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Introduction

The WHO Expert Committee on Biological Standardization met in Geneva from 27 to 31 October 1997. The meeting was opened on behalf of the Director-General by Dr F.S. Antezana, Assistant Director-General.

Dr Antezana welcomed the representatives of nongovernmental organizations and the European Pharmacopoeia Commission of the Council of Europe and also welcomed and introduced the new Committee members.

He underlined the fact that 1997 marked the 50th anniversary of the Committee, which started its activities in June 1947. However, work on international biological standardization and the provision of International Standards had already been going on for 25 years before that under the League of Nations through its Commission on Biological Standardization. The work of the Committee had had a significant impact on improving public health globally. Nevertheless, the increasing complexity and sophistication of biologicals, as well as the increasing number of biological products, presented a considerable challenge, especially for the developing world. Noting these developments, the World Health Assembly in May 1997 had adopted a resolution on the quality of biological products moving in international commerce. The resolution recognized that standardization activities need to be strengthened to meet the challenges of the 21st century and requested that an independent review be undertaken of WHO's activities in this field. Dr Antezana announced that this review was now under way and would recommend steps to strengthen the leadership of WHO in promoting the quality, safety and efficacy of biological and biotechnological products.

He noted that a number of items on the agenda reflected the expansion and increasing diversity of the field of biologicals. In some instances traditional products were being replaced by equivalents derived by recombinant DNA technology; in addition, new possibilities for diagnostics were emerging, such as the use of genome amplification techniques for the viral safety testing of blood and blood products. New approaches were also being explored for control testing, with molecular-based techniques promising a possible reduced reliance on testing in animals. This complexity underlines the importance of the Committee for the exchange of information, and as a source of expertise, on a global scale. Dr Antezana emphasized the need for its decisions and advice to be based on sound scientific principles and common sense.

Finally, he thanked institutions, manufacturers and individuals who donate candidate reference materials for their continued contributions, through their support of WHO's activities in international biological standardization, to global public health.

General

Developments in biological standardization

The Committee stressed that the timely dissemination of its work was essential. Some progress had been made through the publication of summaries of its meetings in the *Weekly epidemiological record*, in scientific journals and on the WHO web site on the Internet (www.who.int). The Committee recommended that the use of other WHO publications such as *WHO drug information* should be investigated, as this would permit a wider dissemination of the Committee's decisions.

The Committee also recognized the need for a more targeted distribution of its reports and recommendations. WHO was encouraged to consider developing a biologicals information publication for this purpose. Concern was also expressed about the premature use of draft requirements and guidelines. The Committee emphasized that these documents have no status until they are formally adopted. In future, such documents will be watermarked with the world "draft" to clarify their status. The Committee also expressed the need for clarification as to when its requirements, guidelines and recommendations "come into effect". The Secretariat explained that the Committee's report contained recommendations, not mandatory requirements, and could therefore be considered effective as soon as adopted. Requirements published by WHO are scientific and advisory in nature and become binding only when adopted by a national control authority as the basis of national regulations.

At the 1996 meeting the Committee had considered draft requirements for acellular pertussis vaccines; these had been adopted as "guidelines" since there was a lack of consensus about the antigenic composition of the vaccines, no unequivocal immunological correlates of protection had yet been demonstrated nor had a generally accepted animal model been validated. It had been recommended that a working group should continue discussion of related issues, such as developments in assay methods and reference preparations, as well as consider how the guidelines could be extended. The Committee agreed that it was timely to establish such a group, which could now take account of data from the most recent Swedish field trial of

acellular pertussis vaccines,[1] as well as the views of Japanese manufacturers and of the Japanese National Institute of Infectious Diseases.

With respect to plasmid DNA vaccines, which had also been discussed at that meeting, the Committee was informed that an international working group on the standardization and control of nucleic acid vaccines had already been established in response to the guidelines published by WHO and the FDA, both of which raise important and complex issues regarding testing and control. The Committee expressed the need for further guidance from this international working group in relation to the preparation and quality control of plasmid DNA vaccines for clinical trials.

