blind randomized controlled trial is not ethically justified or where the clinical end-point requires long-term follow-up (e.g. hepatitis B vaccination in neonates (see B.9.3.1)), or where the number of individuals is too large to follow up (*69*). However, the absence of randomization is a major limitation (*70*). Where the results of these observational cohort studies are the principal or only evidence of efficacy, careful assessment of the quality of the study and the strength of its results is needed. Seeking the advice of experts in the conduct and evaluation of such studies is recommended. In all cases,

the use of supportive studies should be justified and their relevance to the investigation in question considered.

Case–control studies

Case–control studies may be useful when prospective controlled trials are not feasible due to low incidence of disease (see also case–control studies, section B.9.3.2).

B.7.2 *General considerations for efficacy trials*

B.7.2.1 Size of trial

A vaccine efficacy trial may be based on clinical end-points, incidence of the infection (as in the case of HIV) or, if they exist, on immunological correlates of protection. Efficacy trials based on clinical end-points often require large samples; possibly thousands of subjects in each arm. Large numbers of subjects are needed for the precise estimation of vaccine efficacy if the incidence rate of the disease in the study population is expected to be low. For diseases with a higher incidence (e.g. influenza), smaller sample sizes will often suffice. When an immunological end-point that correlates with clinical protection is used as the primary efficacy end-point, the number of subjects required per arm to provide a statistically adequate evaluation may be considerably smaller e.g. several hundreds per group (see Correlates of protection B.7.2.3). In the case of large trials (e.g. 10 000–50 000 subjects) it may take many months to recruit the subjects who might then need to be followed up for a further 2 or 3 years. Large field trials of this type may simulate conditions in clinical public health practice and evaluate large numbers of subjects in a heterogeneous population. However, trials of this size and duration may be logistically difficult. In all cases, the applicant should provide adequate justification of the size and duration of the trial.

B.7.2.2 Choice of control

The choice of control depends on a number of factors as described below and should always be justified. A "placebo" control in vaccine trials usually denotes the use of a comparator arm that does not include the antigen(s) under investigation. If the antigen of interest is incorporated into a combination vaccine, the control arm may utilize a licensed vaccine that contains all the same antigens except that relevant to the efficacy evaluation. A control arm may also be a vaccine (usually already marketed) indicated for a different infectious disease(s). Finally, an active control is a comparator vaccine indicated for the same infectious disease(s).

Placebo control

Demonstrating the protective efficacy of a new vaccine always requires an appropriate control. For monovalent vaccines, an inert placebo or a vaccine that protects against another disease, but gives no protection against the target disease may serve as the control. Combination vaccines involving a new component for a new infectious disease indication require omission of the new component of the vaccine in the control arm of the study. If the new component is an already-licensed vaccine, or one for which efficacy and safety have already been demonstrated, a placebo-controlled study may not be necessary. The new component may be studied in an interference trial, comparing the simultaneous, but separate, administration (at two different sites of administration) of the new component with the combined administration of the combination vaccine with the new component.

Active control

Vaccines containing a new antigen, or an established antigen with a different formulation (e.g. liquid versus lyophilized; changed adjuvant, excipient or preservative; changed dose of antigen) or that involve a new method of administration (e.g. aerosol as opposed to intramuscular administration of an influenza vaccine) may be investigated in a comparative study using an antigenically similar active control vaccine on which adequate information is available (e.g. stability data).

A placebo control arm for internal validation should be considered when there are factors that may influence the stability and validity of the efficacy measure of the active control, such as vaccine quality; antigenic variation; vaccination coverage and other protective measures, or demographic; epidemiological; socioeconomic and other characteristics of the population.

B.7.2.3 Correlates of protection

In clinical trials where prevention of disease is used as an end-point, considerable effort should be made to establish immunological correlates of protection, in addition. Such correlates are also useful, and may be necessary, for situations in which the conduct of clinical trials using prevention of disease as an end-point cannot be practically or ethically justified. Nevertheless, it is important to recognize that correlates of protection may be difficult or impossible to define.

The following section describes a simple definition of correlates of protection. Immune correlates of protection may be population-

based or individual-based (*71*). Validated and standardized laboratory methods for serological assays are essential.

A commonly used measure of population-based correlates of protection requires the identification of a level of antibody that is achieved by most of the subjects in a protected group (i.e. vaccinated) and is not achieved by the majority of a susceptible group (i.e. unvaccinated). The level of protection correlated with the antibody level of vaccinnees is the vaccine efficacy measured in the phase III trial. For a population-based correlate it is only necessary to measure immunogenicity in a representative and statistically adequate sample of the vaccinated and unvaccinated phase III cohort.

The individual-based correlate of protection involves the measurement of pre-immunization and at least one post-immunization antibody level(s) in all study subjects and relating this to whether they subsequently develop the disease. The objective is to identify a threshold level in a vaccinee that predicts protection. For an individual-based correlate, it is necessary to measure post-immunization antibody levels in the entire phase III cohort. An alternative approach for those subjects who have a defined exposure may based on the measurement of early post-exposure antibody levels before boosting.

Immune responses should always be evaluated as part of a phase III clinical protection study with the aim of identifying immunological correlates of protection. For such an evaluation to be clinically meaningful, validated standardized assays are essential. Methods for the validation and standardization of immunological (antibodies and cell-mediated) correlates of protection should be developed and are vital for ensuring comparability of data between one trial and another. To correlate humoral immune responses to a vaccine with protective efficacy, the qualitative and quantitative relationships should be determined. The recommendations concerning the evaluation of immune responses described in phase II (B.6) should also be applied in clinical protection trials.

B.7.3 *Duration of protection and need for booster vaccinations*

Randomized controlled trials may provide an early indication of likely long-term protection and the need for booster vaccination(s). In addition to the course of antibody response and its relation to clinical outcome, longer-term follow-up of antigenically new vaccines should include critical characteristics of the vaccine that serve as prognostic factors for sustained protection. Therefore, in addition to studying the quality and dynamics of the antibody response, informa-

tion should be obtained on the relative importance of antibody titre, the extent of seroconversion and the induction of immunological memory.

When efficacy trials are completed, controlled follow-up of the entire study population (or a subset), which may extend into the post-licensure period, provides the best opportunity to define with confidence the serological correlate(s) of protection, and the need for, and the timing of booster vaccination(s). If efficacy studies were not possible, subsets of recipients may be followed over time for measurement of serological parameters. However, if there is no established correlate of protection, and if induction of memory is thought to be an important component of immunity, these studies may be inconclusive. For the determination of long-term protection and the potential need for booster vaccination, postmarketing serosurveillance studies may be necessary as it may not be possible or appropriate to prolong a trial beyond the point at which efficacy is established.

B.7.4 *Safety evaluations in phase III trials*

Safety evaluation during clinical development and prior to marketing authorization describes and quantifies the safety profile of a vaccine over a period of time, in a manner that is consistent with the intended use. The safety evaluation should include all subjects enrolled in all trials who receive at least one dose of vaccine, and safety surveillance should begin from the start of enrolment. Data on comparisons with antigenically similar active controls (vaccines used to prevent the same infectious disease) should be provided, if available. Safety issues identified during preclinical testing should be specifically addressed in the phase I, II and III clinical trials. Special considerations should be given to the safety concerns raised in animal studies and to environmental concerns related to vaccines based on genetically modified organisms (*72*).

Frequent adverse events must be thoroughly investigated and special features of the product explored (e.g. clinically relevant interference with other vaccines or drugs and factors leading to differences in effect, such as age or epidemiological characteristics). Obtaining such evidence is often the most difficult task of clinical research and requires large-scale randomized trials that employ clinical, epidemiological, biostatistical and laboratory methods. It is important to have a prospective definition and an order of prioritization for adverse outcomes. The difficulty of conducting such trials is usually determined by the incidence of infection and disease and the ability to establish a specific clinical or laboratory diagnosis for the disease in

question. This, together with the expected vaccine efficacy, is what determines sample size.

Randomized studies must have sufficient power to provide reliable rates of common (>1/100 and <1/10) adverse events, and to detect less common, but not necessarily very rare (<1/10 000) adverse events (*30*).

For the earlier phases of the study, a specific monitoring plan with a timetable and methods should be specified in the protocol for all subjects (see methodological considerations). When adequate safety data are available from phase I and II trials, it may be acceptable in the phase III study to actively monitor only a subset of subjects (e.g. several hundred per group) to quantify common and non-serious local and systemic events in the trial participants. For the rest of the phase III participants, active monitoring could focus on the identification of significant and/or unexpected serious events (e.g. hospitalization and death).

B.7.5 *Serious adverse events*

A serious adverse event is an event that is associated with death, admission to hospital, prolongation of a hospital stay, persistent disability or incapacity, or is otherwise life-threatening in connection with the clinical trial. All reported serious adverse events should be described in detail and the following information recorded:

— patient's study number or identification number;
— study identification;
— type of adverse event;
— how long after the vaccination the adverse event occurred;
— patient characteristics, including any underlying diseases, concomitant vaccinations or drugs;
— actions taken, e.g. therapy administered; and
— course of the adverse event including duration, outcome and investigator's assessment of causality.

The possibility of biological plausibility and/or a causal relationship with the vaccination should be considered and investigated in every case, although attributing causality is often difficult for events that occur anyway in the study population background (such as sudden infant death syndrome). Active monitoring of serious adverse events reported after completion of immunization is of major importance, because serious adverse events should be evaluated following a specific pattern.

Prior to licensure, both the applicant and the regulatory authority need to consider whether any reports of adverse drug reactions raise sufficient concern to warrant a suspension (perhaps only temporary) of product development. Additional clinical safety studies may be needed to confirm the relationship between the vaccine and the adverse event, and to establish precise incidence.

The duration of monitoring of study subjects following a serious adverse event depends upon the specific characteristics. Standard case report forms should be drawn up and used to record information on adverse events. Such forms should be used from phase I onwards.

Some serious adverse events following vaccination may be too uncommon to be observed in clinical trial programmes undertaken for marketing approval. Therefore, to obtain a more precise insight into the risk–benefit balance of the vaccine, a postmarketing surveillance programme should be implemented. In addition, specific postmarketing studies are often performed.

B.8 Bridging studies

Bridging studies within the context of this document are studies intended to support the extrapolation of efficacy, safety and immunogenicity data from one formulation, population, formulation and dose regimen to another. The need for performing bridging studies should be considered carefully and justified in the protocol. The end-points for clinical bridging studies are usually the relevant immune responses and clinical safety parameters.

Various methods may be used, depending on the purpose of the study. These are considered below.

B.8.1 *Design and extent of a clinical bridging study*

The clinical bridging studies (to support comparability with respect to the manufacturing process, change in product composition, or a new dose, route or schedule for immunization) should ordinarily be randomized controlled trials. As a minimum these studies should have adequate power to establish comparability of the relevant immune responses (see non-inferiority, section B.3.3.2) and to detect common adverse events. Additional comparative safety data may be needed to support extensive changes, such as a change in antigen composition in a new combination vaccine.

Clinical bridging studies to support extrapolation of efficacy data for a vaccine from one population to another are not randomized. However, for the outcomes to be valid it is important to minimize relevant

confounding variables. The composition and manufacturing process of the vaccine administered to study subjects should be as similar as possible (e.g. using the same lot for all subjects if available). The nature and extent of a bridging study are determined by the likelihood that vaccine efficacy may vary according to ethnic factors, manufacturing changes or changes in dosing schedule. Such studies are not required when it is sufficiently clear from pharmaceutical and preclinical experience that a change in the manufacturing process will not alter clinical efficacy or safety (e.g. specifications for quality control and lot release are not changed and therefore physicochemical characterization may be sufficient).

A controlled immunogenicity study may suffice (provided the serological correlate for clinical protection is validated) if regions are ethnically dissimilar, provided extrinsic factors are similar. An immunogenicity study will also help to select the appropriate schedule (i.e. the most protective) taking into account the incidence of the disease to be prevented (*73*). Controlled bridging trials using clinical endpoints are necessary when there has been a change of manufacturing process or manufacturing site resulting in a new product, the preclinical efficacy and safety data relating to the already-licensed product are no longer applicable; and a serological correlate for protection is not established.

Such studies would also be required in the target region when:

— the vaccine may be influenced by ethnic differences in the target population, and extrinsic factors are dissimilar;
— there is uncertainty regarding the appropriate dose regimen because local immunization schedules and/or antigenic doses differ from those used in trials conducted elsewhere;
— there is insufficient confidence in accepting the results of randomized controlled trials carried out elsewhere; or
— the vaccine is antigenically new in the region of the target population.

To minimize confounding factors related to the assays, the sera from different groups should be tested at the same time using the same assays, personnel and laboratory conditions. For studies that are not randomized or are not blinded with regard to subject enrolment (e.g. population bridging studies), special efforts should be made to avoid bias in sample testing. This may be achieved by appropriate coding of samples which will avoid any identification that distinguishes a separate group and sequential testing by group.

B.8.2 *Situations in which bridging studies may be required*

B.8.2.1 Bridging studies for change in manufacturing process

Changes made to the product composition (e.g. adjuvants or preservatives) or manufacture (process, site or scale) after the efficacy trial and prior to approval, or after licensing, may have a significant impact on safety and/or efficacy. Any proposed change in the production of a vaccine must be shown by the manufacturer to result in a product equivalent to that used in preclinical (or earlier clinical) testing. Such changes should be evaluated on a case-by-case basis to determine the supporting data required to demonstrate comparability of the "new" product with the previous version. An additional clinical study comparing the new version to the previous versions may or may not be required.

B.8.2.2 Bridging studies for new dosing schedules

Comparability with the original vaccine is also a concern when changes have been made in the immunization schedule, dose and/or route of administration (e.g. change from subcutaneous to intramuscular administration). In most cases, these changes should be supported by a clinical bridging study. The vaccine should be studied in the most conservative situation (the most restrictive), i.e. where the least response is expected. The most restrictive schedule should be applied in the initial clinical trials (youngest age at first dose, and smallest interval between doses), to make extrapolation to other schedules possible. This approach will allow the extrapolation to less conservative vaccination schedules without additional trials. For example, it is easier to extrapolate from a 2, 3, 4 schedule to a 3, 4, 5 schedule than the other way around.

B.8.2.3 Bridging studies for a new population

There are many situations in vaccine development where a new population has important differences from the trial population in which efficacy was established. The ability to extrapolate the data is particularly important when it is not feasible to repeat an efficacy trial with clinical end-points.

Population bridging studies address the concern that the safety and/or efficacy profiles of a vaccine in a particular target population may differ from those observed in the population studied in the original efficacy trial. The question of efficacy may be addressed by showing that the relevant vaccine-elicited immune response in the new population is similar to that in the population studied in the original efficacy trial. Thus, retaining sera and other relevant samples from the original efficacy trial for such comparisons is important, and this

requirement should be taken into account in the planning of efficacy trials.

Clinical bridging studies are justified only when ethnic or other factors specific to the target population exist, and when the studies do not unnecessarily duplicate clinical studies or delay the supply of important vaccines to populations requiring them. Ethnic factors may be genetic, physiological (intrinsic), or epidemiological, cultural and environmental (extrinsic). Cultural characteristics include the nature of the health care infrastructure and available resources (*21*).

B.8.2.4 Bridging studies for safety

A bridging study for safety may be necessary when there are special safety concerns in the target population.

- Bridging efficacy studies may provide safety data when the power of the study is sufficient to assess the rates of common adverse events. A limited safety study might precede the clinical bridging study to ensure that serious adverse events do not occur at a high rate.
- A special safety study is required if an efficacy bridging study is not needed, or when the efficacy study does not provide adequate safety information, including when:
  - — there is an index case (the individual in whom the event was first reported) or cases of a serious adverse event in foreign clinical data (generated outside the target region);
  - — there are differences in reporting of adverse events elsewhere;
  - — insufficient data on safety in the target population are available from an efficacy bridging study;
  - — the safety profile cannot be extrapolated from foreign data to the target population; or
  - — immunization schedules and/or antigenic doses differ from those used in foreign trials.

B.9 Post-licensure studies and surveillance

Following licensure, when a vaccine is in use, monitoring of its efficacy, safety and quality is referred to as postmarketing surveillance or postmarketing studies (phase IV studies). The purpose of these studies is to monitor the performance of a vaccine in the large target population under conditions of routine use, to detect adverse reactions and to monitor efficacy and effectiveness. In order to obtain more accurate estimates of adverse events and of effectiveness than those from phase III studies, active surveillance and phase IV studies using carefully designed surveys are used. Resource constraints

usually limit such surveys to a subgroup of the population, although for rare diseases it may be necessary to survey the entire population to obtain statistically valid data. Postmarketing studies are planned in study protocols. Although occasionally the designs may be as used in prelicensure trials, in most cases phase IV studies are set up as observational cohort or case–control studies. Whereas phase I, II and III studies make every attempt to standardize subjects, immunizations, evaluations and laboratory studies, it is usually impossible in phase IV studies.

Postmarketing surveillance and studies may be conducted to investigate:

— the optimal use of a vaccine (e.g. age at vaccination, simultaneous administration of other vaccines, changes in the vaccine strains and interchangeability of vaccines);
— efficacy in certain risk groups (e.g. the elderly, immunocompromised patients and patients with certain diseases); and
— maintenance of long-term efficacy and monitoring of long-term safety.

To ensure adequate postmarketing surveillance marketing authorization holders should be committed to presenting a postmarketing surveillance programme at licensure and all national regulatory authorities should endeavour to put in place a system for pharmacovigilance for vaccines. The outcomes of surveillance (assessments of effectiveness, adverse events and quality) should be reported to the national authorities and/or the marketing authorization holder, and they should be published.

Postmarketing surveillance programmes should be appropriate to the disease epidemiology, infrastructure and resources in the target area. Essential standards of efficacy, safety and quality should always be defined before initiating a postmarketing surveillance programme and the programme should include assessment of:

— the impact of the target disease (morbidity and mortality);
— potential of the disease to cause an epidemic;
— whether the disease is a specific target of a national, regional or international control programme; and
— whether the information to be collected will lead to significant public health action.

Ideally, a postmarketing programme should be based on criteria set for a particular vaccine as a part of marketing approval. The essential standards for these should always be defined. To ensure that an

intervention is conducted to an acceptable standard, to identify areas where special attention is required and to ascertain (in cases of vaccine or programme failure) the possible reasons for this failure, each step should be carefully monitored and described in protocols. Important applications of postmarketing surveillance are in the early stages of use of a novel vaccine, or when circumstances change (e.g. the emergence of new antigenic variants of a pathogen) and doubts are raised about the continuing efficacy of the current formulation.

B.9.1 *Safety evaluation*

Postmarketing surveillance may be the only means of detecting long-term or acute events that occur too infrequently to have been revealed by clinical trials. Under specific circumstances active postmarketing surveillance or phase IV studies should be considered to determine the incidence and significance of infrequent and rare emerging serious events following immunization with the vaccine under investigation. With respect to safety, the intent of a phase IV study is to detect the rarer or unexpected events that may not have been seen in the smaller phase II or phase III studies because of their limited statistical power. Rare events are often idiosyncratic; a causal relationship is difficult to establish and this usually cannot be done prior to licensure.

Surveillance for the collection of safety data may be conducted by active or passive processes, and may be directed at an entire population or at a subgroup. In practice, a mixture of these processes is often used. Voluntary reporting of adverse events (passive surveillance) is the most often used. It is effective in detecting severe or lethal events and unusual clinical responses. The true rate of incidence of adverse events, particularly of those that do not have distinctive manifestations, is likely to be considerably underestimated.

Targeted studies of a specific adverse event are usually case–control studies or retrospective studies on exposure cohorts linked to historical controls (*74*). In retrospective exposure cohorts the event of interest can be studied in a controlled setting using sampled historical data identified prospectively. Postmarketing surveillance for safety evaluation should include information from all possible sources. Databases linked to large patient cohorts are a valuable source of information for investigating serious adverse events (*75*). Collecting data on safety using a structured, planned postmarketing surveillance study may be set as a condition for marketing approval.

B.9.2 *Evaluation of vaccine effectiveness*

Following the evaluation of efficacy in a randomized controlled phase III clinical trial, the effectiveness of a new vaccine in routine practice should be determined (*76*). Studies of effectiveness measure direct and indirect protection (e.g. protection of unvaccinated persons by the vaccinated population (herd immunity)). Vaccine effectiveness is affected by a number of factors, including:

— vaccination coverage of the population;
— immune status of the population;
— correlation of strains used in vaccine production with circulating strains; and
— The incidence of disease due to strains not included in the vaccine following introduction of the vaccine in that population.

If conducted consistently over a prolonged period, postmarketing surveillance allows the longitudinal assessment of efficacy under a range of conditions, and it may disclose variations in vaccine quality. The duration of follow-up of subjects in the postmarketing programme should be described in a protocol. Implementation of an immunization programme in a certain population may necessitate the development of a structured plan for postmarketing serosurveillance to identify changes in disease epidemiology in the target population over time. This may include evaluation of:

— the impact of the programme, through analysis of reported vaccine failures, and (if applicable) assessment of why disease is still occurring;
— whether new immunization strategies are necessary; and
— possible harm caused by replacement disease following the intervention (e.g. other serotypes replacing the serotypes in the vaccine).

A protocol for serosurveillance should be presented at the time of marketing authorization, or implementation of a vaccination programme. A structured plan for executing the programme should be presented, including information on participating institute(s) and intervals of reporting (usually every 6 months, for 5 years).

B.9.3 *Study design*

B.9.3.1 Observational cohort studies

The evaluation of the benefit of a community-based immunization programme requires large-scale surveillance. An observational cohort study, directed at the events, exposures and diseases occurring among vaccinated and unvaccinated members of the target

population under normal conditions may provide an estimate of vaccine effectiveness.

In non-randomized studies, nested household surveys in a random sample of the study population may minimize bias. In some cases randomization from phase III trials may be continued concurrently.

Observational cohort studies may require community-wide sampling. The chosen sample size will depend upon the characteristics of the intervention applied (i.e. whether risk-group intervention, community intervention or traveller immunization).

B.9.3.2 Case–control studies

Case–control studies should be considered in investigating diseases of low incidence or when studying adverse events in response to vaccines when they can be particularly useful (*77*). In order to generate adequate information on vaccine efficacy, population samples should be well defined and representative, and a serological correlate for protection, if available, should be used (see B.7.2.3). The advantages of case–control studies are that they can be small-scale and the follow-up period is short. The main limitations are the potential for (a) selection bias, and (b) information bias. Selection bias is due to lack of randomization and the selection of the control group, especially when the study is not population based. Every effort should be made to include as many cases as possible. All aspects of study design and conduct should be detailed in the study protocol and justified.

B.9.3.3 Stepped wedge design

The stepped wedge design should be considered when previous studies have indicated that the intervention is likely to be beneficial (*51*) and the public health need to introduce the intervention precludes withholding it from a population. The intervention is introduced in phases, group by group, until the entire target population is covered. The groups form the unit of randomization.

B.9.3.4 Outbreak interventions

At the start of an outbreak (or epidemic), the susceptibility of all individuals in the target population to the infecting pathogen is assumed to be equal. The methodological approach chosen to study the effectiveness of the intervention should be appropriate to the size and nature of the outbreak.

- Pre-exposure cohort studies or secondary attack-rate studies are preferred in infections with a high attack rate.
- Case–control studies are useful in studies of diseases with a low incidence or in small isolated outbreaks.

- Community-based cohort studies are unsuitable for short-term evaluation; however, they may be useful for the post hoc evaluation of the performance of a vaccination programme or for long-term follow-up of specific clinical outcomes or safety issues.

In areas where the immunization rate is high, outbreak investigations underestimate vaccine efficacy. The degree of underestimation is related to the extent of the epidemic that triggered the investigation, vaccination coverage in the community and the extent of clustering of vaccination failures in the population.

B.9.4 *Monitoring of postmarketing surveillance*

A postmarketing oversight policy should be established by a national regulatory authority to enable control of product release, periodic inspections, reporting mechanisms, recall of batches, or, if necessary, for revoking marketing approvals, approval of manufacturing changes, and evaluation and approval of new indications and/or dose regimens. General guidelines for continued oversight of vaccines after licensure as described in WHO Technical Report Series 858, should be followed (*1*). Guidance on the operation of epidemiological surveillance and monitoring of adverse events are provided by WHO and other bodies (*37*, *78–80*). Standards for assessment of causality are described in these and other regulatory documents. Targeted monitoring and special studies may be required for certain adverse events (*75*). Monitoring vaccines for use in the Expanded Programme of Immunization should include not only efficacy and safety, but also compatibility with existing vaccines (antigens) used in this programme (*81*). Ideally, this should be considered prior to marketing approval. In addition, the immunization programme and vaccine supply should be considered.

B.10 Special considerations for combination vaccines

A combination vaccine consists of two or more vaccine immunogens in a physically mixed preparation intended to prevent several diseases or to prevent one disease caused by different serotypes (or serogroups) of the same organism (*13*, *14*, *79*). The mixing may occur as a manufacturing step or it may be performed by a health care professional on site before administration according to the package insert instructions. Vaccines mixed ad hoc without regulatory approval are not considered to be combination vaccines.

The main goal of a clinical trial of a combination vaccine is to evaluate the efficacy of each component vaccine, and the safety of the combination, regardless of whether or not the combination consists of

previously marketed or investigational individual component vaccines. The immunogenicity and safety of a new combination should be compared with the effects of simultaneous, but separate, administration of the individual vaccines.

B.10.1 *Efficacy studies*

Once the serological correlates of protection have been validated for each of the antigenic components, consideration should be given to evaluating the efficacy of a new combination vaccine consisting of components already licensed and/or components with proven efficacy using immunogenicity rather than clinical protection end-points. Failing this, prospective controlled clinical studies or alternative approaches such as postmarketing surveillance are required.

Studies of combination vaccines are usually designed and analysed (for efficacy or immunogenicity) as non-inferiority trials, the aim being to demonstrate that the combination is comparable with the individual components. Each of the individual components is expected to add materially to the prophylactic effect of the vaccine (*61*, *79*).

Clinical studies of combination vaccines should:

— have sufficient power to rule out pre-existing differences in response parameters between the study groups;
— use appropriate sample sizes, as for monovalent vaccines (see methodological considerations); and
— consider the clinical consequences of any potential difference observed.

Clinical bridging studies may be needed to facilitate extrapolation of data to a different population or to support a different immunization schedule.

Immunogenicity trials of new combination vaccines to prevent several diseases (multidisease combination vaccines) should be designed to rule out predefined differences in immune responses between the new product and the individual components administered separately. When antibody concentrations following administration of the combined vaccine are less than those observed following separate administration of the individual components or simultaneous administration of the individual of the individual vaccines at different sites, it should be demonstrated that these findings are not clinically relevant. Any change in dose or schedule for individual components should be justified.

For a combination vaccine consisting of several strains or serotypes, the primary end-point for clinical efficacy should be the prevention of disease caused by the different vaccine-type strains, or the ability of the vaccine to modify the course of such disease.

The study should have sufficient power to enable meaningful separate analyses to be made of the prevalent strains or serotypes identified as being of major significance to public health in the target area. The appropriateness of the coverage provided by the individual vaccine components in the target population should be justified e.g., in the case of multivalent vaccine that does not cover all serotypes of the disease such as pneumococcal conjugate vaccine, epidemiological data should be provided to justify the selection of strains for this vaccine. The feasibility of extrapolation from limited numbers of strains or serotypes to other strains or serotypes should be substantiated.