A meeting sponsored by WHO had been held at the National Institute for Biological Standards and Control, Potters Bar, in September 1997 to consider inactivated influenza vaccines produced from mammalian cell lines. Whereas such cell lines had potential advantages, such as increased flexibility for scaling up production, issues of strain selection, vaccine safety and efficacy were raised. The Committee recommended that the Secretariat monitor developments and report back at a future meeting. Consideration should be given to establishing a WHO cell bank for MDCK cells and to the need to isolate virus seed, to produce vaccine virus in validated cell lines and to update the requirements published by WHO.

WHO Informal Consultation on Cytokine Standards

The Committee was informed of developments which had been discussed at the WHO Informal Consultation on Cytokine Standards, held in June 1997. This was the third meeting of the consultative group proposed by the Committee in its forty-fifth report (WHO Technical Report Series, No. 858, 1995, p. 5).

The Committee emphasized the need for proposals to establish reference materials for cytokines and growth factors to be submitted to the consultative group before being submitted to the Committee. It also stressed that more information in support of establishing these reagents should be presented to the consultative group and that a standardized format should be developed. In particular, the proposals ultimately presented to the Expert Committee should include:

- the names and affiliations of all participants in the collaborative study;

[1] *Efficacy trial of acellular pertussis vaccines: technical report, trial II.* Stockholm, Swedish Institute for Infectious Disease Control, June 1997.

- information on the starting materials;
- details of the assay protocol used;
- information on test replicates and reproducibility;
- accelerated and real-time stability data, including the rationale for choosing the testing procedures used.

The Committee was also informed that no conclusions had been reached concerning international standards for interferons alfa because of the very large amount and complexity of the data generated from the WHO collaborative study. Results and recommendations would be discussed at a future meeting of the consultative group.

Quality of biological products moving in international commerce

The Committee noted resolution WHA50.20 adopted by the 1997 World Health Assembly in which the Director-General was requested to extend WHO's assistance to develop and strengthen national control authorities and control laboratories, to eliminate existing inconsistencies in requirements published by WHO, to expand WHO's interactions with other agencies in the preparation of requirements and standards, to ensure timely dissemination of the Expert Committee's decisions and to review the relation between technical reports, requirements and guidelines published by WHO and World Trade Organization agreements.

In addition, the Director-General was requested to convene an independent review of WHO's remit and activities in biological standardization, particularly of the WHO Biologicals unit and how it interacts with other groups with related functions within WHO and externally. The Committee was informed that the first steps in implementing this review had been taken and that a three-person review team had been established, to be supported by a larger consultative panel.

Informal discussions on harmonization of existing and future requirements for vaccine licensing, testing and production

The Committee noted the report of an informal group, established by the Children's Vaccine Initiative in close collaboration with the WHO Biologicals unit and the Global Programme for Vaccines and Immunization to discuss priorities and possibilities for further harmonizing technical requirements for vaccine production and licensing. The meeting had taken place in July 1997, in Brussels, immediately after the fourth International Conference on Harmonisation.

The report underlines that differences in regional and national requirements can limit the availability of certain vaccines in a given

region or country. A number of quality and licensing issues were discussed, including potency requirements for diphtheria and tetanus toxoids, whole cell and acellular pertussis vaccines, the thermostability requirements for measles vaccines, and the abnormal toxicity test, also known as the innocuity or general safety test. The Committee encouraged WHO to take initiatives to make progress on these issues and was pleased to learn that some activities were already under way, including plans to hold a scientific meeting to discuss potency requirements for diphtheria and tetanus toxoids, especially as used in combination vaccines. The Committee considered that any review of thermostability tests should include all live virus vaccines and not just measles vaccines. Plans to review the abnormal toxicity test are discussed later in this report (pp. 10–11).

World Trade Organization (WTO)

The Committee noted and discussed a presentation on the implications for vaccine requirements of one of the multilateral agreements of the WTO, the Agreement on Technical Barriers to Trade (TBT). The agreement authorizes dispute resolution panels to arbitrate related conflicts that arise, but this had not yet been necessary in the field of pharmaceuticals in general or vaccines in particular. In such a case, the requirements, guidelines and reference materials published or established by WHO, because of their global nature, could conceivably be selected for use during arbitration procedures. However, the scientific and advisory nature of such entities may make this difficult. In addition, regions or countries could invoke public health as justification of a given "barrier". The Committee noted that these arbitration procedures were at a very early stage of development and that there was a need for closer contact between WHO and WTO in order to obtain a better mutual understanding of the situation.

Reverse transcriptase activity associated with chicken cells

The Committee was presented with an update on issues related to the detection by very sensitive polymerase-chain-reaction-based methods of low levels of reverse transcriptase activity in live viral vaccines prepared in chicken cells. It had now become clear that this enzymic activity was caused by the presence of endogenous avian retrovirus sequences in the chicken cells used as substrates for the production of certain live viral vaccines. There was no evidence that this activity was associated with safety problems. The Committee proposed that WHO

should host a meeting to review progress in this area and make recommendations.[1]

Regional standardization activities

The Committee heard presentations of ongoing standardization activities in Latin America, south-east Asia, China, Europe, India and USA. These activities included networking among laboratories, the preparation of regional (working) reference materials calibrated against international reference materials, proficiency testing and training programmes.

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

Guidelines for the production and quality control of synthetic peptide vaccines

The Committee noted document BS/96.1844 (Rev.1)[2] which had been amended and circulated for comment to a wide group of experts, as well as to members of WHO's Expert Advisory Panel on Biological Standardization, following the Committee's comments on the first draft. The Committee considered that the document contained very helpful guidance on the production and quality control of candidate synthetic peptide vaccines. The Committee noted that the guidelines were primarily oriented towards achieving consistency and safety in product development since issues regarding clinical efficacy were largely unknown. After making a few minor amendments, the Committee adopted the text as Guidelines for the Production and Quality Control of Synthetic Peptide Vaccines and agreed that it should be annexed to its report (Annex 1).

Requirements for tick-borne encephalitis vaccine (inactivated)

The Committee noted document BS/97.1855 which proposed, for the first time, requirements for tick-borne encephalitis vaccine. The requirements were formulated to take account of current manufacturing practices and control procedures and included guidance on how these practices and procedures could be updated. The Committee was

[1] See *Weekly epidemiological record*, 1998, 73:209–212.

[2] References prefixed "BS/. . ." and "BLG/. . ." are to unpublished working documents of the World Health Organization. They are not issued to the general public, but a limited number of copies may be available to professionally interested persons on application to Quality Assurance and Safety: Biologicals, World Health Organization, 1211 Geneva 27, Switzerland.

informed that the document had been circulated to members of WHO's Expert Advisory Panel on Biological Standardization and that a number of comments had been received.

After making some modifications, the Committee adopted the text as Requirements for Tick-borne Encephalitis Vaccine (Inactivated) and agreed that it should be annexed to its report (Annex 2).

During discussion of these requirements a number of general issues were also raised by the Committee. These included:

- the use of porcine trypsin and its possible contamination with porcine retroviruses;
- a need to explore alternatives to the use of human albumin as a reagent or stabilizer in vaccine manufacture;
- a need to review the scientific basis for specifications regarding completeness of inactivation of virus vaccines;
- a need to encourage neutralization of excess formaldehyde used during inactivation of virus or toxins;
- a need to encourage the use of serologically defined endpoints as opposed to challenge-defined endpoints in potency tests;
- a need to survey global experience with the traditional test for haemadsorbing viruses and the need to use erythrocytes from species other than guinea-pigs.

Requirements for thromboplastins and plasma used to control oral anticoagulant therapy

The Committee noted document BS/97.1856 which reflected a substantial revision of the Requirements for Thromboplastins and Plasma Used to Control Oral Anticoagulant Therapy, published in 1983. The Committee was informed that the changes made to the original text had been agreed following extensive discussion at a WHO/International Society on Thrombosis and Haemostasis (ISTH) consultation and circulated to the members of the ISTH Standardization Subcommittee on Control of Anticoagulation, as well as to the WHO Expert Advisory Panels on Biological Standardization and Human Blood Products and Related Substances. In view of the form of the document and the many important technical details and specifications it contains, the Committee considered that it would best be published as guidelines rather than requirements. Any significant deviation from the methodology described in the guidelines would need thorough validation and/or evaluation as to its impact.