B.10.2 *Safety analysis of combination vaccines*

For the safety evaluation of combination vaccines, as much information as possible should be obtained from randomized, controlled trials. Such studies are usually designed and analysed as non-inferiority trials that aim to demonstrate that the safety of the combination is not inferior to that of the individual components. Where applicable, the controls for the study should be the already marketed vaccines with the same antigen composition. The size chosen for the study groups should take into account differences in rates of common and/or clinically important adverse events. For vaccines intended for infants and children, defining differences in rates of high fever may be especially relevant. Blinding is virtually essential for making valid comparisons and for the accurate determination of the rates of events causally related to vaccination. If blinding of a study is not feasible, the methods used to minimize bias should be described.

The safety and efficacy of new formulations in which reduced doses of some or all of the components of a combination vaccine are necessitated by the volume of the combination of components being too large for safe administration must be demonstrated.

Simultaneous administration of vaccines

For monovalent vaccines intended for simultaneous administration with other vaccines to the target population, any clinically relevant interference with the other vaccines should be ruled out. Immunological interference and adverse safety interactions after simultaneous administration should be compared with the results of separate administration of the (new) vaccine component(s) at different times.

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Appendix

Summary protocol for vaccine evaluations

Title and summary _____

Brief description of the study site(s) _____

Investigators _____

Background and rationale _____

Preclinical and laboratory evaluation of vaccines _____

Summary of product characteristics (details of methods for production and control of candidate vaccine) _____

Primary and secondary objectives _____

Study design _____

— hypothesis _____

— end-points _____

— study plan _____

— trial size _____

— duration of study _____

Study population _____

— inclusion and exclusion criteria _____

Methods and procedures _____

— recruitment of subjects _____

— allocation of subjects _____

— vaccine delivery _____

— follow-up _____

— laboratory methods _____

— statistical plan and analyses _____

Monitoring of the trial _____

— data monitoring _____

— quality assurance of data and laboratory methods _____

Timetable _____

— start and end of recruitment _____

— end of follow-up _____

— date of report _____

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Annex 2

Recommendations for the production and control of meningococcal group C conjugate vaccines

Recommendations published by WHO are intended to be scientific and advisory. Each of the following sections constitutes guidance for national regulatory authorities and for the manufacturers of biological products. If a national regulatory authority so desires, these Recommendations may be adopted as definitive national requirements, or modifications may be justified and made by the national regulatory authority. It is recommended that modifications to these Recommendations be made only on condition that modifications ensure that the vaccine is at least as safe and efficacious as that prepared in accordance with the Recommendations set out below. The parts of each section printed in small type are comments for additional guidance intended for manufacturers and national regulatory authorities which may benefit from those details.

Introduction

The recommendations (formerly known as Requirements) for meningococcal polysaccharide vaccines were adopted by the WHO Expert Committee on Biological Standardization in 1976 (*1*) and amended in 1978 and 1981(*2*, *3*). In clinical studies these vaccines have been shown to have an efficacy in the region of 90% and have proved to be highly effective in public health interventions (*4*). Nevertheless, their inability to elicit protective responses in young infants or to induce good immunological memory has prevented their use in national infant immunization schedules.

Following the successful introduction of the *Haemophilus influenzae* type b conjugate (Hib) vaccines, considerable progress has been made in the development of similar conjugate vaccines based on meningococcal group C capsular polysaccharide. Controlled clinical trials have demonstrated that these vaccines are highly immunogenic in all age groups and, as T-cell dependent antigens, induce immunological memory and affinity maturation of anti-capsular antibodies (*5–8*, *8–17*). Vaccines based on meningococcal group C conjugates have been shown to offer protective immunity following their introduction in the UK (*18*). Glycoconjugate vaccines are both physically and immunobiologically distinct from their unconjugated counterparts emphasizing the need for new recommendations for these products.

General considerations

Neisseria meningitidis is a significant cause of bacterial meningitis and septicaemia. Meningococci are divided into serogroups on the basis of their chemically and serologically distinct capsular polysaccharides, but only organisms belonging to one of the five groups, A, B, C, Y and W135, cause disease (*19*). Group A organisms cause widespread epidemic disease in the so-called "meningitis belt" countries, whereas the other four groups are responsible for endemic disease and localized outbreaks worldwide (*20*, *21*). Disease caused by group C organisms occurs primarily in infants although outbreaks caused by group C organisms in students and military recruits have contributed to an elevated incidence of meningococcal disease in teenagers and young adults.

An ideal vaccine would offer comprehensive protection against all five of the pathogenic serogroups, but its development has faced major obstacles related to the immunobiology of the capsular antigens. Bivalent (A and C) and tetravalent (A, C, Y and W135) polysaccharide vaccines have been widely available since the early 1970s (*4*). Pivotal studies carried out during the 1960s confirmed the critical role of antibody-dependent complement-mediated lysis of the meningococcus as the principal immunological mechanism of protection (*22*, *23*). The polysaccharide vaccines elicit good bactericidal antibody responses in immunologically mature individuals and have been used effectively to manage epidemics and localized outbreaks as well as to offer protection to groups, such as students and military recruits, who are regarded as being at particular risk of the disease (*24–26*). However, vaccines based upon plain polysaccharides have serious drawbacks; their immunogenicity is age-related and they fail to elicit immunological memory, and hence a booster response on subsequent exposure to the polysaccharide (*27–29*). Experience with the Hib vaccine had shown that the immunogenicity of polysaccharides could be improved by chemical conjugation to a protein carrier thereby eliciting a T-cell-dependent antisaccharide response (*28*, *30*, *31*). Several different meningococcal group C conjugates have been developed that demonstrably stimulate T-cell-dependent antibody production (*5*, *7*, *14*, *32*, *33*).

Special considerations

The production and control of conjugate vaccines is more complex than that for their unconjugated capsular polysaccharide counterparts. Polysaccharide vaccines consist of defined chemical substances that, if prepared to the same specifications, can reasonably be ex-

pected to have comparable potencies, regardless of the manufacturer. Effective meningococcal group C conjugate vaccines have been developed that differ both in the nature of the saccharide and of the carrier protein employed. Meningococcal group C capsular polysaccharide is usually *O*-acetylated. However, 5–10% of group C case isolates produce a capsule that is not *O*-acetylated. Conjugate vaccines based on either acetylated or de-*O*-acetylated polysaccharide elicit potent serum bactericidal antibody (SBA) responses against organisms expressing either form of the group C capsule, even though the antibody responses may not be directed against the same epitopes (*6*, *7*). Effective meningococcal group C conjugate vaccines have been manufactured with either the diphtheria toxoid CRM197 or tetanus toxoid as carrier. The manufacturer has a choice of possible carrier proteins providing that the resulting conjugate vaccine is safe and stimulates production of T-cell-dependent protective antibody in infants and young children and boostable immune responses.

As the low burden of group C meningococcal disease made phase III clinical studies unfeasible, the first meningococcal group C conjugate vaccines were licensed in the UK on the basis of their proven immunogenicity rather than their clinical efficacy. The rationale behind licensure was based on:

— the studies by Gotschlich et al. (*24*) that demonstrated that serum bactericidal activity could be taken as an indicator of clinical protection against group C meningococcal disease;
— the evidence from clinical trials that showed that the conjugate vaccine was highly immunogenic and induced immunological memory in all age groups; and
— experience with the Hib vaccines that had already established the safety and advantages of conjugate vaccine technology.

The introduction of the vaccine was phased, first targeting the highest risk groups and providing the opportunity to obtain estimates of vaccine efficacy by comparing the incidence of disease between vaccinated and unvaccinated groups (*18*). The immunogenicity of the vaccine in humans should be assessed before the new vaccine is licensed.

Although it has been widely accepted since the studies by Gotschlich et al. (*24*) that SBA levels correlate with immune protection against group C meningococcal disease (*22*), there has been considerable debate during the development of meningococcal group C conjugate vaccines about the way in which the bactericidal assay should be performed (*34*, *35*). This debate has focused mainly on whether baby rabbit serum could substitute for human serum as the source of complement in the assay, and if so how the results should be interpreted, as meningococci are more sensitive to lysis mediated by baby

rabbit complement than human complement (*36–38*). Whether human or baby rabbit serum is used as the source of complement, the source should be standardized following specific guidelines. Guidance on the methods available for evaluating the immune response will be published as an addendum in the light of emerging data. This guidance was subsequently established by the 53rd meeting, February 2003, of the WHO Expert Committee on Biological Standardization and published as Annex 3 of WHO Technical Report Series 926 (2004).

Because the meningococcal group C conjugate vaccines are manufactured from purified components by a clearly defined chemical process, the strategy for the control of the vaccine relies heavily on evaluation of molecular characterization and of purity to ensure that each vaccine lot is consistent with the specification of the vaccine lots used in the definitive clinical trials that confirmed their safety and immunogenicity (*15*, *39–41*). In addition, the immunogenicity of meningococcal group C conjugate vaccines has been evaluated in mice and such data can provide an indication of the consistency and structural integrity of the vaccine (*42*). However, although immunogenicity testing in animals forms a necessary part of vaccine development, experience gained following the licensure of the meningococcal group C conjugates suggests that a routine animal potency test is not necessary when vaccine consistency has been assured by physicochemical criteria.

Combination vaccines containing meningococcal polysaccharide conjugate components

The introduction of meningococcal group C conjugate vaccines as an additional element of the infant immunization programme in the UK has served to highlight the need to combine paediatric vaccines for effective vaccine delivery (*43*). Vaccine formulations consisting of multiple components that include meningococcal group C conjugates are likely to be developed soon. If a meningococcal group C conjugate vaccine is indicated for concomitant use with other vaccines, possible effects on the clinical performance of each component in the combined vaccine, including the meningococcal group C conjugate vaccine component, should be evaluated in terms of their safety and immunogenicity.

Because of the problems associated with performing physicochemical analyses on complex vaccine formulations, the manufacturer should consider which batch release tests to perform on final bulks and 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 shall be "meningococcal group C conjugate vaccine" translated into the language of the country of use. The use of this name should be limited to vaccines that satisfy the requirements formulated below.

A.1.2 *Descriptive definition*

Meningococcal group C conjugate vaccine is a preparation of capsular polysaccharide from group C *Neisseria meningitidis* that is covalently linked to a carrier protein.

A.1.3 *International reference materials*

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

The following reagents are available through the courtesy of manufacturers and national control or reference laboratories: CDC1992 Reference Serum for the standardization of SBA assays and ELISAs is available from National Institute for Biological Standards and Control (NIBSC) in Europe and Centers for Disease Control in the USA; meningococcal group C polysaccharide and methylated human serum albumin for use in ELISA, and group C specific monoclonal antibody used to confirm the identity of group C polysaccharide are available from NIBSC.

A.1.4 *Terminology*

Master seed lot. A bacterial suspension of *N. meningitidis* 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 should be maintained in the freeze-dried form or be frozen below –45 °C.

Working seed lot. A quantity of live *N. menigitidis* 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, if 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 a 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 meningococcal polysaccharide.

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. The homogeneous preparation 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 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 pharmaceutical (*44*) and biological products (*45*) should be applied to establishments manufacturing meningococcal conjugate vaccines with the addition of the following.

Details of standard operating procedures for the preparation and testing of meningococcal 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. When they are required, proposals for the modification of the manufacturing and control methods should also be submitted for approval to the national regulatory authority before they are implemented.

N. meningitidis is a class 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 (*46*). Standard operating procedures must be developed for

dealing with emergencies arising from the accidental spillage, leakage or other dissemination of meningococcal organisms. Personnel employed in the production and control facilities should be adequately trained. Appropriate protective measures including vaccination against *N. meningitidis* should be implemented. Adherence to the 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 the polysaccharide*

A.3.1.1 Strains of N. meningitidis

The strain of *N. meningitidis* used for preparing the group C polysaccharide should be identified by a record of its history, including the source from which it was obtained and the tests made to determine the characteristics of the strain. The strain should have been shown to be capable of producing group C polysaccharide.

> The strains C11 and C2241 have been shown to be suitable for group C polysaccharide.
>
> ^{1}H nuclear magnetic resonance spectroscopy is a suitable method for the confirmation of the identity of the polysaccharide

A.3.1.2 Seed lot system

The production of meningococcal group C 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, the preservation of strain viability for freeze-drying or for frozen storage, then 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 encephalopathies* (*47*) and should be approved by the national control authorities.

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

A.3.1.3 Culture media for the production of meningococcal polysaccharide

The liquid culture medium used for vaccine production should be free from ingredients that will form a precipitate upon addition of hexadecyl trimethylammonium bromide to a concentration of $1\,g\,l^{-1}$. If materials of animal origin are used 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 encephalopathies (*47*) and should be approved by the national control authorities.

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

A.3.1.4 Single harvests

Consistency of growth of meningococcal organisms should be demonstrated by monitoring growth rate, pH and the final yield of group C polysaccharide.

A.3.1.5 Control of bacterial purity

Samples of the culture should be taken before killing and examined for microbial contamination. The purity of the culture should be verified by suitable methods that should include inoculation on to appropriate culture media. If any contamination is found, the culture and any product derived from it should be discarded. The killing process should similarly be adequately validated.

A.3.1.6 Purified polysaccharide

Each lot of meningococcal group C polysaccharide should be tested for purity. The limits given below are expressed with reference to the polysaccharide in its salt form, corrected for moisture. Each manufacturer must define the limits for its own product and they must be agreed by the national control authority.

Generally, the culture is harvested after killing the organism by heating to 56 °C for 10 minutes or by the use of a suitable inactivating agent; after killing the polysaccharide is partially purified by precipitation with hexadecyl trimethylammonium bromide. Methods used for further purification of this intermediate should be approved by the national regulatory authority. Purified meningococcal polysaccharide and, when necessary, partially purified intermediates, are usually stored at or below –20 °C to ensure stability.

A.3.1.6.1 Identity test

A test should be performed on the purified polysaccharide to verify its identity.

A serological test and/or ^{1}H nuclear magnetic resonance spectroscopy provide convenient methods for this purpose (*39*, *48*, *49*).

A.3.1.6.2 Molecular size distribution

The molecular size distribution of each lot of purified polysaccharide should be estimated. The distribution constant (K_D) should 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 and shown to be consistent from lot to lot for a given product. An acceptable level of consistency should be agreed with the national regulatory authority.

Suitable methods for this purpose are: gel filtration through Sepharose CL-4B or CL-2B (or similar) in a 0.2 molar buffer using a refractive index detector (*3*) or colorimetric assay or high-performance size-exclusion chromatography (HPSEC) either alone or in combination with light-scattering and refractive index detectors (e.g. multiple angle laser light scattering MALLS) (*50*).

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 Polysaccharide composition

The quality of the polysaccharide can be estimated by the determination of the sialic acid content. The sialic acid content should be not less than 80% of the dry weight of the isolated product, as determined by the resorcinol assay, using *N*-acetylneuraminic acid as a standard (*51*).

Other methods, such as high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD), may be used to define the quantitative composition of the purified polysaccharide, but the methods should be validated for the purpose (*42*). If other methods are used, alternative specifications for sialic acid may apply and should be agreed with the national regulatory authority.

A.3.1.6.5 Protein impurity

Each lot of purified polysaccharide should contain not more than 1% by weight of protein, as determined by the method of Lowry et al., using bovine serum albumin as a reference (*3*, *52*), or by another suitable validated method.

Sufficient polysaccharide should be assayed to detect 1% protein contamination accurately.

A.3.1.6.6 Nucleic acid impurity

Each lot of purified polysaccharide should contain not more than 1% by weight of nucleic acid as determined by ultraviolet spectroscopy, on the assumption that the absorbance of a $10\,g\,l^{-1}$ nucleic acid solution contained in a cell of 1 cm path length at 260 nm is 200 (*3*), or by another validated method.

Sufficient polysaccharide should be assayed to detect 1% nucleic acid contamination accurately.

A.3.1.6.7 Endotoxin content

To ensure an acceptable level of pyrogenic activity of the final product, the endotoxin content of the purified polysaccharide should be determined, and shown to be within limits agreed as being acceptable by the national regulatory authority.

> Less than 100 International Units of endotoxin per μg of polysaccharide when measured by the *Limulus* amoebocyte lysate test can be achieved in the production process. Alternatively, a recognized pyrogenicity test can be performed in rabbits.

A.3.1.6.8 *O*-acetyl content

The meningococcal group C polysaccharide used in the conjugate may be either *O*-acetylated or de-*O*-acetylated (*53*). For the *O*-acetylated form, the *O*-acetyl content should be monitored (by colorimetric or other validated assay) to ensure consistency of production. Similarly for the de-*O*-acetylated form, the absence of *O*-acetylation should be demonstrated to ensure consistency of production.

> Bulk group C polysaccharide used for the production of licensed polysaccharide vaccine is suitable for the production of conjugate vaccine (*1*). *O*-acetyl content is conveniently determined by a colorimetric assay or ^{1}H NMR. *O*-acteylated polysaccharide typically contains at least 1.5 mmol/g saccharide.

A.3.1.7 Modified *polysaccharide*

Modified polysaccharide preparations are usually partially depolymerized either before or during the chemical modification.

A.3.1.7.1 Chemical modification

Several methods for the chemical modification of polysaccharides prior to conjugation have been found to be satisfactory. The chosen method should be approved by the national regulatory authority.

> Suitable methods include:
>
> - Production of size-reduced polysaccharides by controlled acid hydrolysis and size fractionation. The resulting oligosaccharide fraction is reductively aminated and activated by coupling through the amine group to a functional linker, bis-*N*-hydroxysuccinamide ester of adipic acid. The reaction between the activated oligosaccharide and the protein generates the conjugate vaccine.
> - Size-reduced polysaccharides are produced by periodate oxidation generating aldehyde groups. Upon mixing with the carrier protein Schiff's bases form between the aldehyde groups of the oligosaccharide and the amino groups of the protein, which can be reduced to form stable covalent bonds by treatment with sodium cyanoborohydride.

As part of the in-process controls, the processed polysaccharide to be used in the conjugation reaction may be assessed for the number of functional groups introduced.

A.3.1.7.2 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 processed polysaccharide should be measured by a suitable method. The size should be specified for each type of conjugate vaccine with appropriate limits for consistency, as the size may affect the reproducibility of the conjugation process.

The molecular size may also be determined by HPSEC using MALLS detection (*54*). Other suitable methods include gel filtration, ion exchange chromatography or high-pressure liquid chromatography (HPLC) used together with an appropriate validated chemical assay.

A.3.2 *Control of the carrier protein*

A.3.2.1 Microorganisms and culture media for production of the 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 *Report of a WHO consultation on medicinal and other products in relation to human and animal transmissible spongiform encephalopathies* (*47*) and should be approved by the national regulatory authority.

Production should be based on a seed lot system and the strains should be 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

Proteins that have been used as carriers in meningococcal conjugate vaccines licensed to date include tetanus toxoid and the non-toxic mutant of diphtheria toxin (CRM197). The 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 control authority.

Proteins and purification methods that might be used include:

- *Tetanus or diptheria toxoid*. This must satisfy the relevant requirements published by WHO (*55*) and be of high purity. The purity should be at least 1500 Lf/mg (Lf = limit of flocculation) protein (nondialysable) nitrogen (*56*).
- *Diphtheria CRM197 protein*. This is a non-toxic mutant of diphtheria toxin, isolated from cultures of *Corynebacterium diphtheriae* C7/β197 (*57*). Protein of purity greater than 90% as determined by HPLC is prepared by column chromatographic methods. When produced in the same facility

as diphtheria toxin, methods must be in place to distinguish the CRM197 protein from the active toxin.

The carrier protein should also be characterized. The identity may be determined serologically. Physicochemical methods that may be used to characterize protein include SDS-PAGE, isoelectric focusing, HPLC, amino acid analysis, amino acid sequencing, circular dichroism, fluorescence spectroscopy, peptide mapping and mass spectrometry as appropriate (*58*).

A.3.3 *Control of bulk purified conjugate*

A number of methods of conjugation are currently in use; 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 once the immunogenicity of a particular meningococcal conjugate vaccine has been demonstrated. The derivatization and conjugation process should be monitored by analysis for unique reaction products or by other suitable means.

Residual unreacted functional groups potentially capable of reacting in vivo may be present following the conjugation process. The manufacturing process should be validated to show that no activated functional groups remain at the conclusion of the manufacturing process.

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.

NMR spectroscopy may be used to confirm the identity and integrity of the saccharide in the conjugate (*59*, *60*)

A.3.3.1 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, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDAC) and phenol should be confirmed by suitable tests or by validation of the purification process.

A.3.3.2 Conjugation markers

Where the chemistry of the conjugation reaction results in the creation of a unique linkage marker (e.g. a unique amino acid), each batch should be assessed to quantify the extent of covalent reaction of the meningococcal polysaccharide with the carrier protein, so that the frequency of the covalent bond is given as a function of the number of polysaccharide repeating units or overall polysaccharide content.

A unique linkage marker could be assessed for each batch or, alternatively, the manufacturing process should be validated to demonstrate that it yields conjugate with a level of substitution that is consistent from batch to batch.

The ratio of saccharide to protein is also a suitable conjugation marker, though not a direct measurement.

A.3.3.3 Capping markers

Each batch should be shown to be free of unreacted functional groups on either the chemically modified polysaccharide or the carrier protein.

Where possible, unreacted functional groups should be assessed for each batch. Alternatively, the product of the capping reaction can be monitored or the capping reaction can be validated to show removal of unreacted functional groups.

A.3.3.4 Polysaccharide content

The content of meningococcal polysaccharide should be chemically determined by means of an appropriate validated assay.

Methods that have been used for the determination of the meningococcal polysaccharide content include the resorcinol assay or HPAEC–PAD detection.

A.3.3.5 Conjugated and unbound (free) polysaccharide

Only the meningococcal polysaccharide that is covalently bound to the carrier protein (i.e. conjugated polysaccharide) is immunologically important for clinical protection and excessive levels of unbound polysaccharide could potentially result in immunological hyporesponsiveness to group C polysaccharide.

Each batch of conjugate should therefore be tested for unbound or free polysaccharide to ensure that the amount present in the purified bulk is within the limits agreed by the national control authority based on that present in lots shown to be clinically safe and efficacious.

Methods that have been used to assay unbound polysaccharide include gel filtration; ultrafiltration and hydrophobic chromatography; ultracentrifugation with HPAEC-PAD, or colorimetric detection (*42*).

A.3.3.6 Protein content

The protein content of the conjugate should be determined with an appropriate validated assay (section A.2). Each batch should be tested for conjugated and unbound protein.

The unconjugated protein content is normally <5%. Appropriate methods for the determination of conjugated and unbound protein include HPLC or capillary electrophoresis.

A.3.3.7 Polysaccharide to protein ratio

The polysaccharide to protein ratio of the conjugate should be calculated. For each conjugate, the ratio should be within the range approved for that particular conjugate by the national regulatory

authority and should be consistent with the ratio in vaccine that have been shown to be effective in clinical trials.

A.3.3.8 Molecular size distribution

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 (*42*). The method should be validated with an emphasis on its 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 that of lots shown to be immunogenic in clinical trials.

> Typically the size of the polysaccharide–protein conjugate may be examined by gel filtration on Sepharose CL-4B. Suitable alternative methods are acceptable.
>
> Since the saccharide: protein ratio is an average value, determination of this ratio over the size distribution can be used to provide further proof of manufacturing consistency (*58*).

A.3.3.9 Sterility

The bulk purified conjugate should be tested for bacterial and mycotic sterility in accordance with the requirements of Part A, sections 5.1 and 5.2, of the revised Requirements for Biological Substances (*61*), 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.3.3.10 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.3.4 ***Final bulk***

A.3.4.1 Preparation

The final bulk is prepared by mixing the adjuvant, a preservative and/or stabilizer (as appropriate) with a suitable quantity of the bulk conjugate so as to meet the specifications of vaccine lots that have been shown to be safe and efficacious in clinical trials.

A.3.4.2 Sterility

Each final bulk should be tested for bacterial and mycotic sterility as indicated in section A.3.3.9.

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* should be applied (*45*).

A.3.6 *Control tests on final product*

A.3.6.1 Identity

An identity test should be performed on each final lot.

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 described in section A.3.3.9.

A.3.6.3 Meningococcal polysaccharide content

The amount of each meningococcal group C conjugate in the final containers should be determined, shown to be within the limits specified by the national regulatory authority, and be plus or minus 20% of the stated content.

The conjugate vaccines produced by different manufacturers differ in formulation. A quantitative assay for the meningococcal polysaccharide in the final container is likely to be product-specific. Colorimetric methods, chromatographic methods (including HPLC), or serological methods 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 the limits of the preparations that have been shown to be adequately stable in the stability studies of the vaccine.

The test should be performed on one vial per 1000 up to a maximum of 10 vials but on no less than five 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 Pyrogen content

The vaccine in the final container should be tested for pyrogenic activity by intravenous injection into rabbits or by a *Limulus* amoebocyte lysate 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 also 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.

> The consistency of adsorption of the antigen to the adjuvant is important and the adsorption of production lots should be demonstrated to be within the range of values measured in vaccine lots shown to be clinically effective.

A.3.6.7 Preservative content

If a preservative has been added to the vaccine, its content 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 meningococcal conjugate vaccine for unexpected toxicity (abnormal toxicity) should be agreed with the national regulatory authority. Such a test may not be required if another animal test (e.g. a test for immunogenicity) is to be performed and the test for unexpected toxicity can 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 shown to be safe and effective for vaccine lots 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 abnormalities such as improper sealing, 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, (Annex 1) should be applied (*45*).

A.5 Retained samples

The recommendations in section 9.5 of good manufacturing practices for biological products (Annex 1) should be applied (*45*).

A.6 Labelling

The recommendations in section 7 of good manufacturing practices for biological products (Annex 1) should be applied with the addition of the following (*45*).

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

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

A.7 Distribution and transport

The recommendations in section 8 of good manufacturing practices for biological products (Annex 1) should be applied (*45*).

A.8 Stability testing, storage and expiry date

A.8.1 *Stability testing*

Adequate stability studies form an essential part of the vaccine development studies. The stability of the polysaccharide before conjugation should be demonstrated. 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 from different bulk conjugates.

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 type of excipients and the conditions of storage. The hydrolysis may result in reduced molecular size of the meningococcal 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 (*42*).

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 unbound polysaccharide or bound polysaccharide content as a percentage of the total polysaccharide should be determined. The vaccine should meet the recommendations for the final product (see Part A, sections A.3.3.5 and A.3.3.8) up to the expiry date.

> Molecular sizing of the final product may be carried out to ensure the integrity of the conjugate.

The desorption of antigen from aluminium-based adjuvants, if used, may take place over time and should be investigated and shown to be within the limits agreed by the national regulatory authority.

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 that appear 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. Group C conjugate vaccines have generally proved to be stable over a wide range of storage temperatures, although some formulations have been shown to be affected by repeated freeze-thawing.

A.8.3 *Expiry date*

The expiry date should be approved by the national control 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. Recommendations 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 (*45*) should be applied.

B.2 Official lot 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 meningococcal group C 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 Reactivity and immunogenicity of vaccine in humans

The national regulatory authority should satisfy itself that adequate control of the meningococcal group C conjugate vaccine has been achieved. Manufacturing consistency for vaccine lots used in the clinical trials should be demonstrated and well documented. These lots should be adequately representative of the formulation intended for marketing. Clinical data may be required to support the demonstration of manufacturing consistency. Such studies may need to be repeated if changes in production are made, or when the meningococcal conjugate is part of a new combination vaccine formulation. The national regulatory authority should ensure that the studies are performed in an adequate number of subjects to obtain statistically valid data on reactivity and immunogenicity. The meningococcal group C 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 control authority, which will decide on a case-by-case basis whether additional clinical data are required. Such a review should take into account the likelihood of changes in production or formulation affecting the quality, the consistency, the structural integrity or the immunoge-

nicity of the product, and should also consider the possible cumulative effect of multiple modifications that individually may be regarded as minor.

Two types of assay are useful for measuring antibody responses to vaccination. The studies by Gotschlich et al. demonstrated that a serum bactericidal titre of ≥1:4 measured with human complement is an indicator of clinical protection against group C meningococcal disease (*22*). The SBA thus provides a good surrogate measure of protective immunity. However, subsequent standardization of this assay uses rabbit complement, which has been shown to increase the titres. Nevertheless, there is a general consensus that when baby rabbit serum is used as the source of complement, SBA titres of <1:8 are predictive of susceptibility to invasive meningococcal disease and titres of 1:128 are highly predictive of protection. Currently, there is uncertainty as to whether titres between 1:8 and 1:64 can be a measure of protective immune response and further serological data should be obtained. In the UK, a combination of additional indicators was used to assess immune response to license a meningococcal group C conjugate vaccine. These included:

— evidence of a fourfold rise in antibody titre between pre- and postimmunization sera;
— demonstration of immunological memory; and
— evidence of increased avidity of serogroup C-specific antibody (*36*).

The ELISA is an antigen-binding assay and has less variability that the SBA which is a bioassay. The ELISA measures total or isotype-specific serum antibody responses. However, the results of the "standardized" ELISA (*62*) frequently have not correlated with measurements of SBA. Modifications of the standardized ELISA including the use of more purified polysaccharide, derivatized antigens, and incorporating chaotropic agents in the serum-diluting buffer can improve the correlation (*63*).

In light of emerging data, guidance on study design for the evaluation of new stand-alone or combined meningococcal group C conjugate vaccines and serological methods will be the subject of a WHO consultation to be published as an addendum to these recommendations[1].

Immunization with meningococcal group C conjugate vaccines also primes for the ability to generate memory antibody responses upon

[1] This Addendum was subsequently approved by the WHO Expert Committee on Biological Standardization at its fifty-third meeting (WHO Technical Report Series, No. 926, 2004).

subsequent exposure to plain meningococcal polysaccharide (*5*). Although unproven, the ability of an immunized person to generate a memory antibody response upon exposure to the pathogen may be an important second mechanism of protection, particularly when serum antibody concentrations are below the protective threshold.

Some Hib polysaccharide–protein conjugate vaccines show lower immunogenicity, compared with administration of Hib conjugate vaccine alone, when given in a manufactured combination vaccine; or when mixed with another vaccine immediately before injection; or when administered at the same time as, but as a separate injection to, certain other vaccines. National regulatory authorities should ensure that the data made available to them are relevant to individual national immunization programmes, so that appropriate recommendations may be made regarding co-administration of vaccines.

For combinations of meningococcal group C conjugate vaccines and other antigens, that are either pre-combined or intended to be given by mixing immediately before injection, the national regulatory authority should ensure that there are adequate studies to demonstrate that there is no clinically significant interference with the induction of immunological memory by the meningococcal group C conjugate component.

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Annex 3
Guidelines for the production and control of inactivated oral cholera vaccines

This document provides information and guidance to national regulatory authorities and vaccine manufacturers concerning the characteristics, production and control of inactivated oral cholera vaccines intended to facilitate progress towards their international licensure and use. The text is presented in the form of Guidelines instead of Recommendations because further work is still needed to develop and standardize appropriate methods and criteria that will assure the consistent quality, safety and stability of these vaccines. Guidelines allow greater flexibility than Recommendations with respect to expected future developments in the field and indicate present deficiencies.

1. Introduction

A parenterally administered, killed whole-cell cholera vaccine has been widely available for many years. The WHO Requirements for this vaccine were first adopted in 1959 and revised in 1968 (*1*); an addendum was incorporated in 1973 (*2*). However, this vaccine offers at best only limited protection of short duration and produces unpleasant side-effects in many vaccinees. In view of these limitations, the vaccine has not been considered satisfactory for general public health use, and in 1973 the twenty-sixth World Health Assembly abolished the requirement in the International Health Regulations for a certificate of vaccination against cholera.

Considerable progress has been made during the past decade in the development of a new generation of oral vaccines against cholera. These have already been licensed in some countries and are now being considered for wider public health application (*3*). Two distinct types of oral cholera vaccine have been developed; those consisting of live attenuated bacteria and those consisting of killed (inactivated) bacterial cells. In some cases, the latter are combined with the purified recombinant DNA-derived B-subunit of the cholera toxin. These positive developments have led to a need for international guidance to assure the quality and safety of this new generation of cholera vaccines. The present guidelines apply only to inactivated oral cholera vaccines.

Because the WHO Requirements (*1*) for the production and control of the killed whole cell parenteral cholera vaccine may not be relevant to the production and control of the new generation of cholera vaccines, and because such a vaccine is no longer recommended for general public health use (although it is still produced in some countries), as well as the potential for confusion with guidelines relating specifically to the new vaccines, the Expert Committee for Biological Standardization, decided at its fiftieth meeting to discontinue those requirements (*4*).

2. General considerations

2.1 The pathogen and the disease

Throughout history, the highly pathogenic waterborne bacterium *Vibrio cholerae* has caused devastating outbreaks of diarrhoeal disease in most parts of the world. Altogether seven cholera pandemics have been recorded, the latest of which started in 1961, and is still continuing. An estimated 120000 deaths worldwide are caused by cholera each year. Humans are the only known natural host for *V. cholerae* and the disease is closely linked to poor sanitation. Despite the availability of oral rehydration treatment, small children and the elderly are particularly susceptible to the extreme dehydration that results from severe cholera. Although oral rehydration therapy may often save lives it has no effect on the course of the disease or on dissemination of the infection.

V. cholerae is a Gram-negative, rod-shaped bacterium that carries a single polar flagellum. It is a non-invasive pathogen that colonizes the epithelium of the small intestine after penetrating the mucus layer. The organism causes diarrhoea through the secretion of cholera toxin, the toxic action of which depends on a specific host receptor, the monosialosyl ganglioside GM1.

Strains of *V. cholerae* are characterized by serogrouping based on the polysaccharides of the somatic O antigen. Epidemics have almost invariably been caused by *V. Cholerae* of the O1 serogroup. Three serotypes (Ogawa, Inaba and Hikojima) and two biotypes (classical and El Tor) have been described, although there is some debate as to whether Hikojima is truly a separate serotype. Until recently, *V. cholerae* of the O1 serogroup accounted for most cases of cholera, but an additional *V. cholerae* serogroup, O139, has now emerged as a major cause of cholera in India and Bangladesh (*5*). Serogroup O139 is closely related to the El Tor biotype and has now spread over a large part of Asia. In the 1990s, cholera returned for the first time in

100 years to Central and South America. The causative agent in Latin America is similar, if not identical, to the agent that caused the seventh pandemic in Asia and Africa, i.e. the El Tor biotype of *V. cholerae* serogroup O1.

2.2 Protection against the disease

The available evidence suggests that protection against cholera is best acquired through oral immunization, either through natural infection, or by use of an oral vaccine. Data from studies in Bangladesh indicate that natural cholera infection is about 90% effective in eliciting protection against subsequent attacks for up to 3 years. Infection with the classical biotype of *V. cholerae* (Inaba or Ogawa) appears to stimulate a more potent, or longer-lasting immunity than infection with the El Tor biotype (*6–8*). The traditional killed parenteral cholera vaccine induces only up to 50% protection for 3–6 months. The limited protection afforded by this vaccine seems to be due mainly to the route of administration. Injected cholera vaccine gives rise to little or no local immune response in the gut where both the pathogen and the toxin it produces exert their action during infection. The pathogenesis of *V. cholerae* involves both the colonization of the intestine and the production of the enterotoxin, cholera toxin (CT), which acts locally to stimulate excessive electrolyte and fluid secretion, primarily from the crypt cells of the small intestine. Cholera toxin acts by inducing increased formation of cyclic adenosine monophosphate (cAMP) and/or cyclic guanosine monophosphate (cGMP) in the epithelial cells resulting in the secretion of chloride and bicarbonate into the lumen of the small intestine. Other enterotoxins, such as zonula occludens toxin (ZOT) and accessory cholera enterotoxin (ACE) may also contribute to pathogenesis, but probably play only a minor role. Protection against cholera may therefore be expected to be provided by immune mechanisms that block colonization and multiplication of the pathogen in the intestine inhibit the toxic activity of the toxin, or both. The ability to stimulate local intestinal immunity is therefore now considered critical if a cholera vaccine is to offer protection against infection and the disease (*3*). In addition, antibodies to *V. cholerae* have been found in breast milk and saliva and may be an indirect measure of intestinal immunity (*9*).

2.3 Candidate antigens

Cholera toxin consists of five identical B-subunit peptides that spontaneously associate to form a ring structure into which the enzymatically active A-subunit peptide is non-covalently inserted. The toxic activity resides in the A-subunit while the five B-subunits mediate

binding of the toxin to specific GM1 receptors on intestinal epithelial cells and are primarily responsible for the immunogenicity of the toxin.

The cholera toxin B-subunit elicits an effective antitoxin response that also offers short-lived protection against disease due to the heat-labile toxin (LT) of *Escherichia coli* (*10–12*). Furthermore, the cholera toxin B-subunit appears to be well-suited as an oral immunogen because it is stable in the intestines and is capable of binding to the intestinal epithelium, including the M-cells of the Peyer's patches, which is important for stimulating mucosal immunity, including local immunological memory (*13*). It is believed that this is important for protection because studies in animals have shown a direct correlation between protection against cholera toxin-induced fluid secretion and intestinal synthesis of secretory immunoglobulin A (sIgA) antibodies, and also between protection and the number of antitoxin-producing cells in the intestines. Thus, locally produced sIgA antibodies are considered important for providing antitoxic immmunity in the gut (*14*).

There are, however, other cellular components of *V. cholerae* that induce potentially protective immune responses. The killed whole cells themselves elicit an antibacterial response that is directed mainly against the lipopolysaccharide (LPS) of the pathogen; LPS is the predominant antigen producing immunity to cholera in an experimental setting (*14*). There is also evidence to suggest that an immune response to toxin-coregulated pili (TCP) may also play a role in host protection. In classical *V. cholerae* O1 organisms, TCP have been shown to play an important role in the colonization of the small intestines (*15*). These pili are rarely found on the El Tor vibrios, although an El Tor-specific type of TCP has been reported to be expressed (*16*, *17*). The El Tor organisms, however, express another type of pili called mannose-sensitive haemagglutinin (MSHA) fimbriae; these are poorly expressed on the surface of the classical vibrios (*16*). There is no evidence to suggest that the MSHA fimbriae enhance the immunogenicity of the killed oral vaccines. However, it has been proposed that TCP, while not an important antigen in itself, may enhance immunity by mediating the attachment of the bacteria to the intestinal cells. The relative importance of TCP and LPS as components of inactivated vaccines is unclear.

The growth conditions required for maximum expression of *V. cholerae* antigens in the laboratory need to be carefully determined and may differ significantly from those expected in vivo; for example, the conditions needed for the production of cholera toxin and TCP

(*18*). Furthermore, studies have shown that *V. cholerae*, like other pathogenic bacteria, express a number of antigens during growth in vivo that are not readily produced by the organism when grown under various conditions in vitro (*19*, *20*). With the development of sophisticated genomic-based technologies, including in vivo expression systems to probe host environments, significant new insights into the complexities of host–pathogen interactions are being gained. These may lead to better control of the expression in vitro of antigens that may be important for vaccine production and host protection. Recent studies using in vivo expression technology have shown cholera toxin and TCP to be expressed sequentially during infection and that full toxin expression occurs only after, and is dependent upon, colonization (*21*). There is a possibility that a quorum-dependent signal is involved in the process. Quorum sensing is a process whereby cell–cell communications are mediated by the synthesis, secretion and detection of small extracellular signal molecules (*22*). Cell density is likely to play a part in this process.

2.4 Inactivated oral vaccines

Two killed (inactivated) oral cholera vaccines have been developed and clinically tested. One vaccine, developed in Sweden, consists of inactivated whole cells of *V. cholerae* in combination with a purified recombinant DNA derived B-subunit (rCTB) of the cholera toxin. In early clinical trials of this vaccine a native B-subunit (CTB) was used. The second vaccine, developed in Vietnam following technology transfer from the Swedish manufacturer, consists of whole inactivated *V. cholerae* cells alone. Large-scale field trials in Bangladesh and Peru (*3*, *23–25*) have shown that a whole-cell killed vaccine containing the B-subunit, and a killed whole-cell preparation alone, both produced by a Swedish company, conferred significant protection on recipients for up to 3–5 years depending on age of the vaccines. In the field trial in Bangladesh, three doses of the vaccine containing the B-subunit resulted in 85% and 50% protection when assessed after 6 months and 3 years, respectively, in all age groups, including children aged less than 5 years. However, protection declined rapidly after the first 6 months of follow-up in children aged 2–5 years and disappeared during the third year after vaccination. In contrast, the vaccine from the Swedish manufacturer lacking the B-subunit, that was assessed in Bangladesh, did not confer significant protection against El Tor cholera in young children. In adults, the oral vaccine lacking the B-subunit gave a somewhat lower initial level of protection than that given by the vaccine containing the B-subunit, but after 6 months the protection afforded by the two vaccines was similar. The protective efficacy

of the inactivated whole-cell vaccine containing the rCTB was reproduced in Peru in military recruits in whom two doses gave 86% short-term protective efficacy (*25*). The second vaccine for which clinical trial results are available was produced in Vietnam. Two oral doses of this killed whole-cell oral vaccine lacking the B-subunit were reported to have an efficacy of 66% 8 months after immunization in all age groups (*26*). A second-generation bivalent vaccine, containing the serogroup O139 in addition to O1, but with no B-subunit component, is being developed and evaluated (*3*).

2.5 Correlates of protection

A problem in the evaluation of cholera vaccines is the identification of appropriate markers of protection. Oral vaccination promotes anti-LPS secretory IgA responses similar to those for infection itself (*14*, *27*) whereas parenteral immunization does not. Similarly, the B-subunit of whereas cholera toxin also elicits high antitoxin secretory IgA responses when given orally (*14*, *28*). To be efficacious, cholera vaccine must stimulate a local immune response in the gut mucosa. Intestinal biopsies have shown that there is an increase in antibody-secreting cells specific to the B-subunit of cholera toxin and to whole cells following oral immunization (*29*). However, serum vibriocidal antibodies may offer an indirect measure of the protective immune response. Vibriocidal antibodies are measured by the degree of bacterial lysis that occurs when serial dilutions of serum are incubated with a large standardized inoculum of *V. cholerae* in the presence of complement. Following natural infection of humans, there is a many-fold rise in titre of serum vibriocidal antibodies. Elevated titres of serum antibodies are correlated with protection if immunization was by the oral route (*30*, *31*). The killed whole-cell parenteral vaccine is also capable of eliciting a high vibriocidal titre in immunized individuals, but this vaccine confers only limited protection for a short time. Vibriocidal titre must therefore be seen only as a marker of the stimulation of an appropriate intestinal immune response and not a goal in itself. Serum vibriocidal antibody responses that occur following the ingestion of live oral antigens, delivered by wild type or attenuated *V. cholerae* have been shown to serve as markers for the stimulation of a potential intestinal immunity that endures long after the serum vibriocidal antibody titres have returned to baseline levels (*3*, *8*). In regions where cholera is endemic, vibriocidal antibody titres are relatively high before vaccination, and rises in titre following oral vaccination are modest in comparison with those obtained by vaccinating people in non-endemic area. The only direct predictor of protection to cholera is the local secretory IgA response in the small

intestine, which is clearly not a practical indicator to measure in the context of a large clinical trial. The serum vibriocidal titre is therefore the most useful marker presently available for indicating an appropriate immune response in humans.

2.6 Production and control of inactivated oral cholera vaccines

The vaccines currently produced typically contain 25–50 $\times$ 10^9 cells per dose of each of the strains of *V. cholerae* representing both Inaba and Ogawa serotypes, as well as classical and El Tor biotypes. Some formulations also contain inactivated *V. cholerae* O139 (50 $\times$ 10^9 cells). The vaccine from Sweden also contains 1 mg per dose of purified rDNA derived B-subunit of the cholera toxin.

The whole-cell components of the vaccines are inactivated individually, before or after washing, either by treatment with formaldehyde or by heating. Inactivated bacterial cultures are then harvested by centrifugation or ultrafiltration, washed, resuspended in buffer and mixed with the B-subunit of cholera toxin, if used, to produce the final bulk from which the final lots are produced.

There is no precedent for controlling this new type of vaccine (i.e. an inactivated killed oral vaccine), and there is as yet no internationally accepted direct method for measuring the potencies of such products that guarantees that protective immunity will be elicited in the target population. At present, there is no animal model that can meaningfully be used to measure or predict the potency of these vaccines in humans. It is not known whether animal potency tests using parenteral administration of vaccine would be a reliable indicator of the protective effect of the same vaccine when administered orally. Additionally, the available evidence on tests using the parenteral administration of vaccine to rabbits suggests that the immunological response does not follow a dose–response relationship; in mice parenteral administration results in a large variability in antibody titres that would necessitate the use of a large number of animals. For this reason an animal potency assay has been omitted from these Guidelines. Research to identify appropriate assays that better predict protective efficacy in humans is strongly encouraged. Such assays should be able to detect sub-potent batches of vaccines.

In the light of these difficulties, it is suggested that emphasis should be placed on the characterization and quantification in vitro of the critical vaccine antigens and components. The characteristics of the various antigens and components claimed to contribute to vaccine efficacy, together with data on vaccine composition and dosage, consistency of production, and conformity with specifications, of the

vaccine used in clinical trials, will give some indication, though not definitive proof, of the ability of a vaccine lot to elicit protective immunity. These antigens and components might include LPS, TCP, which it is suggested could act to enhance the immune response rather than as an antigen in itself, and, where indicated, the B-subunit of the cholera toxin. Thus the immunological, biological and biochemical characterization of the individual components claimed to contribute to vaccine efficacy is critical for demonstrating their structural and/or functional integrity in vaccine production lots. Relevant tests should be performed before any procedure such as detoxification, chemical or heat treatment (which may modify the immunological or biological characteristics of the component), is carried out. This would apply to any component considered to be important to the performance of the vaccine, but that may not easily be tested for following inactivation. Other tests, such as that for residual activity of cholera toxin should be undertaken routinely after detoxification, chemical or heat treatment of vaccine lots, or as part of process validation.

Residual cholera toxin is a possible contaminant of inactivated whole-cell oral vaccines. Rigorous washing of the culture and inactivation using heat or formaldehyde treatment are features of the production process. However, a toxicity test to confirm freedom from toxicity will be necessary, and acceptable limits of cholera toxin activity should be set to confirm consistency of manufacture. The amount of active cholera toxin in a new production lot should not exceed that present in lots shown to be safe in clinical studies. The mouse weight-gain test currently in use to monitor the toxicity of vaccine lots is considered to be insufficiently sensitive and of questionable relevance. A more relevant and validated test should be sought. The potential use of the Y-1 adrenal cell assay for cholera toxin as a more specific test for residual toxicity should be investigated. Such a specific test could be used on a-lot-to-lot basis or to validate the production process.

Should the use of vaccine involve administration in extra buffer to protect against acid conditions in the stomach (as for the vaccine containing the B-subunit) the buffer should be similar to that used in the clinical studies and compatible with the vaccine.

The need for a preservative in multidose presentations of an oral vaccine should be carefully evaluated and consideration given to the use of a non-mercury-based preservative should one be thought necessary. If no preservative is added to multidose containers a time-limit of a maximum of 6 hours should be imposed on the storage of opened containers.

3. Manufacturing recommendations

These Guidelines apply to the production and control of liquid formulations of inactivated cholera vaccine intended for oral administration. The Guidelines emphasize the importance of in-process controls for biologicals and cover the following three areas:

— the starting materials;
— the manufacturing process; and
— the final product.

The general manufacturing recommendations contained in good manufacturing practices for pharmaceutical (*35*) and biological products (*36*) should be applied at establishments manufacturing inactivated oral cholera vaccine.

Production and control of the rDNA-derived B-subunit using a genetically modified strain of *V. cholerae* should be according to the guidelines for assuring the quality of pharmaceutical and biological products prepared by recombinant DNA technology (*32*) and other relevant recommendations (*33*, *34*). The same guidelines would apply equally to the production of rCTB in any other host organism, such as *Escherichia coli*.

V. cholerae is a class 2 pathogen and represents a particular hazard to health through infection by the oral route. It should be handled under appropriate conditions for this class of organism (*37*). Standard operating procedures need to be developed for dealing with emergencies arising from the accidental spillage, leakage or other dissemination of cholera organisms. Personnel employed in the production and control facilities should be adequately trained. Appropriate protective measures including vaccination should be implemented. Adherence to current good manufacturing practice and appropriate biosafety measures are important to the integrity of the product, to protect workers and to protect the environment.

Details of standard operating procedures for the preparation and testing of inactivated oral cholera vaccines adopted by a manufacturer, together with evidence of appropriate validation of each production step, should be submitted for approval to the national regulatory authority. All assay procedures used for quality control of the vaccine and vaccine intermediates should also be validated (*38*). Proposals for modifications of the manufacturing process or control methods should be submitted for approval to the national regulatory authority before they are implemented.

The general recommendations for control laboratories contained in the guidelines for national regulatory authorities on quality assurance

for biological products (*39*) should be applied. A vaccine lot should be released using a batch release procedure and only if it fulfils national requirements.

3.1 Control of starting materials

3.1.1 *Strains of* V. cholerae

The current vaccines consist of classical and El Tor biotypes of Inaba and Ogawa serotype and, in some cases, the O139 serotype may be included. The strains used should have the appropriate morphological, cultural, biochemical, serological and other properties appropriate to the strain. A strain of *V. cholerae* that has been genetically modified to delete cholera toxin A-subunit genes is currently used to produce the rDNA derived B-subunit when this is included in the vaccine.

3.1.2 *Seed-bank system*

The production of *V. cholerae*, including strains containing the plasmid encoding the recombinant B-subunit should be based on a master and working seed lot system. Cultures derived from the working seed lot should have the same characteristics as the cultures of the strain from which the master seed lot was derived. If materials of animal origin are used in the medium for seed production, preservation of strain viability for freeze-drying, or for frozen storage, they should comply with the guidance given in the report of a WHO consultation on medical and other products in relation to human and animal transmissible spongiform encephalopathies (*40*) and should be approved by the national control authorities.

3.1.3 *Culture media for growth of organisms*

Where possible, materials of non-animal origin should be used. If materials of animal origin are used, they should comply with the guidance given in the report of a WHO consultation on medical and other products in relation to human and animal transmissible spongiform encephalopathies (*40*) and should be approved by the national regulatory authorities. Human blood or reagents derived from human blood must not be used in either the culture media used for the production of seed banks or of vaccine. If human albumin is used in any part of the production process, it should meet the requirements for the collection, processing and quality control of blood, blood components and plasma derivatives (*41*) as well as current guidelines in relation to human transmissible encephalopathies.

3.2 Control of the manufacturing process

3.2.1 *Control of production cultures*

Production cultures should be shown to be consistent in respect of growth rate, pH and yield of cells or cell products. Acceptance specifications should be established.

Cultures should be checked at different stages of fermentation for purity, identity and cell density. Unsatisfactory cultures must be discarded. Where a plasmid-containing strain (see section 3.2.2) is used for the production of the recombinant B-subunit, the cultures should be checked for the presence and identity of appropriate genetic markers. Numbers of plasmid copies should be checked routinely at lot release or confirmed during process validation.

At the time of harvest and prior to detoxification, whole cell bulks should be checked for purity, identity, opacity, pH and relevant biochemical and antigenic characteristics. For assessing purity, samples of the culture should be examined by microscopy of Gram-stained smears, by inoculation of appropriate culture media or by another suitable procedure.

Following killing by heat or formaldehyde treatment, the cultures should be checked for viability, purity, opacity, identity and pH. The inactivation process may affect cell morphology or integrity, and opacity measurements may not be a reliable indicator of bacterial numbers. Assays for specific antigen content should be used to determine the concentrations of the monovalent bulks used for formulating vaccines based on killed cells only. Assays for each specific LPS should be employed.

3.2.2 *Control of production of purified rDNA derived B-subunit*

3.2.2.1 Strategy for cloning and expressing the gene

A full description of the host cell and expression vectors used in production should be given. This should include:

— the source, genetic characteristics and details of maintenance of the host strain or strains;
— the construction, genetics and structure of the expression vector;
— the origin and identification of the gene that is being cloned.

The cultural conditions used to promote and control the expression of the cloned gene in the host cell should be described in detail. Agents known to provoke sensitivity reactions in certain individuals, such as penicillin or other beta-lactam antibiotics, should not be used in the fermentation process.

The stability of the expression system during storage and beyond the passage level used in production should be documented and specifications set for plasmid retention during storage of seed and during production. The stability of the host-vector system should either be confirmed during process validation or checked routinely at the end of fermentation. Unstable systems should not be used. The expression system should be approved by the national regulatory authority.

3.2.2.2 Characterization of the recombinant vector

The nucleotide sequence of the gene insert and of adjacent flanking segments of the vector, together with restriction enzyme mapping and/or full sequencing of the vector containing the gene insert should be provided to the national regulatory authority.

3.2.2.3 Purification procedures

The methods used to purify the rDNA B-subunit from culture harvests should be described in detail; the capacity of each stage of the purification procedure to remove or inactivate substances other than the B-subunit should also be determined. In particular, the capacity of the purification process to assure the absence of significant quantities of any holotoxin or other *V. cholerae* toxins, such as zonula occludens toxin or accessory cholera enterotoxin, should be assessed, unless it has been demonstrated that the cloning and expression procedures eliminate all possibility of production of such factors. Limits should be established for the quantities of impurities detected in the purified B-subunit preparation and these impurities should be identified and characterized as appropriate.

3.2.3 ***Characterization of rDNA derived B-subunit***

Rigorous characterization of the rDNA derived B-subunit product should be undertaken using a variety of analytical techniques exploiting several different properties of the molecule, including size, charge and amino acid composition. Techniques suitable for such purposes include SDS-polyacrylamide gel electrophoresis (SDS-PAGE), size-exclusion and reverse-phase chromatography. Sufficient sequence information should be obtained by direct sequencing and by peptide mapping, or another appropriate molecular technique, for example, mass spectrometry, in comparison with the natural material. The identity of the product should be confirmed by at least partial *N*-terminal and *C*-terminal amino acid sequencing. Several lots of the product should be as fully characterized as possible. Several appropriate methods should then be selected for use in routine lot release.

Data should be provided on the consistency of yield in terms of both quantity and quality of product for sequential production runs. The effects of freeze-drying should also be investigated.

The rDNA derived B-subunit should be shown to elicit antibody responses in humans, with the antibodies shown to be functional (e.g. toxin-neutralizing) in a suitable assay.

3.3 Control of final bulk

3.3.1 *Preparation*

For vaccine formulated from killed cells only, the final bulk is prepared by mixing suitable quantities of each monovalent bulk suspended in the appropriate buffer. For vaccines containing the rDNA B-subunit, this component is dissolved in buffer to an appropriate concentration and then mixed with the cell suspension final bulk to achieve a mixture containing each component at the required concentration. Preservative, if used, may be added either to individual monovalent bulks or at the final bulk stage.

3.3.2 *Antigen content*

The concentration of each specific antigen (i.e. total O1 or O139 LPS, TCP as appropriate) that is considered to play a part in protection should be assayed in the final bulk by a suitable immunoassay approved by the national regulatory authority. Similarly, for formulations containing the B-subunit, its concentration in the final bulk should be assayed by an approved method, for example, single radial diffusion. The final concentration of each active component should be within limits that are consistent with those of lots shown to be safe and efficacious in clinical trials.