The Committee adopted the text as Guidelines for Thromboplastins and Plasma Used to Control Oral Anticoagulant Therapy and agreed that it should be annexed to its report (Annex 3). The Committee

therefore discontinued the Requirements for Thromboplastins and Plasma Used to Control Oral Anticoagulant Therapy published in 1983 (Requirements for Biological Substances No. 30). It supported the proposal to publish the guidelines in the *Journal of thrombosis and haemostasis*, the official journal of the International Society on Thrombosis and Haemostasis, once the final editing of the text had been completed.

The Committee noted that the package insert for the International Reference Preparations of thromboplastins had been replaced by different memoranda for each of the three thromboplastin reference materials.

Requirements for hepatitis B vaccines made by recombinant DNA techniques

The Committee noted document BS/97.1870 which proposed an amendment to the Requirements for Hepatitis B Vaccines Made by Recombinant DNA Techniques in order to reflect recent developments in assay methodology. After making some minor changes, the Committee adopted the amendment and agreed that it should be annexed to its report (Annex 4).

Requirements for *Haemophilus* type b conjugate vaccines

The Committee was informed of the need to review and update the Requirements for *Haemophilus* Type b Conjugate Vaccines in the light of experience gained in the production, control and use of *Haemophilus* type b conjugate vaccines. An informal WHO Consultation on the Standardization and Control of *Haemophilus influenzae* Type b Conjugate Vaccines had been held in Brussels during April of 1997, where an agreement had been reached to delete the mouse immunogenicity assay as a routine batch control test and to focus on physicochemical criteria for monitoring the consistency of bulk conjugate production. The Committee endorsed the Secretariat's proposal to organize another consultation to elaborate more specific guidance on physicochemical test methods and to finalize revision of the requirements for consideration at its next meeting.

Requirements for immunoassay kits

In view of the development of many new and different types of immunoassays the Committee decided to discontinue the Requirements for Immunoassay Kits published in 1981, since they were considered to be outdated. It concluded that the general approach taken in those requirements would probably have to be replaced by a product-class-specific approach.

Guidelines for standardization and calibration of cytokine immunoassays

The Committee noted proposed guidelines for the standardization and calibration of cytokine immunoassays (BS/97.1854). However, it considered that the document had been drafted more as a report than as guidelines. It therefore agreed that it should be annexed to its report as "Report on the standardization and calibration of cytokine immunoassays" (Annex 5). WHO was requested to consider further developing the document as guidelines.

Criteria for establishing reference materials

The Committee noted a draft revision of guidelines for the preparation, characterization and establishment of international and other standards and reference reagents for biological substances. A number of accompanying documents and publications on criteria for reference preparations were also presented. The Committee proposed that a working group be formed to review further and update the draft revision. It also recommended that the group take into consideration the nomenclature used for different reference materials. The Committee also heard a presentation on chemical reference substances for pharmacopoeial use. A list of these reference substances and infrared spectra was available on the Internet. Revised "General guidelines for the establishment, maintenance and distribution of chemical reference substances" had recently been adopted by the WHO Expert Committee on Specifications for Pharmaceutical Preparations (WHO Technical Report Series, No. 885, 1999, Annex 3), and could serve as an additional resource for the proposed working group.

The Committee also discussed the need for guidance in setting priorities in establishing reference materials. A checklist of points to consider would be helpful and could be included in the revised guidelines.

Requirements for tetravalent dengue vaccine (live)

The Committee noted preliminary draft requirements for tetravalent dengue vaccine (live), which had been developed by a small drafting group established by WHO. The Committee welcomed this initiative since a live tetravalent dengue vaccine had now reached phase II clinical trials, and many countries had expressed interest. However, the Committee expressed a number of concerns. The vaccine had been developed in primary dog-kidney cells, which were an unconventional substrate for live virus vaccine production. Early discussion of this important issue would have been helpful. The Committee was encouraged to learn of possibilities for change to a more conventional

cell substrate in line with existing Requirements for the Use of Animal Cells as *in vitro* Substrates for the Production of Biologicals. However, a change in the cell substrate would lead to the need for considerable redevelopment and re-evaluation, including new clinical trials. The appropriateness of the safety testing described in the draft requirements, in particular the neurovirulence test, and the need for reference materials were also raised.