3.3.3 *Detoxifying agents*

If formaldehyde or another detoxifying agent is used in the preparation of killed cells, its residual concentration should be determined in the final bulk by a method approved by the national regulatory authority. The final concentration should not exceed the limits established for clinical trial lots that have been shown to be safe and efficacious.

3.3.4 *Sterility*

Each final bulk should be tested for bacterial and fungal sterility in accordance with the requirements of Part A, sections 5.1 and 5.2 of the revised requirement for biological substances (*41*) 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.

3.3.5 *Preservative*

If a preservative has been added, its concentration may be determined at the bulk stage by a method approved by the national regulatory authority. The preservative, its concentration and its limits should be approved by the national regulatory authority.

3.3.6 *Potency/immunogenicity*

At present there is no animal potency or immunogenicity assay that can be recommended for use as a reliable indicator of the protective efficacy of inactivated oral cholera vaccines in humans or for the detection of sub-potent batches (see section 2.6).

3.3.7 *Residual toxin activity*

Cholera toxin should be assayed by a method approved by the national regulatory authority. Alternatively, the production process should be validated to show that the quantities of clinically active cholera toxin present in the product are insignificant. The inactivation process should also be validated to assure the absence of significant quantities of holotoxin or other *V. cholerae* toxins.

3.4 Control of final lot

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

3.4.1 *Appearance*

The final containers should be inspected visually (manually or with automatic inspection systems). After shaking, the vaccine should form a uniform, turbid, white or brownish suspension free of aggregates and extraneous particles. Containers showing abnormalities must be discarded.

3.4.2 *Identity*

An identity test should be performed on at least one labelled container from each final lot. The test used should identify the type of vaccine formulated. For preparations formulated from killed cells alone, a serological test that detects *V. cholerae* O1 and O139 (if present) antigens will suffice. For preparations formulated from killed cells and rDNA B-subunit, the identity test must be able to detect the presence of both types of component. The procedures used should be

approved by the national regulatory authority. The antigen-content assays (see below) could also serve as an identity test.

3.4.3 *Antigen content*

The concentration of each specific antigen (i.e. total O1 or O139 LPS, TCP as appropriate), that is considered to play a part in protection, should be assayed by a suitable immunoassay approved by the national regulatory authority. Similarly, for formulations containing the B-subunit, its concentration should be assayed by an approved method, for example, single radial diffusion. The final concentration of each active component should be within limits that are consistent with those of lots shown to be safe and efficacious in clinical trials.

3.4.4 *Sterility*

Each final lot should be tested for bacterial and fungal sterility as indicated in section 3.3.4.

3.4.5 *Preservative content*

If a preservative is included, each final lot should be assayed for preservative content unless this was done on the final bulk. The assay method used and the preservative content permitted should be approved by the national regulatory authority.

3.4.6 *pH*

The pH should be tested and shown to be within the range of values found suitable for vaccine lots that have been shown to be safe and effective in clinical trials and in stability studies.

3.4.7 *General safety (innocuity)*

No such test is recommended for an oral preparation.

3.5 Stability, storage and expiry date

The stability of the vaccine in its final container, when maintained at the recommended temperature, should be established using real-time studies. These should be conducted on at least three consecutive final lots, derived from separate antigen-production lots.

The content of *V. cholerae* LPS and other specified antigens should remain within specified limits for the duration of the shelf-life. If the formulation contains the B-subunit, its content must also remain within specified limits for the duration of the shelf-life. Accelerated stability studies at elevated temperatures may provide additional evidence of vaccine stability, but cannot replace real-time studies.

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

If monovalent bulks or final bulk products are to be stored, stability studies should be performed and an appropriate shelf-life assigned on the basis of the data obtained.

3.6 Reference materials

No formally established international reference materials are currently available for the standardization of oral cholera vaccines, but their development is under consideration. Manufacturers should set aside, as reference material, a vaccine lot identical with, or demonstrated to be equivalent to, a lot shown to give acceptable performance in clinical trials. It is recommended that the reference lot should be stabilized by a validated procedure, such as freeze-drying, to maintain stability over a long period.

Other reference materials should include a stabilized preparation of the rDNA B-subunit and holotoxin.

Manufacturers and national regulatory authorities, should establish reference antisera against O1 and O139 LPS antigens and, monospecific antisera or monoclonal antibodies to Inaba, Ogawa epitopes and B-subunit.

Authors

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Annex 4
Guidelines on viral inactivation and removal procedures intended to assure the viral safety of human blood plasma products

List of abbreviations and definitions used in this Annex

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

AHF	Antihaemophilic factor. Blood coagulation factor VIII, missing in patients with classic haemophilia.
Blood components	These typically refer to red blood cell concentrates, platelet concentrates and plasma.
BEV	Bovine enterovirus. A non-enveloped, single-stranded RNA virus used as a model for hepatitis A virus.
BVDV	Bovine viral diarrhoea virus. An enveloped, single-stranded RNA virus used as a model for hepatitis C virus.
CMV	Cytomegalovirus. An enveloped, double-stranded DNA virus, typically cell-associated.
Coxsackie virus	A non-enveloped, single-stranded RNA virus.
CPV	Canine parvovirus. A non-enveloped, single-stranded DNA virus.
Donor retested plasma	A process for reducing window period transmissions whereby fresh frozen plasma is held in the inventory for a designated period of time until the donor returns and tests negative for virus exposure. The initial unit is then released for use. Also called *quarantine plasma*.
Dry heat	A process of heating protein following lyophilization, typically at 80 °C or higher.
EBV	Epstein–Barr virus. An enveloped, double-stranded DNA virus, typically cell-associated.
EMCV	Encephalomyocarditis virus. A non-enveloped, single-stranded, RNA virus.
Factor IX	Blood coagulation factor IX, missing in patients with haemophilia B.
Factor VIII	Blood coagulation factor VIII, missing in patients with haemophilia A. Also called antihaemophilic factor.
FFP	Fresh frozen plasma.
Fluence	The total quantity of light delivered. Expressed in J/cm^2.
Gamma-irradiation	A process of virus inactivation or bacterial sterilization using gamma-irradiation of liquid, frozen or lyophilized product.

GE	Genome equivalents. The amount of nucleic acid of a particular virus assessed using nucleic acid testing.
GMPs	Good manufacturing practices. Sometimes referred to as current good manufacturing practices.
HAV	Hepatitis A virus. A non-enveloped, single-stranded RNA virus.
HBsAg	Hepatitis B surface antigen. The antigen on the periphery of hepatitis B virus.
HBV	Hepatitis B virus. An enveloped, double-stranded DNA virus.
HCV	Hepatitis C virus. An enveloped, single-stranded, RNA virus.
HDV	Hepatitis delta virus. A defective virus which requires co-infection by hepatitis B virus.
High purity factor VIII	Factor VIII concentrate with a specific activity typically greater than 100 IU/mg.
HIV	Human immunodeficiency virus. An enveloped, single-stranded RNA virus.
HSV	Herpes simplex virus. An enveloped, double-stranded DNA virus, typically cell-associated.
HTLV 1 and 2	Human T-cell lymphotropic virus, types 1 and 2. Enveloped, single-stranded RNA viruses, typically cell-associated.
ID_{50}	The quantity of virus or other infectious agent that will infect 50% of subjects or tissue cultures. Frequently expressed on a log scale; thus, 6 $\log_{10}$ ID_{50} represents 1 million infectious units.
Immunogenic	Causing the formation of antibody. Harsh processing conditions may modify the structure of a protein so as to make it immunogenic.
Intermediate purity factor VIII	Factor VIII concentrate with a specific activity between 1 and 50 IU/mg.
IVIG	Intravenous immunoglobulin.
Limiting dilution	A way of determining titre by diluting the sample continually until the positive signal is lost.
LRF	Log reduction factor. The quantity of virus, expressed on a log 10 scale, inactivated or removed.

MB-plasma	Methylene blue-treated plasma intended as a substitute for fresh frozen plasma.
Nanofilters	Filters that usually have effective pore sizes of 50 nm or less, designed to remove viruses from protein solutions.
NAT	Nucleic acid testing, using amplification techniques such as polymerase chain reaction.
Pasteurization	A process of heating protein in solution, typically at 60 °C.
Polio virus	A non-enveloped, single-stranded, RNA virus.
PPRV	Porcine pseudorabies virus. An enveloped, double-stranded DNA virus.
PPV	Porcine parvovirus. A non-enveloped, single-stranded DNA virus.
Prion	The infectious particle associated with transmissible spongiform encephalopathies. It is believed to consist only of protein and to contain no nucleic acid.
PRV	Pseudorabies virus. An enveloped, double-stranded DNA virus.
Psoralen	A furocoumarin ring structure, which when exposed to light, cross-links nucleic acid.
Quarantine plasma	*See donor retested plasma*.
RT3	Reovirus type 3. A non-enveloped, double-stranded RNA virus.
Rutin	A flavonoid used as an antioxidant that reduces the action of reactive oxygen species.
Solvent/detergent treatment	A process of treating protein in solution, usually with the organic solvent, tri(*n*-butyl)phosphate, and a detergent such as Tween 80 or Triton X-100.
SD-Plasma	Solvent/detergent-treated plasma intended as a substitute for FFP.
Sindbis virus	An enveloped, single-stranded RNA virus.
SLFV	Semliki forest virus. An enveloped, single-stranded, RNA virus.
Titre	The quantity of virus, typically expressed on a $\log_{10}$ scale. Six logs of virus are equal to 1 million infectious units.
TNBP	Tri(*n*-butyl)phosphate. The organic solvent used with *solvent/detergent treatment*.
Triton X-100	A non-ionic detergent frequently used as part of *solvent/detergent treatment*.

Tween 80	A non-ionic detergent frequently used as part of *solvent/detergent treatment*.
UVC	Ultraviolet irradiation, usually at a wavelength of 254 nm.
Vaccinia virus	An enveloped, double-stranded DNA virus.
Vapour heating	A process of heating protein following lyophilization and then reintroducing moisture normally at 60 °C and in some cases at 80 °C.
Viral inactivation	A process of enhancing viral safety in which virus is intentionally "killed".
Viral removal	A process of enhancing viral safety by removing or separating the virus from the protein(s) of interest.
VSV	Vesicular stomatitis virus. An enveloped, single-stranded RNA virus.
West Nile virus	An enveloped, single-stranded RNA virus.

1. Introduction and scope

Human blood is the source of a wide range of medicinal products used for the prevention and treatment of a variety of often life-threatening injuries and diseases. Despite measures such as donor selection, testing of donations and of plasma pools, the transmission of blood-borne viruses by plasma and purified plasma products is still considered to constitute a risk to patients. Over the past 15–20 years, the transmission of the principal viral threats historically associated with these products — hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus (HIV) — has been greatly reduced or eliminated in many areas of the world. This is a consequence of the more sensitive methods being used to screen donated blood and plasma pools, and of the establishment of manufacturing practices that lead to significant virus inactivation and removal. Several procedures for virus inactivation and removal have proven to be robust and to contribute substantially to blood product safety. Viral inactivation methods should be applied to all blood plasma-derived protein solutions.

Continuing concerns about the quality and safety of plasma-derived medicinal products have resulted in a number of urgent requests from Member States for support and advice from WHO. Moreover, the World Health Assembly Resolution No 50.20, of 13 May 1997 on the "Quality of biological products moving in international commerce", requested WHO to extend the assistance offered to Member States to

develop and to strengthen their national regulatory authorities and control laboratories to increase competence in the area, and to extend efforts to upgrade the quality and safety of all biological products worldwide.

The present WHO Guidelines on viral inactivation and removal procedures intended to assure the viral safety of human blood plasma products were developed to complement the WHO Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives"(*1*), in response to the above requests. These Guidelines pertain to the validation and assessment of the steps for viral inactivation and removal employed in the manufacture of human blood plasma derivatives and virally inactivated plasma for transfusion, prepared either from plasma pools or from individual donations. It is hoped that this document, by summarizing current experience with well recognized methods, will help set expectations, serve as a guide to speed implementation, and ensure that implementation is appropriate.

Inevitably, individual countries may formulate different policies, not only in relation to procedures for validation and control, but also regarding donor selection and methods of blood screening. These Guidelines do not replace the requirements of regulatory authorities in various parts of the world (*2–4*); rather, they are primarily intended to assist those national regulatory authorities and manufacturers that are less familiar with viral decontamination processes.

The document does not address products of animal origin or those manufactured by recombinant techniques.

2. General considerations

Viral safety derives from three complementary approaches during manufacture, i.e. donor selection, testing of donations and plasma pools, and the introduction of viral inactivation and removal procedures in the course of manufacture, each of which requires strict adherence to good manufacturing practices (GMPs). Although these Guidelines address only viral inactivation and removal, no individual approach provides a sufficient level of assurance, and safety will only be achieved by using a combination of the three.

Some of the principles that relate to viral inactivation and removal procedures as applied to purified blood plasma products and to plasma intended for transfusion are listed below.

- Viral contamination can arise from the donor, or, less commonly, from other sources introduced during manufacture (e.g. from the reagents employed).
- Viral validation studies are intended to assess the degree to which virus infectivity is eliminated during manufacture. These studies can only approximate the inactivation and removal that occur during routine manufacture because the model viruses employed in the studies may differ from those present in blood, and it may be difficult or impossible to truly model the conditions employed during manufacture. Thus, the appropriateness of the studies needs to be reviewed on a case-by-case basis, and the manufacturer should justify the choice of viruses and the validation conditions employed.
- Viruses to be studied, where required, include: HIV; a model for hepatitis C such as Sindbis virus or bovine viral diarrhoea virus (BVDV); one or more non-enveloped viruses such as hepatitis A virus, encephalomyocarditis virus (EMCV), or porcine parvovirus; and an enveloped DNA virus such as pseudorabies virus or duck hepatitis B virus.
- The ability of a process to inactivate or remove viruses should take into account:
  — the reduction in virus titre achieved;
  — for inactivation processes, the rate of inactivation and the shape of the inactivation curves; for removal, mass balance;
  — how robust the step is in response to changes in process conditions; and
  — the selectivity of the process for viruses of different classes.

  Data should be analysed using appropriate statistical procedures.
- Virus removal should be distinguished from virus inactivation. This is important in ensuring the accurate modelling of a process step and identifying the parameters that are most effective in reducing infectivity in that process. For example, if a chromatography step removes viruses, flow rates and column dimensions are important process variables, whereas if the buffer used inactivates viruses, temperature and pH are likely to be more significant.
- Purification procedures such as precipitation or chromatography can contribute to virus removal; however, removal depends critically on the protein composition and the separation conditions used, and it is difficult to scale down partition processes for validation purposes. Therefore, all appropriate specifications and accepted tolerances should be stated, and control data provided. For chromatographic columns and media, the conditions of storage, preservation and regeneration should be described.

- Validation studies need to be well documented to ensure proper execution of the procedure. The highest titre of virus that can reasonably be employed should be added (spiked) into the solution to be tested at a ratio not exceeding one part virus to nine parts sample. Virus infectivity starting titre should be measured, ideally after addition to the sample, and then with time during the virus inactivation and removal procedure. Worst case conditions must be studied. Appropriate controls should be run to demonstrate the validity and sensitivity of the assay.
- All viral infectivity tests suffer from the limitation that the ability to detect low viral concentrations depends for statistical reasons on the size of the sample. Consequently, the largest sample size that can be practically assayed should be chosen if the study indicates that all viruses are inactivated or removed.
- Appropriate procedures should be employed throughout the manufacturing process to prevent recontamination following use of a virus inactivation or removal method.
- Priority for validating the viral inactivation steps used in the manufacture of plasma protein solutions should be given to those products with the highest risk potential, such as coagulation factors, proteolytic inhibitors and intravenous immunoglobulins.

3. Infectious agents

3.1 Viruses, viral burden and screening methods

Medicinal products made from human blood include clotting factors, immunoglobulins and albumin among others, have all at some time transmitted serious virus infections to recipients. The object of viral inactivation and removal procedures is to improve viral safety so that such transmissions no longer occur. The viruses of particular concern, HBV, HCV and HIV, have all been transmitted by some plasma products, and all cause life-threatening diseases. Other viruses of concern include hepatitis A virus and parvovirus B19, both of which have been transmitted by clotting factor concentrates. Some of the properties of these viruses are listed in Table 1.

The pathogenicity of a virus may depend on the patient group and on the product being administered. For example parvovirus B19 infects the red blood cell precursors and effectively eliminates them for a period. Parvovirus infections are usually relatively mild in the general population because most people have a substantial buffer of mature red cells. However, in patients with haemolytic anaemias (such as sickle-cell anaemia), parvovirus infections can be fatal because the

Table 1
Selected properties of some plasma-borne viruses

Virus	Genome	Envelope	Size (nm)
Hepatitis B virus	dsDNA	yes	40–45
Hepatitis C virus	ssRNA	yes	40–50
Human immunodeficiency virus	ssRNA	yes	80–130
Hepatitis A virus	ssRNA	no	28–30
Parvovirus B19	ssDNA	no	18–26

ds, double-strand; ss, single-strand

lifespan of mature red cells is shorter. Parvovirus B19 may be of greater concern in Africa where sickle-cell anaemia is relatively more common than in Europe, and it is possible that other agents (e.g. hepatitis E virus) would be significant in other geographical settings depending on their prevalence in the donor population. Other examples include cytomegalovirus and human T lymphotropic virus I and II (HTLV I + II) which are strongly cell-associated and are therefore not considered to pose a significant risk in therapeutic proteins derived from human plasma, although they have been transmitted by cellular components in blood transfusions, and HAV, which can be transmitted by purified coagulation factor concentrates, but is not usually a problem with products such as intravenous immunoglobulin (IVIG) that contain anti-HAV antibodies.

For the product to be safe, the production process must inactivate and/or remove all the virus present. The quantity of virus depends on the number of infected donors contributing to the pooled starting material and the titre (concentration) of infectious virus in those donations. Estimates of the frequency of occurrence of hepatitis viruses, HIV and parvovirus and their titres prior to the implementation of screening tests, in European and US donor populations are given in Table 2. For example, before tests for HCV antibody were developed, approximately 1–2% of donors were unknowingly infected with HCV. Parvovirus is now known to be present in 1/1000–1/7000 blood donors, largely because it is a common infection in the general population, and tests for it are not routinely employed. Most pools of 10 000 or more unscreened donor units would therefore be expected to be contaminated with HCV and parvovirus. When this information is combined with the titre of virus in contaminated units and the number of donors contributing to the plasma pool, the titre in the plasma pool can be calculated (Table 2). Because the titres of HCV RNA in an infected individual may range from 10^4 to 10^6 ge-

Table 2
Viruses in plasma from unscreened donor blood

Virus	Prevalence in donor blood	Viral titre (GE/ml)	Calculated titre in plasma pool (GE/ml)[a]
Hepatitis B virus	1/10000	10^3–10^8	0–10^4
Hepatitis C virus	1/50–1/100	10^4–10^6	10^2–10^4
Human immunodeficiency virus	1/1000–1/10000	10^3–10^7	0–10^4
Hepatitis A virus	1/500000	10^3–10^5	0–10^1
Parvovirus B19	1/1000–1/7000	10^2–10^{12}	0–10^9

[a] Assumes the pooling of 10000 units.

Table 3
Frequency of HCV RNA-positive plasma pools following testing of single donations for anti-HCV antibody

Screening test on individual unit	Number of pools (positive/total)	Percentage hepatitis C virus PCR positive
None	8/8	100
First-generation antibody test	65/85	76
Second-generation antibody test	49/123	39

Source: Nübling, Willkommen & Löwer (*5*).
PCR, polymerase chain reaction.

nome equivalents (GE)/ml and those of parvovirus B19 DNA from 10^2 to 10^{12} GE/ml, plasma pools would be expected to contain 10^2–10^4 GE/ml of HCV and 0–10^9 GE/ml of parvovirus. Put more simply, most pools of 10000 or more unscreened donor units would be expected to be contaminated with HCV and parvovirus, whereas contamination with HBV, HIV and HAV would occur at a lower frequency. Viral titres of HBV, HCV and HIV in the plasma pool can reach 10^4 GE/ml. It should be noted that the incidence of virally infected units depends on several factors including the population from which the donors are drawn and, for parvovirus, on seasonal variations.

A study conducted at the Paul Ehrlich Institute, Germany, determined the frequency of HCV RNA-positive pools before and after screening of donors was introduced, using first- or second-generation tests for HCV antibody (Table 3) (*5*). Although screening reduced the number of antibody-positive pools, it is important to note that the viral titre in those pools that were contaminated was not reduced. This is a consequence of using a test for the antibody rather than for

Table 4
Average window period estimates for HIV, HCV and HBV

Virus	Window period without nucleic acid amplification technology (days)	Window period with minipool nucleic acid amplification technology (days)
Human immunodeficiency virus	22	10
Hepatitis C virus	82	9
Hepatitis B virus	59	49

Sources: Schreiber et al. (*8*); Kleinman et al. (*9*).

the virus and because in the case of HCV, and many other viruses, peak titres occur before the appearance of the antibodies in the circulation (i.e. the so-called window period). Nonetheless, because the screening of donors for markers of infection such as hepatitis B surface antigen or antibodies to HIV or HCV can reduce the number of positive pools and, in certain circumstances, the virus load in the starting material, screening is an important element in assuring viral safety.

Nucleic acid amplification technology (NAT) has been introduced in some instances to detect viral nucleic acid. As nucleic acid is associated with the virus itself rather than the host response to infection, NAT minimizes the window period and reduces the total quantity of virus in the plasma pool (*6*, *7*). Window period estimates are given in Table 4. As an additional measurement of the effectiveness of donor screening, the quantity of viral genomic nucleic acid present in the plasma pool can be assessed by NAT. Even if only carried out intermittently, performing NAT on plasma pools provides a basis for assessing product safety when coupled with the data quantifying virus removal or inactivation.

Finally, it should be recognized that all screening methods are subject to the criticisms that they are unable to detect virus infection below a certain level, and that errors in the screening process may occur, particularly where large numbers of donations are used. Additionally, screening is limited to the viruses being tested for. Thus, while screening helps to ensure that the virus load is kept to a minimum, it is not sufficient to ensure safety in itself, and the ability of the production process to remove or inactivate viruses is a crucial second element. The proportion of potential donors who are infected with viruses will depend on the particular geographical region. In donors from certain areas, HBV or HIV infections may be far more common than in those

from countries where the strategies for ensuring viral safety have evolved. Where this is the case, the ability of the production process to inactivate or remove viruses will be even more important.

3.2 Other infectious agents

Bacteria and parasitic infections including malaria and trypanosomes do not pose a risk in plasma products that have been sterile filtered with a 0.2 µm filter.

Prions, the putative causative agent of the transmissible spongiform encephalopathies including Creutzfeld Jakob Disease (CJD) of humans, are a matter of concern, especially as a result of the occurrence of variant CJD (vCJD) in the United Kingdom following the epidemic of bovine spongiform encephalopathy. The continuing concern stems, in part, from experimental evidence in animal models that infectivity could be present in blood, albeit late in infection and at low levels. However, there has been no increase in the incidence of classic CJD (currently one death per million head of population per year wherever it has been measured), despite the increased transfusion of blood and the extremely hardy nature of the agent. As with CJD, there is no evidence that vCJD has been transmitted by blood, blood components or plasma-derived products in clinical practice. However, since vCJD is a newly emerging disease, it is too early to conclude that there is no risk. Measures to minimize the risks to humans from human- and bovine-derived materials are summarized in the report of a WHO consultation on medicinal and other products in relation to human and animal transmissible spongiform encephalopathies (*10*).

3.3 Validation of viral inactivation and removal procedures

3.3.1 *Selection of relevant and model viruses*

The viruses that may contaminate blood and blood products encompass all of the viral types, including viruses with a DNA or RNA genome, with and without a lipid membrane, and ranging in size from the smallest, such as parvovirus, to the middle range, such as HBV. The processes employed should therefore be shown to be able to remove or inactivate a wide range of viruses if they are to be considered satisfactory; typically, validation studies have involved at least three viruses, chosen to represent different kinds of agent.

Viruses have been selected to resemble those that may be present in the starting material (Table 5). All are laboratory strains that may be grown to high titre and assayed readily. The models for hepatitis C virus include BVDV, Sindbis virus, Semliki forest virus and yellow

Table 5
Plasma-borne viruses and their models

Virus	Examples of viruses used to model inactivation/ removal studies
Hepatitis B virus	Duck hepatitis B virus, pseudorabies virus[a]
Hepatitis C virus	Bovine viral diarrhoea virus, Sindbis virus, Semliki forest virus, Yellow fever virus
Human immunodeficiency virus	Human immunodeficiency virus
Hepatitis A virus	Hepatitis A virus, encephalomyocarditis virus
Parvovirus B19	Canine parvovirus, porcine parvovirus

[a] Because there are no convenient models for hepatitis B virus, pseudorabies virus is frequently used: both pseudorabies virus and hepatitis B virus are enveloped, double-stranded DNA viruses.

fever virus as they share many properties, including a lipid membrane, an RNA genome and a particle size of 40–50 nm. Laboratory strains of HIV or hepatitis A virus are used, and canine and porcine parvovirus have been used as models for parvovirus B19. Suitable models for hepatitis B virus have been more difficult to identify, because few viruses of this family can be grown in culture. Duck hepatitis virus has been used, but the pseudorabies virus has also been employed as a large DNA virus. This list is not exhaustive and other appropriate viruses are acceptable. The main viruses of concern are HIV, HBV and HCV, and laboratory viruses are almost always used to represent them. During the developmental phase, viruses that are particularly resistant to the approach taken often serve as useful surrogates. As an example, the use of vesicular stomatitis virus (VSV) has proven useful when first qualifying a viral inactivation step based on low pH or solvent/detergent treatments. Nonetheless, for product registration, the use of viruses that better resemble those present in the starting material should be used. Precautions needed for the safe handling of the viruses for both human and animal contacts should be taken into account in the design and execution of the studies. Readers are directed to existing guidance documents for additional details on the selection and assay of model viruses (*2*, *3*).

3.3.2 *Modelling (downscaling) the production process*

The production process can be viewed as a series of steps, and it is the obligation of the manufacturer to identify those steps likely to remove or inactivate virus and to demonstrate the degree of virus reduction achieved by following them. Not every step needs to be evaluated. The ability of the steps in a process to remove or inactivate viruses is measured on a laboratory scale and not in the production facility

where it would be inappropriate to introduce infectious virus deliberately. The accuracy of the model is crucial, and should be assessed by comparing the characteristics of the starting material and the product for that step for both laboratory and full-scale processes. In the model of the process, physical factors (e.g. temperature, stirring, column heights and linear flow rates, and sedimentation or filtration conditions) and chemical factors (e.g. pH, ionic strength, moisture and the concentration of inactivating agents) should be equivalent to the real process) where possible. It should be noted that whereas many process steps can be modelled readily, models of ethanol fractionation processes have proved particularly variable, in part because of difficulties in scaling down centrifugational processes and in controlling subzero temperatures on a small scale.

Once the step is accurately modelled, virus is introduced into material derived from the fractionation process just prior to the step being evaluated, and the amount remaining after the modelled process step is measured. The results are conventionally expressed in terms of the logarithm of the reduction in infectivity reported. Total infectivity or viral load is calculated as the infectious titre (infectious units per ml) multiplied by the volume. Viral clearance compares the viral load at the beginning with that at the conclusion of the step being evaluated.[a]

For viral inactivation procedures, both the kinetics and extent of virus inactivation need to be demonstrated. The kinetics of inactivation are important since the rapid kill of large amounts of virus is a further indication of the virucidal potential of the step and, for well-characterized procedures for viral inactivation, enables comparison of a process with similar processes executed by others (see section 4). For viral removal systems, an attempt should be made to show mass balance, i.e. to account for all of the virus added. If the buffers used are virucidal, it is important to distinguish virus inactivation from virus removal.

It is necessary to evaluate the effect of possible variations in the process conditions on the virus clearance observed, for example the effects of changes in temperature or composition of the starting material for the particular step. A robust, effective and reliable process step will be able to remove or inactivate substantial amounts of virus, typically 4 logs or more, be easy to model convincingly and be rela-

[a] For example, if at the start of a step the viral titre is 10^5/ml and the volume is 20 ml and at the conclusion of the step the viral titre is 10^1/ml and the volume is 60 ml, then the viral load at the start is 6.3 logs and at the end is 2.8 logs, and the viral clearance is 3.5 logs.

tively insensitive to changes in process conditions. Steps removing 1 log of virus or less cannot be regarded as significant. A production process that includes two robust steps able to remove or inactivate enveloped viruses is likely to give a safe product, particularly if the steps act by different mechanisms (e.g. inactivation by a chemical treatment followed by a robust physical removal step). Non-enveloped viruses are more difficult to remove or inactivate. A process that includes one robust step effective against non-enveloped viruses may give a safe product; failing this, other approaches including implementing screening procedures, e.g. NAT, may prove helpful in excluding infectious material.

Virus validation studies are subject to a number of limitations. The subdivision of the process into individual steps which are separately assessed assumes that the effects of different procedures can be added up in some way. This is true only if the fraction of virus surviving one step is not resistant to another, which is not always the case. If virus is resistant to a chemical treatment because it is present as an aggregate that the chemical cannot penetrate, it may also be resistant to a second, different, chemical treatment. Care must be taken to not count the same treatment twice, for example if ethanol has a direct inactivating effect on a virus, steps in fractionation involving increasing concentrations of ethanol may all inactivate the virus in the same way, and will therefore have no additive effects. In contrast, if the reduction in viral infectivity results from the removal of virus particles at one ethanol concentration, followed by the inactivation of virus at a higher concentration, the effects may be summed. Care must therefore be taken to provide justification for summing the effects of different steps which, ultimately, is dependent on the steps removing or inactivating viruses by different mechanisms. Other limitations are that the properties of the virus used in the laboratory studies may differ in from that occurring in nature, the plasma may contain antibodies to the virus of interest that may affect virus inactivation or removal in unpredictable ways, there may be fractions of virus that are resistant to a number of steps, and the modelling of the process may be imperfect. The clearance figures obtained are therefore approximate.

The difficulties of establishing an adequate laboratory model for virus inactivation and removal mean that the figures produced are unlikely to fully reflect manufacturing operations. In general, for a product to be safe, the process must remove or inactivate virus infectivity to a much greater extent than the level of virus in the starting materials. The use of two complementary steps for virus inactivation and removal may be especially important if the population of donors con-

tributing to the plasma pool has a high incidence of bloodborne viruses, leading to a high viral load in the material being processed. A second advantage in employing two complementary methods of virus inactivation and removal is the potential to increase the spectrum of viruses covered.

3.3.3 *Other considerations*

In practice many inactivation and removal processes result in a product that is safe. For bacteria, a sterile product is conventionally defined as one having fewer than one infectious organism in one million doses. No comparable figure has been agreed upon for viral sterility because viruses are more difficult to assay in the final product, the titre of virus in the stocks used to spike product is limited, and assessing the ability of a process to remove or inactivate viruses is subject to significant sources of error.

The testing of a final product for viral markers, as part of the routine batch release, has generally been found to contribute little to safety. Commercially available serological tests are generally not designed or validated for use with purified fractions. For most products, the purification process is likely to remove viral antibody or antigen to levels below the limit of sensitivity of the test, and for immune globulin preparations testing by ELISA typically yields a very high rate of false-positive results because of their high immune globulin content. With respect to genomic tests, NAT testing of plasma pools has proven useful; however, NAT cannot distinguish virus that has survived an inactivation step from inactivated virus, and if infectious virus is present, it is likely to be at very low concentration. Therefore NAT testing of final product is not recommended. Should final product testing be performed, the tests used must be shown to be suitable for their intended purpose.

3.3.4 *Measurement of infectivity*

The provision of details on the methods used to measure viral infectivity is beyond the scope of this document, and readers are referred to other available guidelines (see references *2–4*). A sample final report of a viral inactivation study is given in Appendix 1. A few points to consider are given below.

- Care should be taken when preparing virus stocks with high titres to avoid aggregation which may enhance physical removal and decrease inactivation thus distorting the correlation with actual production.

- The virus spike should be added to the product in a small volume so as to not dilute or alter the characteristics of the product. Typically, a spike of 5–10% of the total volume is employed.
- Buffers and product should be evaluated independently for toxicity or interference in viral infectivity assays used to determine viral titres, as these components may adversely affect the indicator cells. If the solutions are toxic to the indicator cells, dilution, adjustment of the pH, dialysis of the buffer, or other steps to eliminate toxicity or interference will be necessary. Sufficient controls to demonstrate the effect of procedures used solely to prepare the sample for assay (e.g. dialysis and storage) on the removal or inactivation of the spiking virus should be included.
- If samples are frozen prior to assay, sufficient controls need to be run to show that the freeze/thaw cycle does not affect virus infectivity. Inactivating agents should be removed prior to freezing.
- The reliability of the viral assays employed needs to be demonstrated. This may necessitate repeat runs of the experiment with or without slight changes in conditions to evaluate the robustness of the procedure, and use of viral assay systems of appropriate statistical reliability. A well controlled in vitro virus assay should have a within-assay 95% confidence interval of plus or minus $0.5 \log_{10}$.

4. Review of well-recognized methods for viral inactivation and removal

The methods described in this section are generally recognized as contributing substantially to viral safety based on the following factors:

— their application to a variety of products;
— use by several manufacturers;
— the availability of a substantial body of preclinical and clinical information; and
— their robust nature.

Well recognized methods of inactivation (pasteurization, dry heat, vapour heat, solvent/detergent and low pH) are described in section 4.1, and well recognized methods of removal (precipitation, chromatography and nanofiltration) are described in section 4.2. The selection of the methods to be employed for viral inactivation and removal depends on the size and lability of the protein being prepared, the method(s) of purification the manufacturer wishes to use, and the nature and titre of the viruses of concern. Each method of inactivation and removal has special characteristics that need to be taken into

account. For example, solvent/detergent is very effective against enveloped viruses, but does not inactivate non-enveloped viruses. If HBV is a principal concern, solvent/detergent may have an advantage over methods that employ heating because HBV is known to be relatively heat stable. On the other hand, several methods of heating have been shown to inactivate 4 logs or more of HAV; therefore if HAV is the virus of concern, heat has an advantage over solvent/detergent. As mentioned above, from a virus safety perspective, the best procedures will use a combination of complementary methods because combinations have the advantage of increasing the spectrum of viruses covered as well as of increasing the total quantity of virus that is eliminated. Whether one or more methods of inactivation and removal are used, the maintenance of protein structure and function is equally as important as viral safety and must be evaluated thoroughly. The general characteristics of well recognized methods of inactivation and removal are listed in Tables 6a and 6b, and examples of the successful application of individual, dedicated viral inactivation and removal procedures to commercialized products are provided in Table 6c. Subsequent sections provide representative data applicable to a variety of products; nonetheless, manufacturers are obligated to evaluate virus inactivation and removal in each of their products.

4.1 Methods of inactivation

4.1.1 *Pasteurization of albumin*

Albumin solutions are heated as a liquid at $60 \pm 0.5\,^{\circ}C$ for 10–11 hours continuously, usually following sterile filtration and dispensing into final containers (glass vials). If pasteurization is conducted before filling, care must be taken to prevent post-treatment contamination, and bacterial sterility may be compromised. To prevent denaturation of albumin, low concentrations of sodium caprylate alone or with *N*-acetyl tryptophan are added prior to sterile filtration. Safety with respect to hepatitis viruses and HIV has been demonstrated for decades, with few exceptions (*11*). Much of this history derives from albumin manufactured using cold ethanol fractionation, which also contributes to safety. The inactivation of model viruses added to 5% albumin solution on heating at $60\,^{\circ}C$ is shown in Figure 1. Infectious virus can no longer be detected after 10 minutes of treatment. Because the conditions of treatment are well established and, in some countries, specified by regulation, manufacturers are not required to validate the effectiveness of the treatment itself; however, they need to demonstrate that the process parameters of temperature and time are met. Homogeneity of temperature is typically achieved by total immersion of the vials in a water-bath or by placing them in a forced-

Table 6a
Characteristics of well recognized virus inactivation procedures

Treatment	Advantages	Points to consider	Most relevant properties to be recorded
Pasteurization	• Inactivates both enveloped and some non-enveloped viruses including HAV • Relatively simple equipment	• Protein stabilizers may also protect viruses • HBV is relatively heat stable • Does not inactivate parvovirus B19 • Process validation required	• Temperature • Temperature homogeneity • Duration • Stabilizer concentration
Terminal dry heat	• Inactivates both enveloped and some non-enveloped viruses including HAV • Treatment applied on the final container	• At least 80 °C usually required for elimination of hepatitis viruses • Does not inactivate parvovirus B19 • Requires strict control of moisture content • Freezing and lyophilization conditions require extensive validation	• Freeze cycle • Lyophilization cycle • Temperature homogeneity • Residual moisture
Vapour heat	• Inactivates both enveloped and some non-enveloped viruses including HAV	• Does not inactivate parvovirus B19 • Freezing and lyophilization conditions require extensive validation • Relatively complex to implement	• Freeze cycle • Initial lyophilization cycle • Temperature homogeneity • Moisture before and after heating
Solvent/detergent	• Very efficient against enveloped viruses • Does not denature proteins • High process recovery • Relatively simple equipment	• Non-enveloped viruses unaffected • Not generally affected by buffers used • Solvent/detergent reagents must be removed	• Temperature • Duration • Reagent concentration
Acid pH	• Effective against enveloped viruses • Relatively simple equipment	• Limited efficacy against non-enveloped viruses • Use largely restricted to IgG • At pH 4, effective virus kill requires elevated temperatures • Process validation required	• pH • Temperature • Duration

HAV, hepatitis A virus; HBV, hepatitis B virus.

Table 6b
Characteristics of well recognized virus removal procedures

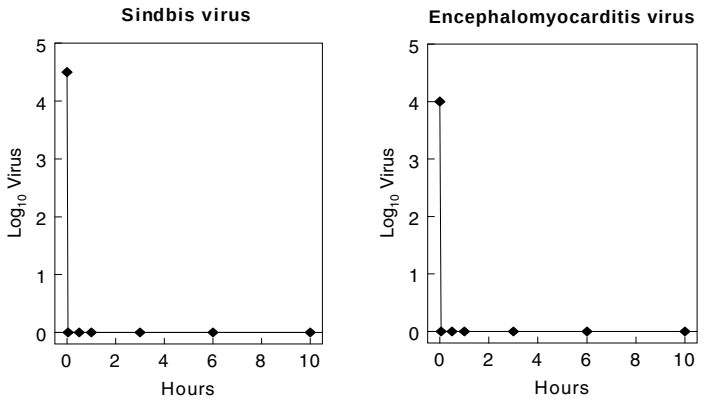
Treatment	Advantages	Points to consider	Most relevant properties to be recorded
Precipitation	• Purifies protein • Can be effective against both enveloped and non-enveloped viruses including HAV and parvovirus B19	• Virus removal usually modest • Difficult to model	• Concentration of precipitation agent(s) • Protein concentration, pH, and possibly ionic strength . • Temperature • Timing for the addition of precipitation agent and for precipitate ageing • Degree of contamination of precipitate with supernatant (or vice versa)
Chromatography	• Purifies protein • Can be effective against both enveloped and non-enveloped viruses including HAV and parvovirus B19	• Virus removal highly dependent on choice of resin, protein solution and buffers • May be highly variable from one virus to another • Degree of virus removal may change as resin ages • Resin must be sanitized between lots	• Resin packing by e.g. HETP measurements • Protein elution profile • Flow rate and buffer volumes • Number of cycles of resin use
Nanofiltration	• Effective against enveloped viruses • Can be effective against non-enveloped viruses including HAV and parvovirus B19 • Does not denature proteins • High recovery of “smaller” proteins such as coagulation factor IX • Risk of downstream contamination limited when performed just prior to aseptic filling	• Degree of virus removal depends on the pore size of filter used • Elimination of small viruses may be incomplete • Filter defects may not be detected by integrity testing	• Pressure • Flow-rate • Filter integrity • Protein concentration • Ratio of product volume to filter surface area

HAV, hepatitis A virus; HETP, height-equivalent theoretical plates.

Table 6c
Established applications of dedicated viral inactivation and removal procedures to marketed plasma products

Treatment	Product type
In-process	
Solvent/detergent treatment	• IgG • Coagulation factors (e.g. factor VIII, factor IX, prothrombin complex, fibrin sealant) • Protease inhibitors (e.g. antithrombin III) • Plasma
Pasteurization	• IgG • Coagulation factors (e.g. factor VIII, factor IX, von Willebrand factor, prothrombin complex, fibrin sealant) • Protease inhibitors (e.g. antithrombin III and alpha-1-proteinase inhibitor)
Steam-treatment	• Coagulation factors (e.g. factor VIII, factor IX, fibrin sealant) • Protease inhibitors (e.g. C1-inhibitor)
Incubation at pH 4	• IgG
Nanofiltration (35 nm or less)	• IgG • Coagulation factors (e.g. factor VIII, factor IX, von Willebrand factor, prothrombin complex) • Protease inhibitors (e.g. antithrombin III)
Terminal (final container)	
Terminal pasteurization	• Albumin
Terminal dry-heat treatment	• Coagulation factors (e.g. factor VIII, factor IX and factor XI)

Figure 1
Rate of virus inactivation on pasteurization of 5% albumin at 60 °C[1]



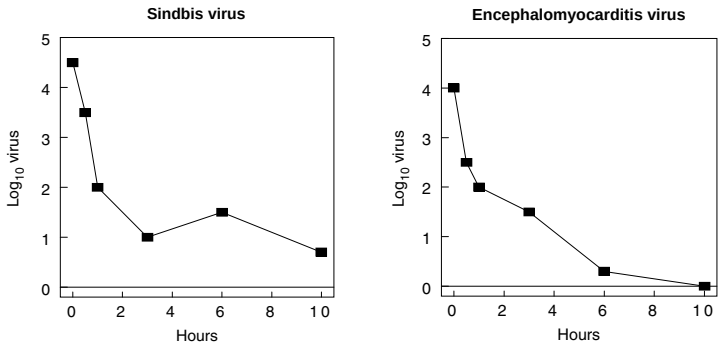
[1] On this and other similar graphs, "0" indicates that no infectious virus was detected.
Source: Horowitz et al. (*12*).

air oven. In both cases, temperature-mapping studies are required to demonstrate homogeneity, including measurements of both the temperature of the water or air and of the product itself. These studies must be performed with representative loads. Once validated, temperature probes are placed at strategic points in the water-bath or oven during each pasteurization run. Albumin used to stabilize other parenteral drugs should conform to the same requirements as albumin for therapeutic use.

4.1.2 *Pasteurization of other protein solutions*

Most proteins denature when heated in solution at 60 °C. To maintain the biological function of the more labile proteins, general stabilizers such as amino acids, sugars or citrate are added. Because these may also stabilize viruses, virus inactivation procedures need to be validated in model studies for each product under the conditions of treatment specified by the manufacturer. Following pasteurization, the stabilizers usually need to be removed. This is typically accomplished by diafiltration, size exclusion chromatography, or positive adsorption chromatography where the protein of interest binds to a chromatographic resin. Pasteurization has been used successfully with a variety of plasma protein products including coagulation factors and immune globulin solutions, although in rare instances transmission of HBV has been reported (*13*). A common method of preparing factor VIII is to heat it at 60 °C for 10 hours in the presence of high concentrations of glycine and sucrose or selected salts. Published results

Figure 2
Rate of virus inactivation on pasteurization of blood coagulation factor VIII at 60 °C in the presence of 50% sucrose and 2.75 M glycine



Source: Horowitz et al. (*12*).

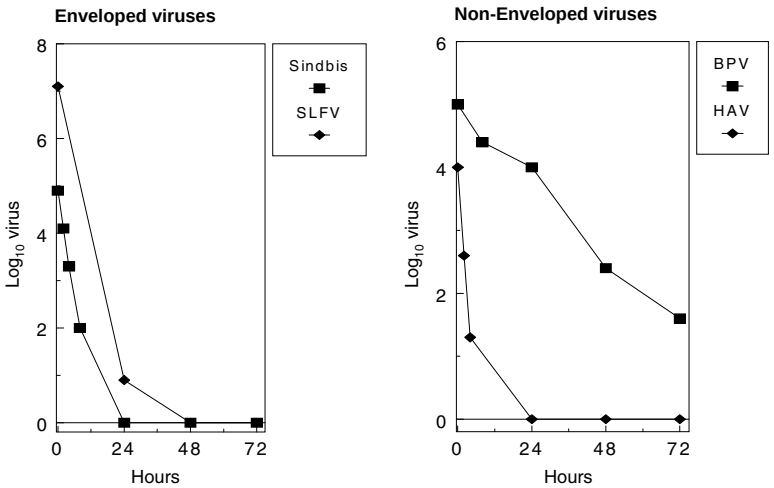
showing the extent and rate of virus inactivation of blood coagulation factor VIII are illustrated in Figures 2 and 3.

Prior to heating, the solution is typically filtered through a 1 µm or finer filter to eliminate particles that might entrap and further stabilize viruses. Heating is conducted in a jacketed tank and the solution is usually stirred throughout the heating cycle. Temperature-mapping studies are conducted to ensure that the temperatures at all points in the tank are within the range specified by the process record. Care must be taken to ensure that all parts of the tank, including the lid, where solution might splash, are heated. Viral inactivation studies, conducted under worst-case conditions, are performed at the lowest temperature that might be encountered in an acceptable production run. Protein recovery should be monitored during virus inactivation studies and should be comparable to that achieved at scale.

4.1.3 *Heating of dry (lyophilized) products*

Proteins can withstand being heated at temperatures of 60–80 °C or higher when they are first lyophilized to remove water. Heating at 60–68 °C for up to 72 hours has generally not been found to eliminate hepatitis transmission (*15*), whereas heating at 80 °C has produced favourable results with respect to transmission of HBV, HCV, HIV and HAV. (*16*) Recently, at least one manufacturer has been treating its coagulation factor VIII with solvent/detergent and also heats final product for 30 minutes at 100 °C. All HAV (≥5 logs) was inactivated within 4 minutes (*17*). Since viruses may be more stable following lyophilization, virus inactivation needs to be validated for each

Figure 3
Rate of inactivation of enveloped and non-enveloped viruses during dry-heat treatment of blood coagulation factor VIII at 80°C



BPV, bovine parvovirus; HAV, hepatitis A virus; SLFV, Semliki forest virus.
Results generously provided by the Scottish National Blood Transfusion Service.

product under the conditions of treatment specified by the manufacturer. Viral inactivation is influenced by residual moisture, the formulation (e.g. content of protein, sugars, salts and amino acids), and by the freezing and lyophilization cycles. Residual moisture is influenced by the lyophilization cycle and may be introduced inadvertently by the rubber stoppers.

Since virus inactivation is very sensitive to residual moisture content, the setting of upper and lower limits for moisture should be based on viral validation studies, and the variation of moisture content between vials should be within the limits set. To ensure reproducibility, one manufacturer has stipulated that, during the freeze-drying process, the temperature in three or more product vials, the shelf coolant temperature and the chamber pressure must remain within defined limits for each timed phase of the lyophilization cycle for every batch manufactured. Following freeze-drying, vials are stoppered under sterile, dry nitrogen at atmospheric pressure to ensure a constant atmosphere from vial-to-vial during dry-heat treatment. In addition, from every lyophilization run, the residual moisture content of five vials out of a lot of 1500 is measured following heat treatment. The moisture contents of these vials are used to calculate the 95% confi-

Table 7
Treatment of a solution of blood coagulation factor VIII by pasteurization

Virus	Extent of inactivation (log ID_{50})	Inactivation time (hours required)
Human immunodeficiency virus	≥5.0	1.0
Cytomegalovirus	≥6.0	8
Epstein–Barr virus	≥3.3	0.5
Herpes simplex virus	≥5.9	4
Poliovirus	≥7.1	10
Vaccinia virus	6.2	10

Source: Hilfenhaus, et al. (*14*).

dence interval for the batch, and this interval must be within the upper and lower limits of moisture defined for the product.

Again using the specifications of one manufacturer, the dry-heat treatment, itself, is performed at 80.25 ± 0.75 °C for 72 hours. Process monitoring during heat treatment is carried out by means of temperature sensors located in 10 vials distributed throughout the load and two "air" probes located at the previously determined warmest and coldest points in the oven. All temperature sensors (both those in the vials and those measuring air temperature) must reach 79.5 °C before the cycle timer starts. Temperatures recorded by all sensors should remain stable between 79.5 °C and 81 °C for a continuous period of 72 hours. In addition, the dry-heat ovens are validated every 6 months, when a further 12 independent probes (10 in vials and two "air" probes) linked to a separate chart recorder are included to increase the temperature coverage to 24 points. In this way the temperature control is tested and the temperature spread within the cabinets established. The cycle time on the automatic control is also checked for accuracy.

Typical results achieved by heating factor VIII at 80 °C are given in Table 8 and Figure 3.

4.1.4 *Heating of lyophilized products under humidified conditions (vapour heating)*

At equivalent temperatures, a higher level of virus inactivation can be achieved by the addition of water vapour before initiating the heat cycle. To assure proper application of this approach, the material to be heated, the addition of moisture and the heat cycle need to be tightly controlled. In one case, freeze-dried intermediate bulk product is homogenized by a combination of sieving and milling. After deter-

Table 8
Treatment of lyophilized blood coagulation factor VIII at 80°C for 72 hours

Virus	Extent of inactivation (log ID_{50})	Inactivation time (hours required)
Sindbis virus	8	72
Human immunodeficiency virus	≥6.4	72
Vaccinia virus	2.6–3.3	72
Herpes simplex virus	2.2	48
Semliki forest virus	≥6.9	24
Hepatitis A virus	≥4.3	24
Canine parvovirus	≥2.1	48

Sources: Knevelman et al. (*18*) Winkelman et al. (*19*) and Hart et al. (*20*).

mination of the residual water content, the freeze-dried intermediate is transferred into a stainless steel tank where an amount of water vapour, that has been predetermined based on the weight and the residual water content of the lyophilized product, is slowly added to adjust the water content to 7–8% (w/w). After an equilibration period, the water content is measured again before the product is ready for vapour heating. The intermediate product is transferred to a stainless steel cylinder. The cylinder is flushed with dry nitrogen to remove oxygen, and a pressure test is performed to ensure that the cylinder is airtight. This cylinder is then transferred to a heating cabinet equipped with an electric heater and a fan to ensure even temperature distribution. The intermediate product within the cylinder is heated according to the temperature regimen specified for the particular product. The cylinder is subjected to an oscillating rotation, changing direction every half-turn, until the end of vapour heating. During the heating process the pressure inside the vessel rises due to heating of the enclosed nitrogen, which cannot expand in the closed cylinder, and also due to evaporating water vapour from the moist intermediate product. After vapour heating, the heating cabinet is opened from the other side, and the product is further processed in a different and isolated manufacturing zone to prevent cross-contamination from non-inactivated product.

To assure consistency from lot-to-lot, the ranges for protein, salt and water content are set on the basis of the results of preliminary viral infectivity and protein functional studies. Additionally, the ratio of product weight to cylinder volume is specified for each product. A pressure test is performed before the start of vapour heating to ensure that the cylinder is airtight. During heating, product temperature and air temperature (one temperature sensor each) and pressure within

Table 9
Virus inactivation by Vapour heating at 60 °C for 10 hours

Product	Virus	Extent of inactivation (log ID_{50})	Inactivation time (hours required)
Factor VIII: intermediate purity	HAV	>3.3	8
	HIV	>6.8	10
	PRV	5.9	10
Factor VIII: high purity	HAV	3.9	10
	HIV	6.7	10
	PRV	5.6	10
Factor IX: intermediate purity	HAV	>5.7	6
	HIV	>6.5	6
	PRV	>7.1	8
Factor IX: high purity	HAV	>6.7	3
	HIV	>7.9	8
	PRV	>6.8	8

Data and process information provided courtesy of Baxter/Immuno. See also Barrett et al. (*24*) and Dorner and Barrett (*25*)
HAV, Hepatitis virus A; HIV, Human immunodeficiency virus; PRV, pseudorabies virus.

the cylinder are measured continuously and must conform to the specifications set for each. Following vapour heating, the water content of the intermediate is measured again.

Although historical reports indicate some cases of transmission of enveloped virus (*21*, *22*), the preponderance of clinical data indicate safety with respect to to transmission hepatitis viruses and HIV. (*23*) It should be noted that some products are heated at 60 °C for 10 hours and others are additionally heated at 80 °C for 1 hour; however, this cannot be considered as the use of two independent steps and the viral kill observed cannot be summed. Typical results achieved by vapour heating are given for several products in Table 9.

4.1.5 *Solvent/detergent treatment*

Organic solvent/detergent mixtures disrupt the lipid membrane of enveloped viruses. Once disrupted, the virus can no longer bind to and infect cells. Non-enveloped viruses are not inactivated. The conditions typically used are 0.3% tri(*n*-butyl) phosphate (TNBP) and 1% nonionic detergent, either Tween 80 or Triton X-100, at 24 °C for a minimum of 4 hours with Triton X-100, or 6 hours with Tween 80. When using TNBP and Triton X-100, some preparations can be treated successfully at 4 °C. Since high lipid content can adversely affect virus inactivation, the final selection of treatment conditions must be based on studies demonstrating virus inactivation under

worst-case conditions; i.e. lowest permitted temperature and reagent concentration and the highest permitted product concentration. Prior to treatment, solutions are filtered through a 1-μm filter to eliminate virus entrapped in particles. Alternatively, if filtration is performed after addition of the reagents, the process should be demonstrated to not alter the levels of solvent and detergent added. The solution is stirred gently throughout the incubation period. When implementing the process in a manufacturing environment, physical validation should be used to confirm that mixing achieves a homogeneous solution and that the target temperature is maintained throughout the designated incubation period. Mixture homogeneity is best verified by measuring the concentrations of TNBP or detergent at different locations within the tank, although measuring dye distribution might be an acceptable substitute. To ensure that every droplet containing virus comes into contact with the reagents, an initial incubation for 30–60 minutes is typically conducted in one tank after which the solution is transferred into a second tank where the remainder of the incubation takes place. In this manner, any droplet on the lid or a surface of the first tank that might not have come into contact with the solvent/detergent reagents is excluded. The use of a static mixer where reagents and plasma product are mixed before being added to the tank is an acceptable alternative. The tank in which viral inactivation is completed is located in a separate room in order to limit the opportunity for post-treatment contamination. This room typically has its own dedicated equipment and may have its own air supply. When the treatment is complete, the solvent/detergent reagents must be removed. This is usually accomplished by extraction with 5% vegetable oil, positive adsorption chromatography (where the protein of interest binds to a chromatographic resin), or adsorption of the reagents on a C-18 hydrophobic resin. Depending on the volume of product infused and the frequency of infusion, the permitted residual levels of TNBP, Tween 80 and Triton X-100 are generally, 3–25, 10–100 and 3–25 ppm, respectively.