WHO Consultation on Medicinal and Other Products in Relation to Human and Animal Transmissible Spongiform Encephalopathies

The Committee considered *Report of a WHO Consultation on Medicinal and Other Products in Relation to Human and Animal Transmissible Spongiform Encephalopathies*, the report of a consultation held in Geneva during March 1997. For bovine substances used as active principles, excipients or production reagents in human medicines, the source and type of material, method of collection and production processes involved were considered to be the main issues for risk assessment regarding transmissible spongiform encephalopathies. Since the WHO meeting, the European Commission had decided to ban all "specified risk materials" from human medicines. These materials include the brain, eyes, tonsils and spinal cord of bovine animals aged over 12 months. Discussions were under way within the European Union as to the impact of this decision on the availability of some medicinal products such as vaccines.

Regarding the use of substances of human origin and the risk of transmission of Creutzfeldt-Jakob Disease (CJD), either the "classical" form or the "new variant", the Committee discussed experimental data obtained using small animals which had shown infectivity in blood cells and some plasma fractions, and human epidemiological data which had not revealed any cases of blood-borne transmission to date. Because of the rapidity of developments in this area, the Committee requested the Secretariat to monitor the situation and to consider convening a further meeting at an appropriate time to evaluate the global consequences of the scientific evidence. This would help unify divergent approaches to the issue in different countries.

Alternatives to animal testing

The Committee was informed of a general interest in the use of alternatives to animal testing in quality control procedures and of the progress that had been made in Europe to replace, refine or reduce the use of animals for this purpose.

For example, requirements published by WHO as well as pharmacopoeial monographs for most, if not all, vaccines include an abnormal

toxicity test (sometimes called innocuity or general safety test) carried out in mice and guinea-pigs. These requirements and pharmacopoeial monographs generally do not indicate the strain of animals to be used, although this can greatly influence the outcome of the test. Furthermore, test interpretation can sometimes give rise to problems. The Committee was informed that the European Pharmacopoeia had recently deleted the abnormal toxicity test from its requirements for certain vaccines. This decision had been taken on evidence that the test contributed little to the safety of the product and was unnecessarily wasteful of animals. As the test had originally been introduced to identify production errors, the implementation of Good Manufacturing Practices (GMP) should now provide for this eventuality.

The Committee was interested to learn from experience in Europe and requested the Secretariat to begin collecting global data on the value of the abnormal toxicity test.

International Conference on Harmonisation (ICH) guidelines on stability testing

The Committee was informed that the International Conference on Harmonisation had prepared guidelines on the stability testing of biotechnological and biological products. The Committee considered that the information in the guidelines was helpful.

Noting that biological and biotechnological products can be particularly sensitive to environmental factors, and that the analysis of their stability can require complex methodologies, the guidelines outline a useful approach to the stability testing of such products, which include well-characterized proteins and polypeptides, their derivatives and products of which they are components, as well as products isolated from tissues, body fluids or cell cultures, or produced using recombinant DNA technology. The guidelines cover testing of the bulk product, any intermediates and the product in its final containers. A number of important issues regarding sample selection, testing frequency, relevant parameters of storage (e.g. light, heat, humidity), and testing for potency, purity and/or contamination as well as other product characteristics are discussed. Useful recommendations are made regarding the development of a stability specification, special procedures for freeze-dried or lyophilized products and product labelling.[1]

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[1] *Quality of biotechnological products: stability testing of biotechnological/biological products.* Geneva, International Conference on Harmonisation (ICH), 1995 (available on request from ICH Secretariat, International Federation of Pharmaceutical Manufacturers Associations, 30 rue de St-Jean, 1211 Geneva 18, Switzerland).

The Committee also agreed that a document detailing the key steps in designing and interpreting stability studies would be extremely useful and recommended that WHO should pursue this idea.

Training manual for inspection of manufacturers of biological products

The Committee was informed that WHO had issued a training manual for the inspection of vaccine manufacturers, entitled *Guide for inspection of manufacturers of biological products*, for use in the global training network of WHO's Global Programme for Vaccines and Immunization. The Committee noted that the manual incorporated certain information from "Good manufacturing practices for biological products" (WHO Technical Report Series, No. 822, 1992, Annex 1). While recognizing the potential of the manual for use in training, the Committee emphasized that its value for other purposes had not been assessed or subjected to review.

To avoid misunderstanding concerning the purpose of the document, the Committee recommended that the title should be revised to "Training manual for the inspection of manufacturers of vaccines" in any future versions.

Procedures for assessing the acceptability in principle of vaccines for purchase by United Nations agencies

The Committee noted a revised document entitled *Procedures for assessing the acceptability in principle of vaccines for purchase by United Nations agencies*.[1] The document replaces that published in the WHO Technical Report Series (No. 786, 1989), which was therefore discontinued.

Requirements for oral poliomyelitis vaccine

The Committee was informed of a series of informal WHO consultations during September 1997 on developments in quality control of oral poliomyelitis vaccine.

Excellent progress was reported from a project to develop a neurovirulence test in transgenic mice (strain TgPVR21) as an alternative to the assay currently carried out in monkeys. A collaborative study showed that nine laboratories had successfully implemented the technique. Further collaborative study was under way on a regulatory decision-making model. A candidate reference material would be

[1] Available on request from Vaccines and Other Biologicals, World Health Organization, 1211 Geneva 27, Switzerland.

included in these studies and, if supported by the results, a proposal would be made to establish a new international reference material. Once the procedure was validated, manufacturers and national control authorities would be encouraged to apply it to vaccine lots previously tested in monkeys. A panel of experts would then review the data and, if appropriate, draft proposals for the regulatory use of the transgenic mouse neurovirulence test. After wide consultation, these would be submitted to a future meeting of the Expert Committee on Biological Standardization, with a proposal to alter the requirements for oral poliomyelitis vaccine to allow neurovirulence tests in transgenic mice as an alternative. The Committee noted that work was most advanced for vaccine prepared from type 3 Sabin virus and encouraged further work to develop appropriate models for type 1 and 2 Sabin virus.

An assay using mutant analysis by polymerase chain reaction and restriction enzyme cleavage (MAPREC assay) is now well established for the quantitation of trace amounts of neurovirulence mutants in batches of vaccine prepared from type 3 poliovirus (Sabin), and is being widely used by both manufacturers and national control authorities. An informal WHO consultation had supported a proposal to establish two new reference materials, in addition to that already established in 1996 (International Standard for MAPREC Analysis of Poliovirus Type 3), to be used as validation controls for the assay of type 3 oral poliomyelitis vaccine (see pp. 22–23). The meeting had also recommended that a comprehensive database of MAPREC results for type 3 vaccine be established and reviewed by experts. Subject to a satisfactory outcome of this review, a proposal would be made to revise the Requirements for Poliomyelitis Vaccine, Oral, to introduce the MAPREC assay as a new test for type 3 vaccine. The Committee encouraged the development of appropriate reference materials for type 1 and 2 vaccine and likewise encouraged continuation of the work on the MAPREC assay.

The Committee was also informed of an informal consultation convened by WHO to review the effectiveness of measures in requirements published by WHO for oral poliomyelitis vaccine to exclude Simian virus 40 (SV40). This consultation was in response to findings of SV40-like sequences in some human tumour tissues. Application of new and highly sensitive polymerase-chain-reaction techniques for SV40 to over 160 batches of oral poliomyelitis vaccine from several manufacturers showed that SV40 had been effectively excluded from these vaccines for over 30 years. However, the consultation considered it prudent to test additional seed viruses from other manufacturers, and the Committee endorsed these plans. Furthermore the

informal consultation had recommended that a proposal be made to the 1998 meeting of the Expert Committee to add a requirement that seed viruses for oral poliomyelitis vaccine be free of SV40 sequences.

Finally the Committee was informed of a WHO working group that had considered containment measures for laboratory stocks of wild polioviruses. Circulation of wild poliovirus was much reduced because of the success of the WHO initiative for global polio eradication. As laboratory stocks of wild polioviruses are a potential source of reintroduction of poliomyelitis into a community, a process and timetable for their containment had been prepared. As part of this process the working group requested the Committee to examine Requirements for Poliomyelitis Vaccine, Oral, and Requirements for Poliomyelitis Vaccine (Inactivated) to determine whether Sabin strains could be substituted for wild polioviruses in the quality control tests that use wild polioviruses. Review of the relevant requirements, for consideration in due course by the Committee, would therefore be initiated to take into account the above-mentioned developments.