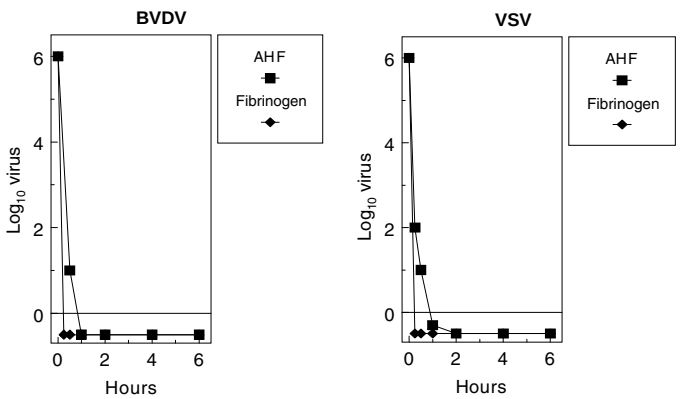
When performing viral validation studies, the reaction is stopped either by dilution or, in some cases, adsorption of the TNBP and Triton X-100 by a C18 hydrophobic resin. An appropriate control needs to be run to establish that virus inactivation does not continue following the use of the stop procedure. Safety with respect to HBV, HCV and HIV has been demonstrated in numerous clinical studies that reflect the high level of virus inactivation demonstrated in both laboratory and chimpanzee studies. Typical results achieved on treating a coagulation factor VIII concentrate and fibrinogen at 24 °C are given in Table 10 and Figure 4.

Table 10
Treatment of blood coagulation factor VIII solution with 0.3% TNBP and Tween 80

Virus	Extent of inactivation (log ID_{50})	Inactivation time (hours required)
Vesicular stomatitis virus	≥4.5	2
Sindbis virus	≥5.5	1
Sendai virus	≥6.0	1
Hepatitis B virus	≥6.0	6[a]
Hepatitis C virus	≥5.0	6[a]
Hepatitis D virus	≥4.0	6[a]
Human immunodeficiency virus-1	≥6.0	0.25

Sources: Horowitz (*26*) and Horowitz et al. (*27*).
[a] These studies were conducted in the chimpanzee model; 6 hours was the only time-point tested.

Figure 4
Treatment of AHF and fibrinogen by solvent/detergent[a]

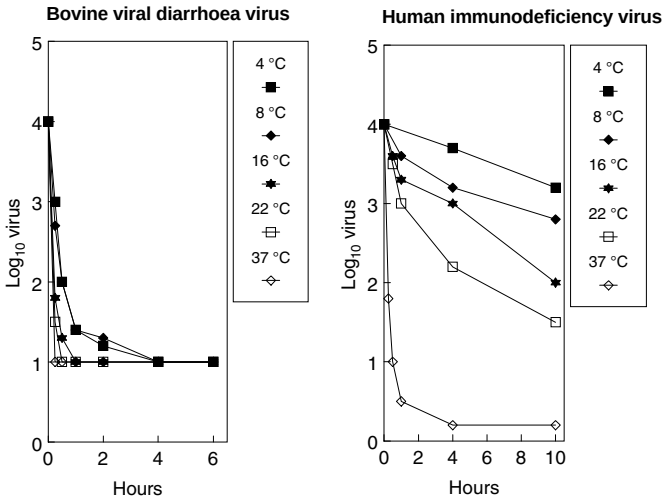


AHF, blood coagulation factor VIII; BVDV, bovine viral diarrhoea virus; TNBP, tri(*n*-butyl) phosphate; VSV, vesicular stomatitis virus.
[a] AHF was treated with 0.3% TNBP and 1% Tween 80 at 24°C and fibrinogen was treated with 0.3% TNBP and 1% Triton X-100 at 24°C. At the time-points indicated, BVDV and VSV infectivity were measured.
Data provided courtesy of V.I. Technologies.

4.1.6 *Low pH*

Most proteins are damaged by exposure to the acidic conditions needed to kill viruses. For example, few viruses are killed at pH 5.0–5.5, a condition known to inactivate factor VIII. Immune globulin solutions are an exception. Various studies have shown that low pH, such as in the pH 4-treatment used in the preparation of

Figure 5
Inactivation of viruses in IgG at pH 4 in the presence of pepsin



Source: Omar et al. (*28*).

immunoglobulins, inactivates several enveloped viruses. (*28*) The presence of trace concentrations of pepsin added to reduce anticomplementary activity during this procedure has been shown to contribute little to virus inactivation. Since acid treatment was originally designed to reduce IgG aggregation and anticomplementary activity, a number of variants of this procedure have been developed; hence, the conditions being used may or may not inactivate virus efficiently. Each manufacturer's process needs to be validated separately because virus inactivation is influenced by pH, time, temperature, pepsin content, protein content and solute content. As an example, the effects of time and temperature on the inactivation of BVDV and HIV in one preparation are given in Figure 5. On the basis of these and other results, one manufacturer incubates its immunoglobulin preparation at pH 4.0 for at least 6 hours at 37 °C whereas another follows solvent/detergent treatment by incubating in the container at pH 4.25 for a minimum of 21 days at 20 °C.

4.2 Methods of virus removal

Before the 1980s, conditions for the fractionation of plasma were selected largely on the basis of considerations of protein purification and less on the capacity of the process to remove virus. Modern purification procedures frequently consider both protein purification and virus removal. For example, an ion-exchange or monoclonal

antibody column may be selected for the degree of protein purification provided, but also be characterized fully with respect to virus removal. Based on this characterization, additional wash buffers or greater volumes of wash buffer may be used to increase the degree of virus removal. Additionally, in the last few years, specific removal methods such as nanofiltration have been developed, and others, such as viral affinity adsorbents, are under development. Such methods are intended to remove viruses. Where virus removal is believed or claimed to be an important consideration for a particular purification step, whether intended or not, the same discipline in validating and implementing that step should be used as is applied to a virus inactivation step.

4.2.1 *Precipitation*

Precipitation with ethanol is the single most widely used plasma fractionation tool worldwide, although other reagents have been used. In addition to its use as a precipitant, ethanol is also a disinfectant. Unfortunately, it acts as a disinfectant mostly at room temperature or above, whereas plasma fractionation is carried out at a low temperature to avoid protein denaturation. The contribution of ethanol to viral safety through inactivation is, therefore, marginal at best. Nonetheless, ethanol can also partially separate virus from protein. Viruses, as large structures, tend to precipitate at the beginning of the fractionation process when the ethanol concentration is still relatively low. As with any other precipitation reaction, the distribution of viruses between precipitate and supernatant is never absolute.

The following log reduction factors (LRFs) were reported for three distinct steps in albumin production by cold-ethanol precipitation (Table 11; the designations of the steps correspond to the Kistler/Nitschmann fractionation scheme) and for the production of immunoglobulin (Table 12). (Note that LRFs should not be summed across

Table 11
Log reduction factors for four different viruses and for three precipitation steps used during the manufacture of albumin

Step	Ethanol %	pH	Log reduction factor			
			HIV	PRV	Sindbis	BEV
Step A	19	5.85	3.3	3.7	4.2	4.2
Step IV	40	5.85	4.4	5.7	5.4	3.6
Step D	10	4.60	0.9	1.7	3.1	1.2

BEV, bovine enterovirus; HIV, human immunodeficiency virus; PRV, pseudorabies virus.
Source: reference *29*.

Table 12
Log reduction factors for five different viruses and for four precipitation steps used during the manufacture of intravenous immunoglobulin

Step	Ethanol %	pH	Log reduction factor				
			HIV	PRV	Sindbis	SFV	BEV
Step A	19	5.85	4.0	3.6	3.2	3.6	3.4
Step B	12	5.10	5.3	4.7	4.6	2.2	4.1
Step C	25	7.00	4.0	4.7	2.9	3.5	3.8
Step D	—	—	2.2	3.0	1.7	—	2.8

Source: reference *30*.
BEV, bovine enterovirus; HIV, human immunodeficiency virus; PRV, pseudorabies virus; SFV, semliki forest virus.

steps unless the mechanism of action has been shown to be independent, or other data demonstrate that the summing is legitimate.)

Because the result of any precipitation step is a partitioning of components between a solid and a liquid phase it should be borne in mind that, in the absence of inactivation, fractionation results in distribution of viruses between these phases. Therefore, if viruses are indeed removed from one fraction, the bulk of virus will be found in another fraction, which may or may not be that used for making the final product. Many manufacturers separate the precipitated proteins by centrifugation whereas others have introduced filtration as an alternative. To prevent clogging of the filters, filtration is carried out using filter aids. Because these substances (diatomaceous earth or similar products) may also adsorb virus, it is often possible to remove more of the viral infectivity from the supernatant than would be expected based on precipitation alone. This may also explain some of the discrepancies found in the literature. Some authors concluded that BVDV, as a model for HCV, was not removed to any significant extent by Cohn–Oncley fractionation, (*31*) whereas others found substantial partitioning in several steps of cold-ethanol fractionation when separation was carried out in the presence of filter aids, as shown for one step in Table 13.

When virus inactivation steps are implemented, it is usually relatively easy to ensure that every drop of a large batch is treated in exactly the same way, e.g. by thorough mixing or by transfer of the whole volume from one tank to another (see above). This is much more difficult to achieve for precipitation; the first volumes that come into contact with a filter press encounter an environment that is quite different from that encountered by the last volumes of the same batch. Although it is probable that these changes occur in a reproducible way in each

Table 13
Removal of various viruses from an immunoglobulin solution by filtration in the presence of Celite

Virus	Log reduction factor
Semliki forest virus	3.4
Vesicular stomatitis virus	2.5
Bovine viral diarrhoea virus	3.1
Pseudorabies virus	3.4
Sindbis virus	4.1
Human immunodeficiency virus	5.4
Coxsackie virus	>6
Bovine parvovirus	3.4
Bovine enterovirus	4.1

Source: Omar & Morgenthaler, (*32*).

batch, this could be difficult to prove. Similarly, model experiments are relatively easy to perform in a homogeneous system, as may be the case during chemical or physical inactivation. However, large-scale centrifugation is usually done in continuous-flow machines and although the could be reduced in size to laboratory scale, parameters such as path lengths and residence times are unlikely to the same. Filtration is not any easier to model on a small scale. In either case, manufacturers need to show with carefully selected parameters (e.g. protein composition and enzyme activity) that both large-scale and small-scale processes achieve the same level of phase separation. Demonstration that the downscaled method provides a similar product to that achieved at full scale is at least as important as the demonstration of virus removal.

In spite of all the problems associated with precipitation as a means of removing viruses, ethanol precipitation has proven its value over the years. There can be little doubt that partitioning though precipitation has contributed substantially to the safety of some plasma-based products, e.g. intravenous and intramuscular immunoglobulins, which have very rarely transmitted viral diseases although until very recently the manufacturing processes for these products did not include a dedicated virus inactivation step. Nonetheless, reliance on virus removal alone is not recommended because small changes in process conditions can affect virus partitioning and safety. As an example, HCV partitioning was modified on introduction of anti-HCV screening, with the result that an IVIG became infectious (*33*).

Chromatography

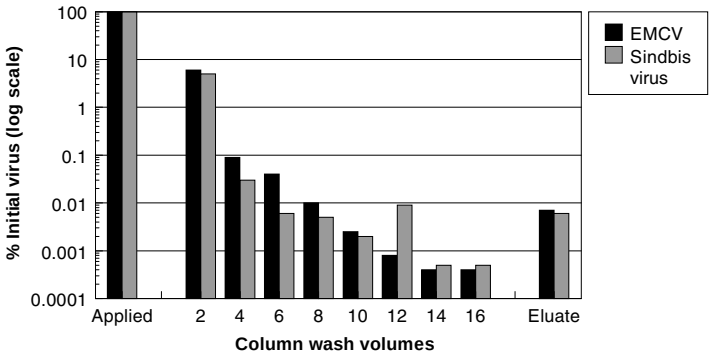
Chromatography has been designed to separate closely related molecules; some variants of chromatography, e.g. affinity chromatography, are specific for a single molecular species. The logical expectation would therefore be the chromatography is a good way to physically separate viruses from therapeutic proteins. Both enveloped and non-enveloped viruses can be removed. The log reduction factors are usually of the order of 2–3 for ion exchange chromatography and may reach 5 for very specific steps, e.g. affinity chromatography. However, because viruses can bind to protein or to the resin backbone, success in removing viruses by chromatography is influenced by a number of factors, including column geometry, the composition and flow rate of the buffers used, intermediate wash steps, the protein composition of the preparation and the ageing of the chromatographic resin. All of these factors need to be defined and controlled.

Relatively modest reduction factors were reported for three consecutive chromatographic purfication steps used in an albumin isolation scheme. LRFs of <0.3, 0.3 and 1.5 were reported for removal of HBsAg during chromatography on DEAE-Sepharose FF, CM-Sepharose FF and Sephacryl S200 HR, respectively (*34*). The same group recorded LRFs of 5.3, 1.5 and 4.2 for HAV for the same three steps (*35*). In another study, the first two chromatographic steps of the same process were investigated for their potential to remove poliovirus and canine parvovirus from albumin. When the two steps were conducted in sequence, overall LRFs of 5.3 and 1.8 were obtained for poliovirus type 1 and canine parvovirus, respectively (*36*).

A second and more commonly applied approach is the use of affinity chromatography, frequently antibody-mediated, of the protein of interest. In the preparation of monoclonal antibody-purified factor VIII, approximately 4 $\log_{10}$ of EMCV and Sindbis virus were removed. Extensive washing of the column prior to eluting factor VIII contributed to the overall removal factor (Figure 6).

Sanitization of resins and associated chromatography equipment between runs is essential because viruses tend to stick to resins and a complete wash-out is often impossible. Discarding used resin is, for financial reasons, normally not a practical option. Many resins withstand chemical or physical treatments that inactivate viruses. Typical treatments include overnight incubation with 0.1–1 N sodium hydroxide or hydrochloric acid, oxidizing conditions such as provided by sodium hypochlorite; very high temperatures, or autoclaving. The selection of a sanitization procedure depends on the column matrix in use. For example, silica backbones are degraded on exposure to

Figure 6
The reduction of model viruses during method M immunoaffinity purification of factor VIII[a]



EMCV, encephalomyocarditis virus; TNBP, tri (*n*-butyl) phosphate.
[a] For EMCV, TNBP and Triton X-100 were present; for Sindbis virus, they were omitted due to the rapid inactivation that would otherwise occur.
Source: Griffith (*37*).

alkali, and immobilized antibody used in affinity chromatography can be degraded by harsh chemical treatments (and by enzymes present in the material being purified).

Since sanitization is an essential part of the production process, it must be validated to the same extent as virus inactivation or elimination steps. The aim of the validation is to prove that there is no cross-contamination from one batch to the next. If it can be shown convincingly that at least one of the solutions used during the regeneration cycle completely inactivates all relevant viruses under the conditions used during cleaning, validation will be relatively simple and can be limited to demonstrating that the column material and all associated equipment has been exposed to the cleaning solution. However, in most cases, inactivation of certain viruses will be incomplete. In such cases, wash-out of viruses during the sanitization cycle needs to be monitored. If necessary, washing may be prolonged until no more virus is removed from the column. Finally, an attempt should be made to demonstrate that no infectious virus remains on the resin, usually by subjecting it to the next purification cycle. These validation experiments need to be done with fresh resin as well as with resin that has been used for the specified maximum number of cycles.

4.2.2 *Nanofiltration*

Nanofiltration is a technique that is specifically designed to remove viruses. Simplistically, nanofiltration removes viruses according to

their size while permitting flow-through of the desired protein. However, large proteins — particularly those that tend to form aggregates — are as large as or larger than small viruses so nanofiltration cannot be used with all products. Effective removal requires that the pore size of the filter be smaller than the effective diameter of the virus. Filters with a pore size that exceeds the virus diameter may still remove some virus if it is aggregated such as by inclusion in antibody/antigen or lipid complexes. In reality, nanofiltration is a more complex process. Apart from sieving effects, adsorption of the virus to the filter surface may also contribute to virus removal, though this will be strongly influenced by the intrinsic characteristics of the solution being filtered. Only a careful validation of the down-scaled process with several virus species will reveal the potential of the method for specific applications.

Nanofilters are usually available in many different sizes (surface areas), which makes it easy to increase to production scale and to decrease to laboratory scale for validation experiments. Careful monitoring of the performance of the nanofilters in every run is mandatory. Filter integrity should be ascertained before and after use, and every filter manufacturer offers test methods that have been developed specifically for this purpose. If a filter fails the integrity test after use, the filtration step has to be repeated. So far, nanofilters may be used only once.

Although nanofiltration is a gentle method, proteins are subjected to shear forces that may damage their integrity and functionality. Appropriate tests should be conducted during the development phase to rule out this possibility, keeping in mind that several filters may be used in series.

Membranes with 15 and 35 nm pore size were reported to remove 6–7 $\log_{10}$ of murine xenotropic retrovirus, SV40 and pseudorabies virus from IgG and IgM solutions (*38*). Troccoli and coworkers found that all viruses larger than 35 nm spiked into an IVIG-solution were completely removed by cascade filtration through one 75 nm pre-filter, followed by two 35 nm virus removal filters; the pre-filter was used to increase the capacity of the small-pore filters. Even smaller viruses like EMCV, HAV and PPV were removed to a significant extent (LRFs were 4.3, >4.7 and 2.6, respectively). The removal of some small viruses (BPV, Sindbis and SV40) could not be evaluated due to neutralization by cross-reacting antibodies (*39*). A single dead-end filtration was able to remove HIV, BVDV, PPRV, RT3 and SV40 with LRFs of >5.7 to >7.8, when these viruses were added to high-purity factor IX and factor XI concentrates (*40*). Numerous other

studies have also demonstrated the efficiency of virus removal with appropriate membranes, either with model solutions or in the presence of purified plasma proteins. Protein recovery has almost always been reported to be excellent. It should however be borne in mind that the virus stocks used in validation studies may be artifactually aggregated as a consequence of achieving high titres in culture systems or of the concentration methods used.

4.3 Protein issues

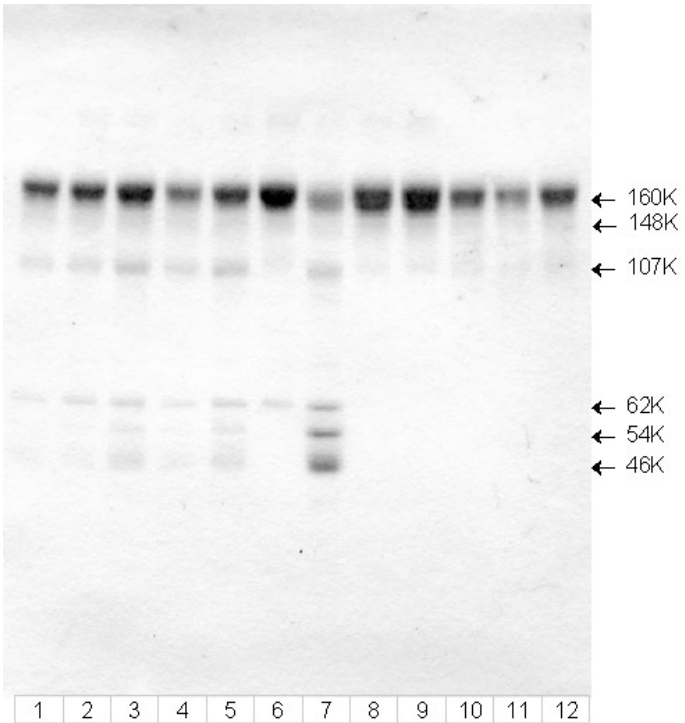
When considering processes that inactivate or remove viruses, just as with other manufacturing procedures, manufacturing consistency and the integrity of the final product with respect to protein function and structure must be demonstrated. Several analytical tests are typically applied to in-process samples and to the final product. These almost always include total protein, one or more functional assays for the protein of interest, and an assessment of its aggregation/fragmentation. Additional final product protein assays are occasionally employed depending on the product being manufactured. For example, anticomplementary activity in IVIG and activated coagulation factor activity in prothrombin complex concentrate are usually measured in every production lot, while the in vivo measurement of the thrombogenic potential of prothrombin complex concentrates is usually assessed during process development.

4.3.1 *Measurements of protein structure*

Depending on the experience with the process methods being employed, laboratory and animal studies such as those described below can be of value for characterizing products under development.

Electrophoresis is a fast and easy method for evaluating the overall integrity of a protein. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) is particularly useful for analysing protein composition, aggregation and fragmentation. The procedure separates proteins approximately according to their relative molecular mass, although protein shape and glycosylation can affect migration. Under nonreducing conditions, disulfide-linked protein chains usually remain together. For instance, under nonreducing conditions, immunoglobulins migrate as a single molecule with a relative molecular mass of approximately 160 kD while under reducing conditions, the chains that were linked by disulfide bridges fall apart, producing two bands with approximate relative molecular masses of 50 kD (heavy chain) and 25 kD (light chain). Cleavage in the primary sequence of proteins is usually easily detected (Figure 7). SDS-PAGE

Figure 7
SDS-PAGE of intramuscular and intravenous immunoglobulin products[a]



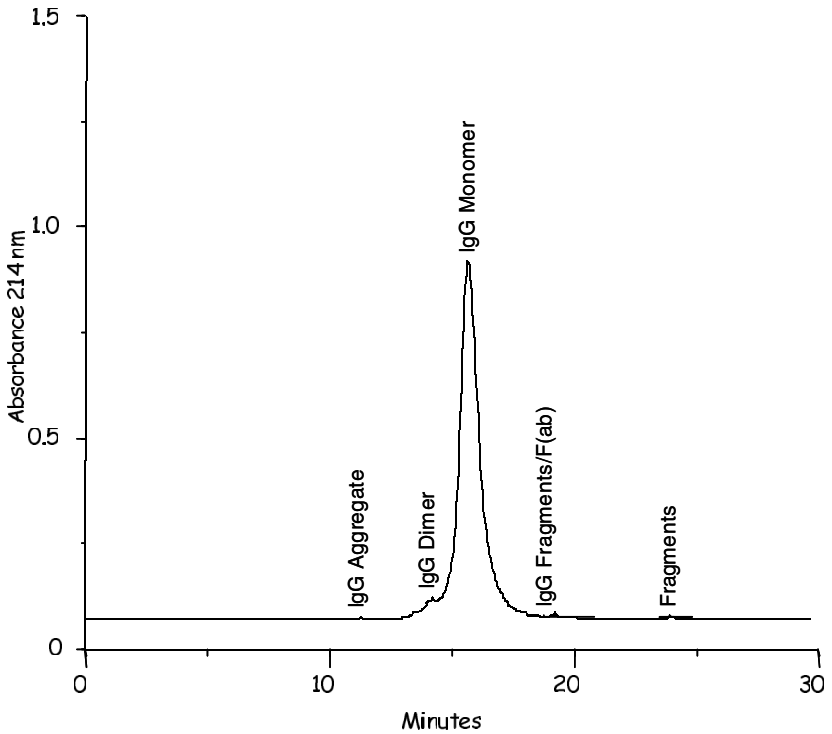
SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.
[a] The products analysed in lanes 1–7 show variable amounts of IgG fragments, whereas those in lanes 8–12 show little, if any evidence of fragmentation.
Figure reproduced with the kind permission of R. Thorpe, National Institute of Biological Standards and Control, England.

will not normally reveal changes in higher-order structures or covalent modifications of amino acids.

Capillary electrophoresis has recently been introduced as an adjunct method to PAGE. Although it is more amenable to automation, and therefore useful for high-throughput analyses, it does not yield substantially more information than PAGE and requires sophisticated equipment.

Size-exclusion gel chromatography separates proteins according to their overall size and shape. The use of size-exclusion high performance liquid chromatography (SE-HPLC) allows rapid analysis and high resolution of protein components and also better reproducibility than that obtained using conventional gel permeation chromatography. Fragmentation and/or aggregation of plasma proteins are usually

Figure 8
SE-HPLC of an intravenous immunoglobulin product showing the expected distribution of molecular species[a]



[a] The product consists primarily of IgG monomers with small amounts of dimer and trace amounts of fragments and aggregates.
Figure reproduced with the kind permission of R. Thorpe, National Institute of Biological Standards and Control, England.

easily demonstrated and quantifiable and gross modifications to the molecular shape of the protein may also be detectable. More subtle changes may not be detected, and the method is insensitive to chemical modifications of amino acids. An example of SE–HPLC analysis of IVIG is given in Figure 8.

Isoelectric focusing separates proteins according to their isoelectric point. Separation is normally performed in the presence of a supporting matrix (e.g. polyacrylamide), and the proteins may be subjected to this method either in a native or denatured state. As covalent modification of amino acids usually changes their electric charge, it also affects their isoelectric point and therefore the protein's position in an isoelectric focusing gel. Isoelectric focusing combined with PAGE is a very powerful tool for the detection of even small differences in

protein structures and properties, although the gel patterns can be very complex depending on the purity of the sample.

Antigen/activity ratio. During process qualification, it may be useful to measure protein functional activity and antigen concentration simultaneously in an immunoassay. A constant ratio of activity to antigen during the isolation process and before and after virus inactivation provides evidence that protein structure was not affected while a decline in this ratio is indicative of detrimental effects.

Neoimmunogenicity may be regarded as a special case of changes to the higher-order structure of proteins, which do not necessarily impair the protein's functionality, but result an immune response in the recipient. Products made with current methods of viral inactivation and removal do not generally stimulate an antibody response in humans. There are, however, two documented instances (one involving a pasteurized product and the other a product treated with solvent/detergent combined with pasteurization) where treated products had unexpected immunogenicity and were therefore withdrawn from the market (*41–43*).

The detection of neoimmunogenicity preclinically is very difficult, and several animal models have been developed. One approach is to immunize one group of laboratory animals (e.g. rabbits) with the preparation to be tested and another group with the same preparation in which the viral inactivation step has been omitted, or a similar preparation with proven lack of neoimmunogenicity. The resulting antisera are compared with one another in a cross-over experiment; if the antibodies raised against the new preparation are completely adsorbed by the old preparation, the preparation under test is not likely to contain neoantigens. However, these experiments have to be conducted in a heterologous system and there is no guarantee that the human immune system recognizes the same epitopes as those recognized by the immune systems of laboratory animals.

Because the models are not believed to adequately predict human response, animal neoimmunogenicity studies are not generally required for products manufactured using well recognized techniques for viral inactivation and removal. If the manufacturing conditions differ substantially from well recognized treatment conditions, such as the use of a higher temperature than that normally employed during solvent/detergent or heat treatments, new combinations of treatments, or the use of a new method of virus inactivation and removal, then animal neoimmunogenicity studies using one of the available models should precede first use of the product in humans.

The best proof of absence of neoantigens is derived from careful clinical studies involving a number of patients determined on a case-by-case basis. The determination of circulatory recovery and half-life in repeatedly infused subjects can be very useful and such measurements are typically made prior to licensure. A full assessment of immunogenicity is best monitored over the long term and is, therefore, is typically monitored in humans following licensure of the product. This has proven to be especially important in the care of patients with haemophilia, and recommendations for the conduct of such studies have been published (*44*). If there is no increase in the appearance of clinically relevant antibodies or of other adverse immunological reactions in patients (as compared with the incidence expected from earlier studies, when available), it is reasonable to assume that the newly developed product does not exhibit neoantigens.

The following are not usually applied to well-established procedures, products and processes, but may prove valuable with new viral inactivation and removal procedures.