International reference materials

Review of existing reference materials

The Committee was informed of the successful transfer in 1997 of all Statens Seruminstitut (SSI) reference materials to the National Institute for Biological Standardization and Control, Potters Bar (NIBSC). The Committee was also informed that agreement had been reached for the transfer of stocks of veterinary reference materials from the WHO International Laboratory for Biological Standards, Central Veterinary Laboratory, Weybridge, England, to NIBSC with effect from January 1998.

The Committee discussed a report (BS/97.1872) that presented the results of a comprehensive review of the international reference materials distributed by NIBSC and that identified some materials for discontinuation, replacement or revision of nomenclature. The Committee underlined the importance of notifying all interested parties that discontinuation of an International Standard also meant discontinuation of the International Unit for that material.

The general distribution of International Standards for *Clostridium botulinum* equine antitoxins types C, D and F, previously used for typing purposes, had stopped. Only equine antitoxins types A, B and E are still produced for therapeutic use, and stocks of these reference materials are low. The Committee proposed to take no action for the time being and requested WHO to establish a working group to

evaluate the need for production of botulinum toxoids and antitoxins for use as therapeutic agents, as well as the need for reagents for their standardization.

The Committee agreed to discontinue the fourth International Reference Preparation of Cardiolipin, the fourth International Reference Preparation of Lecithin (Egg), the International Reference Preparation of Poliomyelitis Vaccine (Inactivated), the second International Standard for Diphtheria Toxoid, Plain, and the International Standard for Tetanus Toxoid.[1] The international reference materials for toxoids for use in flocculation tests, as well as those for antitoxins, are retained.

Concerning the International Standard for Hyaluronidase, the Committee proposed to notify formally the International Centre for Pharmaceutical Enzymes (ICPE) of the International Pharmaceutical Federation (FIP) that it was intended to discontinue the standard and that, as a consequence, the ICPE preparation would become the primary international standard. If agreement was reached, WHO would inform the appropriate parties of the decision.

The Committee was informed that stocks of the International Reference Preparation for Opacity, consisting of a plastic rod, are low. After discussion the Committee agreed that work should start to characterize a new standard, to evaluate for which type of products the standard was appropriate and to review the use of the standard in requirements published by WHO. The Committee also recommended a collaborative study to compare the visual evaluation method with more sophisticated methodologies. A final decision on the need for this type of standard would depend on the outcome of such a study. The Committee requested the Secretariat to monitor the utilization rates of the international reference materials for the following substances in order to evaluate the need for their replacement:

- Anti-Q-fever serum, bovine;
- Anti-streptolysin O, human;
- Anti-typhoid serum, equine;
- Gas-gangrene antitoxin (*Clostridium histolyticum*), equine;
- Gas-gangrene antitoxin (*Clostridium sordellii*), equine;
- Scarlet fever streptococcus antitoxin, equine;
- Syphilitic serum, human.

The Committee requested the Secretariat to begin work to establish replacements for the following international reference materials:

[1] Commonly referred to as "tetanus toxoid, plain."

- International Reference Preparation of Anti-Yellow Fever Serum;
- Third International Standard for Anti-Toxoplasma Serum, Human.

The Committee also requested the Secretariat to survey the utilization rates of the International Reference Preparations of Cholera Vaccine (Inaba and Ogawa) and to evaluate the use of Requirements for Cholera Vaccine published by WHO.

The Committee noted that the International Reference Reagent for Anti-trichinella Human Serum had been found to test positive by polymerase chain reaction for hepatitis C virus RNA. Distribution of the reagent would be continued on an interim basis until a replacement was obtained. The Committee proposed that an additional warning about the positivity of the material for hepatitis C virus should be given in the information accompanying the material. The Committee was informed that the International Reference Preparation of Hepatitis A Immunoglobulin had similarly been found to be positive for hepatitis C virus. It endorsed a proposal to continue to distribute the material on an interim basis and noted that work to obtain a replacement was already under way. A warning like that distributed with the International Reference Reagent for Anti-trichinella Human Serum should be included on distribution of the reference preparation.