- ***Amino acid analysis*** determines the overall quantitative composition of a protein. It may help to detect changes that were inadvertently introduced, e.g. covalent modifications of amino acids.
- ***Amino acid terminus analysis*** may identify changes in the covalent structure of proteins because cleavage of protein chains may produce new terminal sequences that can be identified and located by alignment with the native sequence (if it is known) or when comparing pretreatment to post-treatment samples.
- ***Cleavage with proteolytic enzymes*** can be used to assess protein integrity because denatured proteins or proteins with altered conformation often contain new sites that are now recognized by sequence-specific proteases. Comparing the fragmentation patterns produced by addition of proteolytic enzyme(s) before and after virus inactivation and removal may give clues to subtle changes that have occurred during treatment. The degradation patterns may be analysed by several of the methods already mentioned such as SDS-PAGE and size-exclusion gel chromatography (*45*).
- ***Circulatory survival*** may be considered as an in vivo variant of using proteolytic enzymes, albeit a difficult, time-consuming and expensive variant. It is carried out by injecting the protein intravenously into a suitable animal (e.g. rat or rabbit) and comparing the half-life with that of a reference preparation of the same protein, possibly with the protein in its native state, i.e. in plasma. The kinetics of removal of a foreign protein from circulation are quite sensitive to minor changes in protein structure. As a demonstration of the utility of this method, the circulatory half-life of human

albumin prepared by standard procedures was shown to be unaltered whereas that of chemically modified albumin was halved (*46*). Analogous experiments performed with virally inactivated (by solvent/detergent) immunoglobulins and coagulation factors demonstrated no change from historical controls (*47*).

- ***Other methods that could be indicative of overall changes in shape*** include measurements of sedimentation and diffusion coefficients, viscosity, circular dichroism and optical rotatory dispersion. Most of these methods are difficult and slow to perform. They are of limited value because their results are hard to interpret and can only be evaluated by comparison with a difficult to establish standard preparation of the same protein.

4.3.2 *Final product characterization*

The specifications for many plasma products are provided in several pharmacopoeias, national regulations and in the WHO requirements for the collection, processing and quality control of blood, blood components and plasma derivatives (*48*). Common tests that are generally considered in the characterization of plasma derivatives in final product are listed in Figure 9. Tests to be conducted on each final bulk solution or filling lot should comply with methods and specifications approved by the national regulatory authority.

4.3.3 *Stability assessment*

The purpose of stability studies is to prove that a product remains stable, safe and efficacious during the shelf-life that is claimed for it by the manufacturer. A set of relevant parameters has to be chosen and measured at regular, predefined intervals. These parameters will include measures of potency as well as indicators of protein integrity. Limits for these parameters that may not be exceeded are generally predefined.

True stability tests can only be conducted in real time. Since most plasma-protein products have a shelf-life of 2–3 years, stability tests are usually incomplete at the time of licensure. Real-time stability studies need to be done under worst-case conditions. For example, if the storage conditions for a particular product are specified to be within a temperature range of 2–8 °C, the minimum testing would be carried out within the specified temperature range and at some other higher temperature.

To obtain an indication of product stability before the data from real-time stability studies are available, it is possible to conduct accelerated stability studies (*49*, *50*). For these, the product is exposed to

Figure 9
Tests commonly applied to final product characterization

Albumin

- Protein composition (albumin content)
- Molecular size distribution (polymers, aggregates)

Normal and specific immunoglobulins

Intramuscular administration

- Protein composition (IgG content)
- Molecular size distribution (aggregates, dimers, monomers, fragments)
- Potency tests of antibody reactivity against selected antigens

Intravenous administration

- Protein composition (IgG content)
- Molecular size distribution (aggregates, dimers, monomers, fragments)
- Anti-complementary activity
- Potency tests of antibody reactivity against selected antigens

Coagulation factor concentrates

Anti-haemophilic factor

- Factor VIII coagulant activity
- von Willebrand factor activity (if required)

Prothrombin complex / factor IX

- Factor IX coagulant activity
- Factor II, VII, X coagulant activities
- Measurement of activated clotting factors

Tests common to all products

- Total protein
- Moisture and solubility (if lyophilized)
- pH

more severe conditions than are normally expected to be encountered during routine storage and shipping, e.g. higher temperatures, and stability is assessed over a shorter period than that used for the real-time study. The data can be used to predict stability under the prescribed storage conditions, but cannot replace real-time studies because predictions from accelerated studies do not always correlate with what occurs during real time. Other stress factors that are often incorporated into an accelerated stability test include humidity, light,

mechanical stress (shaking) and combinations of these. Parameters that are identified as critical during accelerated testing will receive particular attention during real-time testing.

In both accelerated and real-time studies, time points need to be chosen such that both transient and permanent deviations from the initial value will be recognized. Should the predefined limits for any parameter be exceeded, a reconsideration of the storage conditions will be unavoidable.

4.4 Clinical trials to assess safety

Historically, the role of clinical trials has been to assess efficacy, both general and viral safety, and immunogenicity. Trial design for established products is a subject of considerable discussion with an overall trend towards simplifying design and reducing the number of patients required. Viral safety is assessed principally by the review of donor demographics, test procedures and process validation. Within the EU, there is a trend towards assessment of viral safety in humans after, rather than before, receipt of marketing authorization (postmarketing surveillance). This trend takes into account the safety exhibited by current products, recent reductions in viral loads, the universal use of well validated methods of virus removal and inactivation, and the relative insensitivity of small clinical trials.

Special circumstances in individual countries and the diverse medical uses of the established products makes the setting of generally applicable guidelines a daunting task. Prior to licensure, all products typically undergo safety evaluation in a minimum of 5–10 volunteers, and in many cases, 25 or more. More patients are included in testing of products made by new processes.

4.5 Implementation in a manufacturing setting

A set of measures should be implemented to ensure that virus inactivation and removal procedures are correctly carried out in a manufacturing process and that cross-contamination following these procedures is avoided. The examples of viral reduction treatment practices given below should not be understood as requirements, but rather as general points for consideration. They are not the only acceptable way of conducting viral reduction treatments but provide examples of the solutions employed by some manufacturers when addressing this issue.

4.5.1 *Overall process design*

When planning to implement a new viral inactivation and removal treatment, the following conditions should be set ahead of time to facilitate their implementation:

— batch-size or volume at the stage of the viral reduction step, and potential up-scaling in the future;
— floor area in the manufacturing facility required for the viral reduction step itself, and for downstream processing (e.g. for removal of stabilizers or chemicals);
— the possibility of creating a "safety area" where successive production steps are arranged in a clear and logical way so as to avoid cross-contamination from a consideration of how the various flows (operators, product, equipment, wastes) will be organized during and after the viral reduction step.
— Whether cleaning or sanitation procedures will be in that place or executed in another location.

4.5.2 *Equipment specifications*

Because viral reduction treatments are critical to product safety, the specifications of the equipment employed in these steps are of particular importance. The following examples are illustrative:

In-process/bulk virus inactivation (e.g. solvent/detergent, low pH, pasteurization)

- Ideally, incubation vessels should be fully enclosed fitted with an appropriate mixing device. Usually, these are temperature-controlled vessels in which the source of heat is a jacket or a heating coil. They often have hygienic, polished internal finishes, flush-fitting valves, hygienic entry ports for the addition of reagents and sampling (e.g. to control pH and osmolality), and probe ports for relevant in-process monitoring (such as measurement of temperature).
- There should be no "dead points" i.e. areas where the temperature defined in the specification or where homogeneity of the mixture cannot be ensured.
- For heat inactivation processes, temperature monitoring equipment should provide a continuous, accurate and permanent record of temperature during the treatment cycle.

Terminal virus inactivation (e.g. pasteurization or dry heat in the final container)

- The heating device (such as a water-bath, steam autoclave, or forced-air oven) should provide even temperature distribution

across the range of batch sizes encountered. This should be demonstrated as part of the equipment qualification.

- The temperature-monitoring equipment should be capable of providing a continuous, accurate and permanent record of the heat-treatment cycle.

4.5.3 *Pre-qualification and validation*

Once the equipment for the virus inactivation or removal step has been received, the following steps are usually followed prior to routine use.

- The installation qualification verifies that the viral inactivation and removal equipment conforms to the predefined technical specifications and relevant good manufacturing practices regulations applicable at the time of installation in the manufacturing environment. This includes confirmation that the required services (e.g. voltage, cooling/heating fluid and steam) are available and appropriate.
- The operational qualification demonstrates experimentally, typically without product, that the equipment for the inactivation and removal of viruses functions within the specified limits and under the requirements for good manufacturing practices in the manufacturing environment.
- The performance qualification establishes that the equipment for the inactivation and removal of viruses operates to the predetermined performance requirements in the presence of product under routine manufacturing conditions.
- Product validation provides evidence that intermediate and/or final product prepared with the newly installed equipment reproducibly meets its specifications.

4.5.4 *Process design and layout*

The benefit of viral inactivation and removal will be negated if the plasma fractions from preceding steps are permitted to recontaminate the intermediates or products that follow; thus, the manufacturer must describe how the operating procedures reduce the likelihood of cross-contamination. Usually, decisions are made after a multidisciplinary team consisting of responsible staff from manufacturing, engineering, quality assurance and microbiology has made its recommendations.

The simplest and best solution to the problem of cross-contamination, from a facility management perspective, is to transfer product from one room to the next in the course of the specific inactivation and

removal procedure. This serves to create different safety zones, which, when arranged in a clear and logical way, help avoid cross-contamination. In the best implementations, every zone has its own dedicated staff, equipment, entrance, air-handling and other services. When this arrangement is not practical, the same effect can be achieved through appropriate management practices. For example, some facilities utilize the same staff in both downstream and upstream areas, and personnel moving into a safer zone must change their outer overalls, shoes or shoe covers, gloves, etc before entering. Equipment must also be decontaminated when moving it into a safer zone. Preferably, the equipment in one safety zone should not be shared with a second zone. Strict segregation has generally been adopted for continuous flow centrifuges, column chromatography matrices and ultrafiltration membranes which are notoriously difficult to decontaminate with the methods that are currently available.

The following points illustrate how some manufacturers have addressed these cross-contamination issues.

In-process/bulk virus inactivation (e.g. solvent/detergent, low pH, pasteurization)

- Inactivation procedures are usually carried out in two stages. For example, the first stage may be a treatment at acid pH 4 which takes place in a normal production room, followed by a second incubation in another tank located in a segregated, contained area.
- For solvent/detergent treatment, most of the inactivation is usually during the first 30–60 minutes of the 4–6 hour total treatment time.
- If bacterial growth during virus inactivation is a consideration, the solution is sterile-filtered (pore size 0.45 μm or less) before treatment.
- Samples are usually taken to confirm that the process conditions for inactivation meet the specified limits (e.g. for pH, stabilizer concentrations and concentration of virus inactivating agent).
- On completion of the first stage of inactivation, the product is aseptically transferred (sterile coupling) into the second vessel, which is located in a safety zone, for completion of the second stage of viral inactivation.
- Ideally, the "safety area" has an independent air-handling system, designated controlled clothing for personnel, and defined routes of entry for all equipment, reagents (including process buffers) and consumables.
- The process water and the reagents supplied to the safety area are of water for injection (WFI) standard or demonstrated to be free of infectious agents.

- All processing after virus inactivation and prior to sterile filtration and dispensing (e.g. removal of solvent/detergent or stabilizers and further purification steps) are carried out in the safety area.
- All the equipment used in the safety area that is in contact with the product is dedicated, or decontaminated in a manner that can be shown to inactivate any remaining virus.
- In some cases, a dedicated aseptic filling area is used for virus-inactivated products while a separate dispensing area is used for products that have not been virally inactivated during the purification process and are treated at the end of the process. Alternatively, products that will be inactivated in the final container can also undergo a preliminary virus inactivation in bulk, or the filling line is cleaned by procedures that can be shown to inactivate virus.

In-process virus removal (e.g. nanofiltration, specified purification steps)

The principles relating to product segregation described above also apply to procedures for virus removal.

Terminal virus inactivation (e.g. pasteurization or dry heat in the final container)

Inherently, terminal virus inactivation procedures greatly reduce the likelihood of recontamination.

- Temperature is monitored at several locations throughout the load including the previously determined locations at which the highest and lowest temperatures occur.
- The temperature control probe is independent from the probes used to monitor product temperature during the heat treatment.
- A maximum time is specified for the temperature to reach its set point.
- The specified temperature is maintained by all probes for the required period.

4.5.5 *Process control*

Quality assurance is a critical part of the manufacturing process because completeness of virus inactivation and removal cannot be guaranteed by testing the final product. It is the responsibility of quality assurance to ensure that the execution of virus inactivation and removal methods in a production setting conforms to the conditions that were validated in the virus spiking studies. Additionally, it is their responsibility to ensure that the procedures that are designed to avoid cross-contamination are strictly followed. In the case of any departure

from the standard, specified manufacturing processes or in environmental conditions, the independent quality assurance team, typically with the assistance of a select committee, will conduct a deviation investigation to determine whether or not the product can be released. Generally, the quality assurance team has final authority to release or reject product.

The following points should be taken into consideration.

- As with all other procedures, viral inactivation and removal procedures should be described in approved standard operating procedures.
- The standard operating procedures should contain critical process limits for the viral inactivation and removal methods.

Because of the critical importance of the viral inactivation and removal step, quality assurance personnel may review and approve the recorded conditions for viral inactivation and removal while the batch is being processed; i.e. not just as part of the final overall review of the batch file.

5. Virally inactivated plasma for transfusion

In the past, plasma has been used to treat a variety of haemostatic disturbances and immune deficiencies and even to provide a source of nutrients. This has led to a significant increase in the often inappropriate use of fresh frozen plasma (FFP). For many of those applications, alternative, safer and more economical treatments are now a better choice than FFP. According to the recommendations of consensus development conferences in various countries, there are a limited number of indications for the use of FFP (*51–53*). These include patients who require massive transfusions, patients with multiple coagulation factor deficits who are bleeding or who need an invasive procedure, patients with thrombotic thrombocytopenic purpura and patients with protein-losing enteropathy. In addition, FFP is indicated where there are no concentrates or purified preparations available such as for congenital coagulation factor deficiencies and immune deficiencies.

Regulatory approvals have been granted to three approaches designed to enhance the viral safety of transfusion plasma, namely:

— quarantine or donor-retested plasma;
— solvent/detergent-treated plasma; and
— methylene blue-treated plasma.

Each of the three approaches is described below, and all have been recently reviewed (*54*). All transfusion plasma options, including the continued use of FFP from well-screened donors, have advantages and disadvantages, and it is up to the local medical community and relevant regulatory bodies to determine which option is preferable and most suitable for the particular setting. Implementation should adhere to the applicable measures described in section 4.5.

5.1 Quarantine or donor-retested plasma

One approach to reducing window-period transmissions is to hold donor units in quarantine for a suitable period of time until the donor returns and can be retested. This method is useful only for the viruses being tested for, although interviewing the donor at the time of the second test may help to identify any transient illnesses that occurred between the two donations. The length of the quarantine period is related to estimates of the window period, which differs for each virus. To reduce transmission of HIV, HBV and HCV, a sufficient hold period should be chosen to give a 95% confidence level of not releasing a product during a window period. Periods of 3–4 months have typically been considered to prevent almost all window-period transmissions. The option to quarantine is made possible by the relatively long outdating (shelf-life) period of FFP, typically 1 year.

Although the transmission of HBV, HCV and HIV will be greatly reduced by use of quarantine plasma, it will not have been eliminated. For example, HCV has been reported to have been transmitted by quarantine plasma (*55*), blood donations that are not screened by genomic techniques continue to harbour HIV and other viruses of concern (*56*), and quarantine has little or no impact on viruses that are not tested for. However, the advantages of this method are that the plasma itself is unchanged and thus has the same properties and indications as FFP, and no sophisticated equipment, other than that used for donor tracking, is required. On the other hand, supply logistics may prove difficult in some circumstances where a large number of donors need to re-donate well before the expiry date of the initial FFP unit. This is of special concern in an environment based on blood donations volunteers, where many donors give blood infrequently, with consequent losses of plasma units.

Implementation requires systems that correctly match donated units with the returning donors and that prevent premature release of units labelled as being either “quarantined” or “donor retested”. Although manual systems may be used, computerization greatly facilitates this process and provides improved security.

Table 14
Inactivation of viruses on treatment of plasma with 1% tri(*n*-butyl) phosphate and 1% Triton X-100 at 30 °C for 4 hours

Virus	Inactivation (log_{10})	Inactivation time (hours required)
Vesicular stomatitis virus	≥7.5	0.25
Sindbis virus	≥6.9	0.25
Duck hepatitis B virus	≥7.3	2.5
Bovine viral diarrhoea virus	≥6.1	0.25
Human immunodeficiency virus	≥7.2	0.25
Hepatitis B virus	≥6.0	4[a]
Hepatitis C virus	≥5.0	4[a]

[a] Only one time point tested.

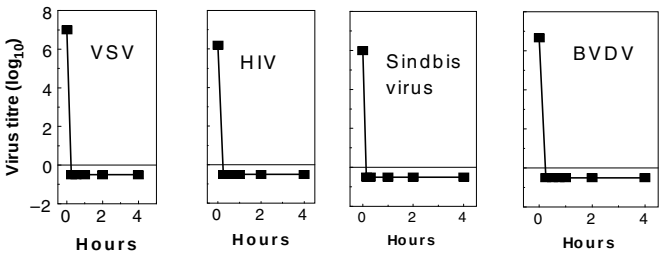
5.2 Solvent/detergent-treated plasma

Routinely collected source, recovered, or FFP is pooled and treated with 1% TNBP and 1% Triton X-100 at 30 °C for a minimum of 4 hours to inactivate enveloped viruses. The reagents are removed by hydrophobic chromatography to near undetectable levels (*57*). The compounds used are non-mutagenic and have an overall benign toxicology profile. Leukocytes, bacteria and parasites are removed by sterile filtration. The final product is available frozen and, in some countries, also in a lyophilized form. Inactivation of HIV, HBV and HCV and of many other enveloped viruses has been demonstrated (Table 14, Figure 10). To reduce the risk from non-enveloped viruses, the application of NAT can eliminate positive pools. Little change is observed in the level of most procoagulant factors, and bag-to-bag consistency is ensured through the pooling process.

Clinical trials conducted in both Europe and the USA have shown that solvent/detergent (SD)-treated-plasma can replace FFP in all of its indications, including the replacement of coagulation factors and the treatment of thrombotic thrombocytopenic purpura (*58–61*). More recently, several deaths were reported in liver transplant patients who received a product provided by one manufacturer in the USA.[1] Although the link with this product or with reduced levels of some anticoagulant proteins in SD-plasma is uncertain, the US manufacturer's product label has been amended to indicate that this product should not be used in patients undergoing liver transplant or in patients with severe liver disease and known coagulopathies.[1]

[1] Information available from the FDA/CBER web site (http://www.fda.gov/medwatch/safety2002) became available after the fifty-second meeting of the WHO Expert Committee on Biological Standardization. This information was added during editing of the Guidelines.

Figure 10
Rate of virus inactivation on solvent/detergent treatment of plasma



BVDV, bovine viral diarrhoeavirus; HIV, human immunodeficiency virus; VSV, vesicular stomatitis virus. Data generously provided by V.I. Technologies, Inc.

Additionally, the coagulation status of patients receiving large volumes of SD-plasma should be monitored for evidence of thrombosis, excessive bleeding or exacerbation of disseminated vascular coagulation.

The same parameters need to be defined and controlled as for other solvent/detergent-treated products. In addition, some regulatory bodies have instituted a maximum for the number of donors that can contribute to an individual lot; the maximum number permitted ranges from 100 to 2500.

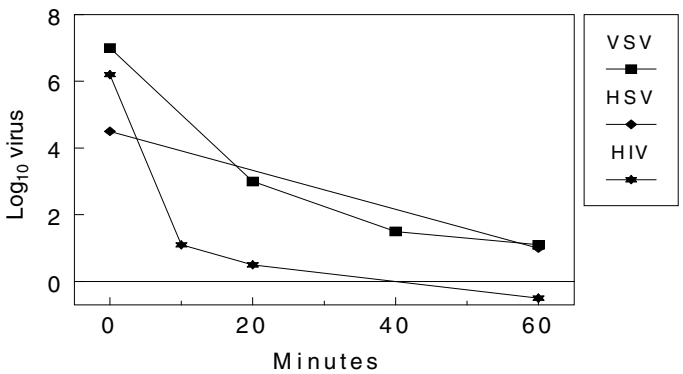
5.3 Methylene blue and visible light

Methylene blue is a photosensitizer, and in conjunction with light and in the presence of oxygen it can inactivate biological systems. The virucidal action of methylene blue is well known (*62*) but the mechanism of action is not entirely clear. Nucleic acid damage usually results from photosensitization with methylene blue. This was ruled out as the cause of virus kill in one case (*63*), but not in others (*64*). In the current procedure, individual plasma units are treated with 1 µM methylene blue and white fluorescent light for 1 h at 45 000 lux (*65*) or with low-pressure sodium lamps at 200 Joules/cm^2 for 20 minutes. The individual units are re-frozen and stored for later use. Added methylene blue is not usually removed although special filters for its removal are being developed (*66*). Model enveloped viruses and cell-free HIV are inactivated effectively, but non-enveloped viruses, (Table 15 and Figure 11) (*67–68*) cell-associated HIV and other cell-associated viruses are less affected. The latter must be removed completely by filtration or other means. A recent study has suggested that parvovirus may be inactivated (*69*). The in vitro coagulation capacity of plasma treated with methylene blue is well maintained, but the

Table 15
Inactivation of viruses on treatment of plasma with 1μM methylene blue and white light for 1 hour

Virus	Inactivation (log_{10})	Inactivation time (minutes required)
Vesicular stomatitis virus	5.0	60
Simian immunodeficiency virus	≥6.3	—
Semliki forest virus	≥7.0	10
Herpes simplex virus	≥5.5	60
West Nile virus	≥6.5	—
Sindbis virus	≥9.7	—
Bovine viral diarrhoea virus	≥5.9	2
Human immunodeficiency virus (extracellular)	≥6.3	10–30
Human immunodeficiency virus (cellular)	0	—
Duck Hepatitis B virus	3.9	60
Hepatitis A virus	0	60
Porcine parvovirus	0	60

Figure 11
Rate of virus inactivation on methylene blue treatment of plasma



HIV, human immunodeficiency virus; HSV, herpes simplex virus; VSV, vesicular stomatitis virus.
Results generously provided by H Mohr, DRK Blutspenddienst, Springe.

activities of fibrinogen and factor VIII are reduced (*70*). Photodynamic methylene blue treatment of plasma resulted in no adverse reactions in a controlled clinical study (*71*) and there is no evidence of neoantigen formation (*72*). The advantage of this approach compared with solvent/detergent-treatment (see above) is the absence of pooling, i.e. recipients would receive plasma from individual donations,

rather than from a plasma pool made from hundreds or thousands of donations. Because it is well known that methylene blue and its reaction products are mutagenic (genotoxic) in bacteria, some regulatory authorities in Europe have requested additional data on the mutagenic potential of these substances in mammals and/or on the validated use of filters to efficiently remove them from treated plasma units.

On the basis of the above considerations, the following factors are likely to affect outcome and therefore need to be defined and controlled:

— the volume of plasma being treated;
— the geometry of the sample;
— the light intensity and duration of exposure;
— the effect of residual cells;
— the transparency of the bag;
— mixing efficiency; and
— residual levels of reagent and its photoproducts.

Some of this information may be available from the manufacturer of the specialty equipment employed during this procedure.

6. Review of newer viral inactivation methods under development

Several new viral inactivation procedures are being investigated, with the principal objectives of providing broader viral coverage, complementing existing methods, reducing cost and/or improved applicability to FFP. Several of these newer approaches are reviewed here, but it should be noted that in many cases, there is little or no clinical experience with these methods.

6.1 Psoralen-treated fresh frozen plasma

The use of the psoralen, S-59, together with ultraviolet (UVA) irradiation is being investigated with both FFP and platelet concentrates. Published data on viral kill are provided in Table 16. The amount of virus killed by S-59 treatment of platelet concentrates is somewhat greater than that in plasma because of its lower protein content. In phase 1 studies involving six healthy volunteers, infusion of up to 1 l of plasma resulted in no adverse events and no significant clinical changes in blood chemistries or haematological measurements (*73*). Three phase 3 trials are under way. In an open-label trial in patients (to date, $n = 34$) with congenital deficiencies in blood clotting factors,

Table 16
Inactivation of viruses on treatment of plasma with 150 μM psoralen S-59 and 3 J/cm2 UVA

Virus	Inactivation (log_{10})
Duck hepatitis B virus	5.4
Hepatitis B virus	≥4.5
Hepatitis C virus	≥4.5
Bovine viral diarrhoea virus	≥6.7
Human immunodeficiency virus	≥5.9
Human immunodeficiency virus (cellular)	6.4

infusion of S-59-treated plasma resulted in a similar increase in coagulation factor levels to those reported with untreated plasma (*74*) in historical data.

Based on the above considerations, the following factors are likely to affect outcome and therefore need to be defined and controlled:

— the volume of plasma being treated;
— the geometry of the sample;
— the light intensity and duration of exposure;
— the effect of residual cells;
— the transparency of the bag;
— mixing efficiency; and
— residual levels of the reagent and its photoproducts.

Some of this information may be available from the manufacturer of the specialty equipment employed during this procedure.

6.2 Irradiation with ultraviolet light (UVC)

Ultraviolet irradiation, typically at a wavelength of 254 nm (UVC) targets nucleic acid, thus a wide variety of viruses are inactivated irrespective of the nature of their envelope. Viruses containing single-stranded nucleic acids are more sensitive, because they are unable to repair damage in the absence of a complementary strand, and sensitivity increases with genome size (*75*), because a larger target is hit more often. Attempts to use UVC in the 1950s failed to prevent hepatitis transmission by whole plasma, but this probably reflects the relatively high titre of HBV present in donor plasma at that time and the fact that HBV is a double-stranded DNA virus. Based on these principles, HAV and parvovirus should be relatively sensitive to UVC. Following the early efforts, considerable thought was given to the factors that affect UVC efficacy, particularly to the various ways

in which a uniform thin film can be formed in continuous flow. For most protein solutions thin films are necessary to ensure complete penetration of the UVC light because the protein solutions at least partially absorb UVC energy. The difficulty in assuring maintenance of an appropriate thin film may be the reason that that a prothrombin complex concentrate treated with UVC was reported to transmit HIV (*76*). UVC has also been shown to damage protein. For example, albumin prepared from whole plasma irradiated with UVC was reported to be appreciably less stable during storage than albumin prepared from unirradiated plasma (*77*, *78*).

The most practical applications use a light source that emits at 254 nm. With such a source, Hart et al. (*79*) have shown that both albumin and IVIG solutions could be treated with 5000 Joules/m^2 UVC before an unacceptable level of IgG aggregates was observed. Non-enveloped and heat and/or acid-resistant viruses (e.g. polio 2, T4 phage and vaccinia) were effectively inactivated. The results of validation studies performed with albumin appear encouraging (*80*). Horowitz et al. have shown that the addition of quenchers of reactive oxygen species enhances the specificity of virus inactivation by UVC in protein solutions. By adding the plant flavonoid rutin to the protein solution prior to treatment with UVC, these investigators found that the inactivation of several viruses was largely unaffected (Figure 12), but that several coagulation factors were protected against UVC-induced damage (*81*). The beneficial effect of including rutin during UVC treatment was also observed with fibrinogen incorporated into a fibrin sealant, albumin and IVIG (*82*).

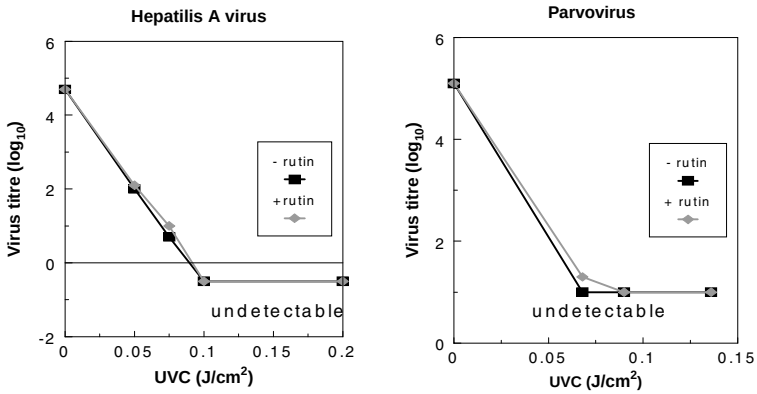
The above mechanistic considerations and experimental findings indicate that the following factors are likely to affect outcome and therefore need to be defined and controlled:

— UVC dose;
— uniformity of dose over time;
— flow rate; and
— optical density of the material being treated.

6.3 Gamma-irradiation

The use of gamma irradiation has been studied extensively for a range of applications from sterilizing hospital supplies to reducing bacterial and viral contamination of meats, other foods and sewage sludge. In most installations, ^{60}cobalt serves as the source. Gamma irradiation can act by two different mechanisms. The first is the direct rupture of covalent bonds in target molecules including both proteins and nucleic acids. The second is an indirect, mechanism, such as with

Figure 12
Inactivation of non-enveloped viruses added to a concentrate of coagulation factor VIII with UVC

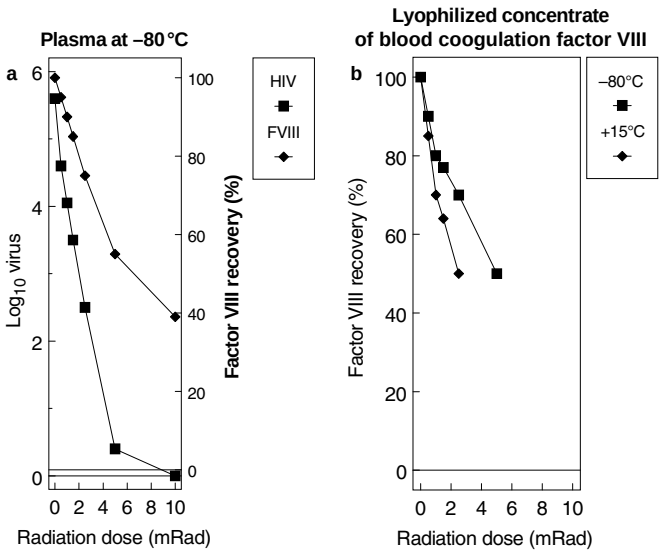


0.5 mM rutin was either present or absent
Source: Chin et al. (*81*).

water, producing reactive free radicals and other active, radiolytic products, which in turn can react with a variety of macromolecules including both proteins and nucleic acids. Indirect reactions can be reduced by adding radical scavengers, removing water by lyophilization, and/or working at cold temperatures. More recently, for the same total dose of radiation, reducing the dose rate has been reported to improve the balance between protein recovery and virus inactivation. The kinetics of viral kill are typically linear in a semi-logarithmic plot of virus titre versus radiation dose, suggesting that inactivation occurs with a single hit of radiation that is absored of directly by the nucleic acid is the likely basis of the inactivation.

The principal challenge in using gamma irradiation is the inactivation of the desired quantity of virus while maintaining the structural and functional integrity of protein. For example, Hiemstra et al. showed that on treating plasma, the inactivation of 5–6 logs of HIV required 5–10 mRad, whereas recovery of at least 85% of factor VIII demanded that the dose not exceed 1.5 mRad (Figure 13). Coagulation activity present in a lyophilized blood coagulation factor VIII, concentrate was even more sensitive whether the treatment was at –80 °C or +15 °C. Moreover, following irradiation of either lyophilized antihaemophilic factor or lyophilized prothrombin concentrates, high-pressure size-exclusion chromatography revealed protein changes at doses as low as 0.5–1 mRad.

Figure 13
Gamma irradiation of plasma and of blood coagulation factor VIII



FVIII, factor VIII; HIV, human immunodeficiency virus.
a Effect on HIV (squares) and factor VIII activity (diamonds) on treatment of frozen plasma. **b** Effect on factor VIII activity in a lyophilized concentrate of blood coagulation factor VIII.
Source: Hiemstra et al. (*83*).

These results contrast with those of Kitchen et al. (*84*), who reported a recovery of 85% for factor VIII and of 77% for factor IX on treatment of frozen plasma with 4 mRad gamma irradiation. This dose of radiation resulted in the inactivation of 4.3 logs of HIV and more than 4 logs of several other viruses including polio and measles. It has not yet been possible to explain the different findings in these two studies.

More recently, Miekka et al. (*85*) reported that treatment of lyophilized preparations of blood coagulation factor VIII with 2–3 mRad of gamma irradiation resulted in the inactivation of 4 logs of porcine parvovirus while retaining 93% of fibrinogen solubility, 67% of factor VIII activity and over 80% of α-1-proteinase inhibitor activity. The dose rate may have been an important variable in these studies. Since then, Drohan et al. have reported that treatment of a monoclonal antibody preparation in the presence of an antioxidant protein protection cocktail resulted in the inactivation of ≥4.8 $\log_{10}$ of PPV. The retention of antigen-binding activity was improved 3- to 4-fold by the presence of the protectant cocktail.

On the basis of the above mechanistic considerations and experimental findings, the following factors are likely to affect outcome and therefore need to be defined and controlled:

— total dose;
— dose rate and dose uniformity;
— composition;
— oxygen content;
— temperature; and
— (for lyophilized products) residual moisture.

6.4 Iodine

Iodine is a strong oxidizing agent and, as a result, is a powerful microbicidal agent. However, in its free form iodine is not sufficiently selective. When bound to polymers, such as polyvinylpyrrolidone (*86*), cross-linked starch (*87*), or dextran chromatographic medium such as Sephadex, the virucidal action of iodine is more controlled. The iodine in these bound forms is slowly released into the protein solution, and virus inactivation occurs over the course of hours. For example, starch-bound iodine at a concentration of 1.05 mg/ml resulted in more than 7 $\log_{10}$ inactivation of model lipid enveloped and non-enveloped viruses while more than 70% of the activity of the clotting factors in plasma was retained. In another implementation, protein was passed through a bed of iodine–Sephadex followed immediately by a bed of Sephadex used to trap and remove free iodine.

Based on the above mechanistic considerations and experimental findings, the following factors are likely to affect outcome and therefore need to be defined and controlled:

— iodine concentration;
— age of iodine–Sepharose;
— temperature;
— contact and incubation times; and
— composition of protein solution being treated.

In addition, careful studies evaluating the covalent incorporation of iodine into macromolecules are required.

6.5 Pasteurized fresh frozen plasma

A system for pasteurizing pooled plasma in bulk at 60 °C for 10 hours with 80–90% retention of coagulation factor activity has been described (*88*). Added stabilizers are removed by diafiltration. Data on viral kill are provided in Table 17. No changes in blood pressure or

Table 17
Inactivation of viruses on pasteurization of plasma at 60 °C for 10 hours[a]

Virus	Inactivation (log_{10})
Human immunodeficiency virus-1	≥5.0
Vaccinia	≥4.3
Pseudorabies	≥4.1
Parainfluenza type 3	≥6.3
Sindbis	≥5.7
Polio Sabin 1	≥6.2
Reovirus	3.2

[a] Stabilized with 1300 g/l sorbitol, 514 g/l sucrose, 4 mM calcium gluconate, 15 mM trisodium citrate, 5 g/l L-lysine and 5 g/L L-arginine.
Source (*83*)

heart rate were observed when the treated plasma was infused in rat at 4 ml/kg body weight, and there was no sign of toxicity on infusion of a single dose of 25 mL/kg body weight of treated plasma into mice. Clinical studies have not been initiated. One alternative that does not require a manufacturing plant, described in a preliminary report, is to heat plasma from a single donor at 50 °C for 3 hours in the presence of lower concentrations of stabilizers, thus avoiding the need for diafiltration (*89*). Although this approach results in lower levels of virus inactivation with some viruses, complete inactivation of HIV (≥6.6 logs) was achieved.

The same factors need to be defined and controlled as for other pasteurized products. In addition, if single units of plasma are treated, the effect of varying the ratio of plasma volume to stabilizer mixture needs to be evaluated.

7. Summary

A number procedures for the inactivation and removal of viruses are now in common use and are well recognized as contributing substantially to the virus safety of plasma products and plasma for transfusion. Adoption of these or equivalent methods is encouraged. For the virus inactivation and removal procedures commonly employed, the information above should help define criteria for acceptance often based on a decade or more of experience. For new products or products from new manufacturers, the rate of virus kill and the extent of virus kill or removal should match those shown for products with good safety records. Assuming this requirement is met for selected viruses, the details of how a process is installed in the production

facility, including staff training, equipment selection, steps taken to monitor the process and process controls, and measures taken to prevent recontamination, probably deserve more emphasis than increasing the number of different viruses studied or the number of slight variations explored.

Which method is most appropriate depends on a variety of factors such as the type of virus, the nature of the product and the characteristics of the production process. The method selected needs to guarantee both viral and general safety without affecting clinical effectiveness, and full safety may require the use of more than one method. The use of more than one robust virus inactivation and removal procedure may be especially important if the viral load present in plasma is substantially higher than that encountered in the countries where the strategies for ensuring viral safety have evolved.

National regulatory authorities frequently need to address the question of how much viral and protein data should be required prior to initiation of clinical trials or routine clinical use. No definitive answer to this question is yet available. Decisions of this nature need to take local circumstances into consideration. For example, to initiate clinical trials, the US Food and Drug Administration usually limits its virus requirements to studies demonstrating the adequate inactivation and removal of HIV, a model for HCV such as BVDV, and a single non-enveloped virus such as parvovirus or HAV.

This guidance document is intended to define the scientific principles that should be taken into consideration as a common basis in the evaluation of the safety of a plasma-derived product, both by the regulatory authorities and the manufacturer. The following principles should be applied.

- Viral inactivation and removal are part of an integrated process designed to guarantee product safety; they cannot replace other safety measures such as donor selection, donation screening or overall compliance with current good manufacturing practices.
- The preparation of all purified plasma products should incorporate two independent and complementary methods able to eliminate enveloped viruses, at least one of which is a viral inactivation step.
- The inactivation and removal of nonenveloped viruses with current methods is frequently incomplete. Manufacturers are therefore encouraged to develop procedures to deal with such viruses.
- Studies that assess viral clearance are required for all products. An exception can be made for albumin produced by the established methods using ethanol fractionation followed by pasteurization. This means that even if the manufacturing process, including virus

inactivation and removal, has been validated by other manufacturers and has a history of use, additional viral validation by the new manufacturer is still required.

- When validating virus inactivation and removal, viruses should not be brought into the production facility.
- When applying established methods of viral inactivation to a particular product, the kinetics and extent of viral inactivation should be assessed with reference to existing data derived from products with a history of safety in which viral inactivation has been carried out by the same or similar procedures.
- When applying established methods of virus removal to a particular product, the extent of removal should be assessed with reference to existing data derived from products with a history of safety that have been manufactured by the same or similar procedures. Studies should include an attempt to show mass balance, i.e. to account for the entire quantity of virus added.
- A robust, effective, reliable process step will be able to remove or inactivate substantial amounts of virus, typically 4 logs or more, be easy to model convincingly and be relatively insensitive to changes in process conditions.
- Final product testing for viral markers, as part of the routine batch release, is not recommended as the outcome is generally of very limited value in determining viral safety. The results of such tests (both serological and NAT) can often be misleading and difficult to interpret.
- The manufacturer should demonstrate, using appropriate methodologies, that the viral inactivation step(s) has (have) not adversely affected the required characteristics of the product.
- Manufacturing aspects such as facility layout, equipment, product flow, staff training and standard operating procedures need to comply with current good manufacturing practices, including measures to prevent the recontamination of product or intermediates.
- Regulations can be established only by the national regulatory authority. Products imported into a country should comply with both the requirements in the country of origin and in the country where the product will be used. Batches of plasma derivatives recalled or withdrawn in one country should under no circumstances be exported to another country.

8. Authors

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Appendix

Example of a study on the inactivation of human immunodeficiency virus-1 by treating a therapeutic plasma protein preparation with tri(n-butyl)phosphate and Tween 80

A solvent/detergent procedure was evaluated for its ability to inactivate human immunodeficiency virus type 1 (HIV-1) added to a therapeutic plasma protein preparation. The study evaluated the rate and extent of HIV-1 inactivation under "worst-case" conditions in that the concentrations of tri(n-butyl)phosphate (TNBP) and Tween 80 were 85% of that specified and the temperature was at the minimum specified under routine manufacturing conditions. Samples were titrated by 50% tissue culture infectious dose ($TCID_{50}$) end-point assay using C8166 cells.

The calculated log reduction factor for the solvent/detergent procedure was:

$$\geq 6.00 \pm 0.31 \log_{10} TCID_{50}.$$

Validation study report

Objective

The objective of this viral validation study is to provide information concerning the inactivation of HIV-1 on treatment of a therapeutic plasma protein (hereinafter "Test Article") with a solvent/detergent procedure.

Testing facility

Responsibilities for preparing the spiking virus, performing the scale-down process, performing the virus titration, writing the final report and maintaining an archive with the raw data were defined. The validation studies were reviewed by the quality assurance unit.

The following records were stored in the archives: virus spiking records, sample records, cell culture records, culture treatment records, virus titration records, dilution records, inoculation records and records of examination of cells.

Selection criteria for viruses

Validation of virus removal and inactivation should include relevant viruses that are known to, or likely to, contaminate the source mate-

Table 1
Virus used in this viral clearance study

Virus	Genome	Envelope	Family	Size (nm)	Resistance to physicochemical reagents
HIV-1	RNA	Yes	Retro	80–110	Low

rial. The virus proposed for this study is HIV-1, a potential contaminant of human blood products. The characteristics of HIV-1 are given in Table 1.

Equipment and supplies

All equipment and supplies required for this study, including pipettes, pH meters, water-bath, biohazard hoods and incubators were provided. All had been calibrated and certified within the past 6 months.

Test article

Responsibilities for the preparation, stability, purity and concentration of the Test Article were defined. The Test article was sampled from the point in manufacture just prior to virus inactivation, frozen at –70 °C or below, and shipped to the testing facility on dry ice. Once received, the test Article was stored at –70 °C or below.

Virus preparation

Stock virus was prepared at the testing facility. Its titre was determined with three independent assays of its $TCID_{50}$ using 5-fold dilutions and eight replicates per dilution. The certified titre was the average of these three determinations.

Cytotoxicity and viral interference

A previous study had been conducted to determine whether the test article, in the presence or absence of the solvent/detergent reagents, was cytotoxic to the indicator cells used in assessing infectivity of the virus, or interfered with its detection. The results indicated that cytotoxicity could be overcome by diluting the Test Article 81-fold (3^4) with RPMI-1640 + 10% FBS (culture medium) and that, at this dilution, the Test Article did not interfere with the detection of 100 $TCID_{50}$ of HIV-1.

Protocol

1. On the day of testing, the Test Article was thawed in a water bath at 37 °C for approximately 1 hour and clarified by centrifugation at

5000×*g* for 10 minutes and the precipitate discarded. The pH following centrifugation was 7.2, and the A_{280} was 25.6.

2. (a) HIV-1 stock (1 ml) was added to Test Article (19 ml) at a dilution of 1:20 and mixed thoroughly. This was divided into two aliquots, one of 15 ml (to receive solvent/detergent) and one of 5 ml (to receive water). Both were brought to 21 ± 1 °C in a shaking water-bath.
   (b) An additional aliquot (50 µl) of HIV-1 stock was diluted 1000-fold into culture medium containing 10% FBS to serve as the positive control. This was placed on ice during the remainder of the experiment.
3. (a) To the 15 ml aliquot was added 667 µl of 20% Tween 80 followed immediately (after mixing) by 40 µl of TNBP.
   (b) To the 5 ml aliquot was added 222 µl water for injection.
   (c) Both were returned to the shaking water-bath set at 21 ± 1 °C[(1)].
4. (a) From the vessel to which solvent/detergent had been added (the +SD vessel), 0.5 ml was removed after 0, 15, 30, 60, 120 and 240 minutes and diluted immediately 81-fold with culture medium containing 10% FBS. Following dilution, the samples were placed on ice.
   (b) From the –SD vessel, 0.5 ml was removed after 0 and 240 minutes and diluted immediately 81-fold with culture medium containing 10% FBS. Following dilution, the samples were placed on ice.

Assay of infectivity

1. Samples were assayed for HIV infectivity on the day of sampling using C8166 as indicator cells, 3-fold serial dilutions with eight replicates per dilution and 50 µl/well. In addition, to increase the sensitivity of the assay, the +SD, 240-minute time point was also assayed in "large volume" using 800 replicates without further dilution and 50 µl/well. Excess samples of the original dilution (approximately 10 ml) were placed on ice and stored at –70 °C or below until completion of the study in case additional assays were required.
2. For the test to be valid, the titre of the positive control must be within ±1 log of the certified titre.
3. Calculation of titre

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(1) The final concentrations of TNBP and Tween 80 were 0.255% and 0.85%, respectively. These are intentionally 85% of that specified for use during manufacture to test the worst case likely to be encountered. Similarly, the temperature specified during manufacture is 24 ± 2 °C; thus, the use of 21 ± 1 °C should also represent worst case conditions.

The following formula for the calculation of $TCID_{50}$ is based on the Karber method:

$$LT = LT_{min} + (\log SDF)/2 + \log SDF \, \Sigma P_i$$

where:

LT = log titre for the sample volume tested
LT_{min} = log of smallest dosage causing infection in all cultures
SDF = serial dilution factor (usually 3, 5 or 10)
ΣP_i = the sum of the proportion of positive results observed at all dilutions greater than that causing infection in all cultures.

4. Calculation of 95% confidence interval
The 95% confidence interval was calculated using the following formula:

$$SE^2 = (\log SDF)^2 \times \Sigma\{(P_i(1 - P_i))/(n_i - 1)\};$$

and the 95% confidence interval is: $\pm 1.96 \times SE$

where:

SE = the standard error
SDF = serial dilution factor (usually 3, 5 or 10)
P_i = proportion of positive results at dilution i
n_i = the number of replicates at dilution i
Σ = the summation over all dilutions

5. Calculation of viral reduction factor (RF)

$$RF = \log_{10} \frac{\text{input virus titre (per unit volume)} \times \text{input volume}}{\text{output virus titre (per unit volume)} \times \text{output volume}}$$

For example:

$$RF = \log_{10} \frac{10^8 \text{ IU/ml} \times 10 \text{ ml}}{10^2 \text{ IU/ml} \times 20 \text{ ml}}$$

Results

The controls met the criteria for a valid test. The positive control was within ±1 log of the certified titre of the stock virus, and the negative control did not elicit any cytopathology during the test period. The raw data recorded are given in Table 2.

The $TCID_{50}$ titres of the samples tested were as shown in Tables 3 and 4.

Table 2
Raw data

Sample	No. of wells positive for HIV-1/total[a]																	
Serial dilution factor (3^x)	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Positive control	8/8	8/8	8/8	8/8	8/8	8/8	8/8	7/8	1/8	1/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
Negative control	0/8	0/8	0/8	0/8	0/8	0/8												
+SD T = 0 min	8/8	8/8	8/8	8/8	8/8	3/8	1/8	0/8	0/8	0/8	0/8							
+SD T = 15 min	8/8	4/8	3/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8							
+SD T = 30 min	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8									
+SD T = 60 min	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8									
+SD T = 120 min	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8									
+SD T = 240 min	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8									
+SD T = 240 min (large volume)	0/800																	
–SD T = 0 min				8/8	8/8	8/8	7/8	5/8	2/8	0/8	1/8	1/8	0/8	0/8	0/8	0/8	0/8	0/8
–SD T = 240 min				8/8	8/8	8/8	8/8	7/8	3/8	3/8	1/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8

+SD, solvent/detergent present; –SD, solvent/detergent absent.
[a] 50 µl were tested per well.

Table 3
Calculation of $TCID_{50}$ values

Sample	Titre ± 95% CI (log_{10} $TCID_{50}$/ml)	Volume (ml)	Volume correction (dilution prior to titration)	Viral load (log_{10} $TCID_{50}$)
Certified titre of spiking virus	8.45 ± 0.24			
Positive control	4.94 ± 0.20		1000	7.94 ± 0.20
Negative control	no virus detected			
Solvent/detergent treatment				
+SD; T = 0 min	3.69 ± 0.21	15.67	81	6.79 ± 0.21
+SD; T = 15 min	1.96 ± 0.25	15.67	81	5.06 ± 0.25
+SD; T = 30 min	≤0.70[a]	15.67	81	≤3.80
+SD; T = 60 min	≤0.70[a]	15.67	81	≤3.80
+SD; T = 120 min	≤0.70[a]	15.67	81	≤3.80
+SD; T = 240 min	≤0.70[a]	15.67	81	≤3.80
+SD; T = 240 min (large volume)	≤–1.12[a]	15.67	81	≤1.98
–SD; T = 0 min	4.88 ± 0.31	15.67	81	7.98 ± 0.31
–SD; T = 240 min	5.24 ± 0.29	15.67	81	8.34 ± 0.29

+SD, solvent/detergent present; –SD, solvent/detergent absent; $TCID_{50}$, 50% tissue culture infectious dose.
[a] No virus detected. The theoretical titre was based on the Poisson distribution.

Table 4
Reduction factors (viral clearance)

Process step	Initial load (log_{10} $TCID_{50}$)	Output load (log_{10} $TCID_{50}$)	Log_{10} reduction
Solvent/detergent treatment (240 min)	7.98 ± 0.31	≤1.98	≥6.00 ± 0.31

$TCID_{50}$, 50% tissue culture infectious dose.

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Annex 5
Biological substances: international standards and reference reagents

At its meeting in November 2001, the WHO Expert Committee on Biological Standardization made a number of changes to the previously published list,[a] which are set out below. A list of all current International Biological Reference Preparations, including the present changes, is available on the Internet at http://www.who.int/biologicals. These materials are held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, Potters Bar, Herts., England.

Additions

Preparation	*Activity*	*Status*
Blood products		
Blood coagulation factors II, VII, IX and X, plasma	0.91 IU/ampoule for factor II 1.00 IU/ampoule for factor VII 0.86 IU/ampoule for factor IX 0.93 IU/ampoule for factor X	Third International Standard 2001
von Willebrand factor (vWF)	11.0 IU/ampoule for vWF: antigen 9.4 IU/ampoule for vWF: ristocetin cofactor activity	First International Standard 2001
Streptokinase	1030 IU/ampoule	Third International Standard 2001
Cytokines, growth factors and endocrinological substances		
Intact chorionic gonadotrophin, human	1.88 nmol/ampoule	First WHO Reference Reagent for immunoassay of intact hCG 2001
Nicked chorionic gonadotrophin, human	0.78 nmol/ampoule	First WHO Reference Reagent for immunoassay of nicked hCG 2001
Chorionic gonadotrophin-β, human	0.88 nmol/ampoule	First WHO Reference Reagent for immunoassay of hCG-β 2001

[a] International Biological Reference Preparations 1998. Geneva, World Health Organization, 2000.

Chorionic gonadotrophin-α, human	0.84 nmol/ampoule	First WHO Reference Reagent for immunoassay of hCG-α 2001
Chorionic gonadotrophin-βcf, human	1.02 nmol/ampoule	First WHO Reference Reagent for immunoassay of hCG-βcf
Chorionic gonadotrophin-βn, human	0.33 nmol/ampoule	First WHO Reference Reagent for immunoassay of hCG-βn
Ciliary neurotrophic factor	6.5 µg/ampoule (8000 IU-bioassay)	First WHO Reference Reagent for bioassay, 2001
Prolactin, human, recombinant	24.5 µg/ampoule (1400 mU-bioassay)	First WHO Reference Reagent for Prolactin, recombinant, human 2001
Prolactin human, recombinant, glycosylated	5.5 µg/ampoule (88 mU-bioassay)	First WHO Reference Reagent for Prolactin, recombinant human, glycosylated 2001
Prolactin human, recombinant, non-glycosylated	10.5 µg/ampoule (670 mU-bioassay)	First WHO Reference Reagent for Prolactin, recombinant human, non-glycosylated 2001

Discontinuations

The WHO Expert Committee on Biological Standardization, at its fifty-second meeting discontinued the following reference materials.

Preparation	*Activity*	*Status*
Amykacin	50 600 IU/ampoule	First International Standard 1983
Calcitonin, porcine	0.8 IU/ampoule	Second International Standard 1991
Capreomycin	920 IU/mg	First International Reference Preparation 1967
Chlortetracycline	1000 IU/mg	Second International Standard 1969
Kininogenase, porcine	22.5 IU/ampoule	First International Standard 1982
Lymecycline	948 IU/mg	Second International Reference Preparation 1971
Methacycline	924 IU/mg	First International Reference Preparation 1969
Novobiocin	970 IU/mg	First International Standard 1965

Paromomycin	75 mg	First International Reference Preparation 1966
Prolactin, ovine	22 IU/mg	Second International Standard 1962
Thyrotropin, bovine	0.074 IU/mg	First International Standard 1983
Naja (*Naja & Hemachatus* species) antivenin, equine	300 IU/ampoule	First International Standard 1964

Relabelling of existing standard

The Fourth International Standard for Blood Coagulation Factor VIII and von Willebrand factor, plasma, human, is relabelled with the newly assigned value of 0.83 IU/ampoule for collagen-binding activity in addition to the existing values of 0.89 IU/ampoule of factor VIII antigen, 0.73 IU/ampoule of von Willebrand antigen and 0.73 IU/ampoule of von Willebrand ristocetin cofactor activity.

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Annex 6
Recommendations and guidelines for biological substances used in medicine and other documents

The recommendations 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 22 791 24 76
Fax: +41 22 791 48 57
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 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)
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 (2004)
Cholera Vaccine (Inactivated, oral)	Adopted 2001, TRS 924 (2004)
Diphtheria, Tetanus, Pertussis and Combined Vaccines	Revised 1989, TRS 800 (1990)
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 1990, TRS 814 (1991)
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)
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)
Meningococcal C conjugate vaccines	Adopted 2001, TRS 924 (2004)
Monoclonal Antibodies	Adopted 1991, TRS 822 (1992)
Poliomyelitis Vaccine (Inactivated)	Revised 2000, TRS 910 (2002)
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	Adopted 1966, TRS 323 (1966)
Sterility of Biological Substances	Revised 1973, TRS 530 (1973); Amendment 1995, TRS 872 (1998)
Synthetic Peptide Vaccines	Adopted 1997, TRS 889 (1999)
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)
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**
A review of tests on virus vaccines	TRS 673 (1982)
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 and reference reagents for biological substances	TRS 800 (1990)
Guidelines for quality assessment of antitumour antibiotics	TRS 658 (1981)
Laboratories approved by WHO for the production of yellow fever vaccine, revised 1995	TRS 872 (1998)
Procedure for approval by WHO of yellow fever vaccines in connection with the issue of international vaccination certificates	TRS 658 (1981)
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 of a WHO Consultation on Medicinal and other Products in Relation to Human and Animal Transmissible Spongiform Encephalopathies (2003)	WHO/BCT/QSD/2003.1
Report of a WHO meeting on hepatitis B Vaccines Produced by Recombinant DNA Techniques	TRS 760 (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)