The Committee noted that there was a need to clarify the nomenclature of the fifth International Standard for Rabies Vaccine. The potency of the material had been established on the basis of immunogenicity in mice. The same preparation had also been established and assigned potencies as the International Standard for Rabies Virus PM-Glycoprotein and as the International Standard for Rabies Virus PM-Ribonucleoprotein.[1] This had given rise to confusion. The Committee recommended that the names first International Standard for Rabies Virus PM-Glycoprotein and first International Standard for Rabies Virus PM-Ribonucleoprotein be abandoned, to make it clear that one International Standard for Rabies Vaccine existed, which had been assigned different potencies with respect to three major rabies virus antigenic activities. The potency of the standard with respect to PM-glycoprotein and PM-ribonucleoprotein was accordingly maintained.

The Committee expressed the need to update the existing catalogue of International Standards and Reference Reagents (*Biological substances*. Geneva, World Health Organization, 1990) and the need to explore alternative and complementary ways of publication, such as the Internet and CD-ROM.

[1] PM = Pitman-Moore.

Antibodies

Antibody to human platelet antigen 1a

The Committee noted the report on a preparation of antibody to human platelet antigen 1a (BS/97.1862) and established the preparation, coded 93/710, as an interim Reference Reagent for Antibody to Human Platelet Antigen 1a, for use as a minimum sensitivity reagent. No unitage was assigned. In the memorandum accompanying the material a statement will indicate that the reagent should not be used other than as a minimum sensitivity reagent.

Anti-hepatitis E serum, human

The Committee noted the report of a collaborative study performed by seven laboratories in five countries on a candidate reference material for human anti-hepatitis E serum (BS/97.1869).

The Committee was aware that assays for anti-hepatitis E were at an early stage of development and recognized that full assessment of new antibody assays requires panels of sera. Nevertheless, it felt that the availability of a common reference material would help evaluate interlaboratory variation and would aid developments in the serology of hepatitis E virus. The Committee therefore established the preparation, coded 95/584, as an interim Reference Reagent for Anti-Hepatitis E Serum, Human, and assigned a value of 50 units per ampoule.

Antibiotics

Proposed discontinuation of certain antibiotic standards

The Committee noted the report on the enquiry into the use of certain antibiotics (BS/97.1872) carried out by the Secretariat in response to requests made at the 1996 meeting. The Committee further noted that official requirements still exist for capreomycin, lymecycline and novobiocin and took no further action with respect to the relevant reference materials.

In view of the information provided, the Committee considered that the international reference materials for doxycycline, demeclocycline, minocycline, oxytetracycline and tetracycline could probably be discontinued. These antibiotics are used very extensively and two of them are included in the WHO Model List of Essential Drugs. In view of this, the Committee requested WHO to announce the proposed discontinuation of the international reference materials for these five antibiotics in *WHO drug information*, to explain the consequences for

the calibration of assays and to seek comments. The Committee agreed to review the responses at its next meeting and make a decision on discontinuation at that time.

Antigens

Swine erysipelas vaccine

The Committee was informed of recent evidence of the apparent deterioration of the International Standard for Swine Erysipelas Vaccine and the low level of remaining stocks. In view of the changes reported, the Committee discontinued the International Standard for Swine Erysipelas Vaccine and requested the Secretariat to inform the WHO International Laboratory for Biological Standards, Central Veterinary Laboratory, Weybridge, England.

The Committee agreed that its role in the establishment of reference materials for purely veterinary use should be considered during the independent review of WHO's activities in the biologicals field (see p. 1). The Committee deferred a decision on whether the discontinued standard should be replaced until the review team had reported.

Blood products and related substances

Antithrombin concentrate

The Committee noted that a collaborative study by 18 laboratories in 10 countries had been performed to calibrate a candidate replacement for the International Standard for Antithrombin III Concentrate (BS/97.1863).

On the basis of the results, the Committee established the material studied, in ampoules coded 96/520, as the second International Standard for Antithrombin Concentrate.[1] It was decided to assign 4.7 International Units of functional activity and 5.1 International Units of antigenic activity to each ampoule.

Further information on the starting materials was requested and would be added as an addendum to document BS/97.1863.

Serum amyloid A protein

The Committee noted the report (BS/97.1860) on a collaborative study of a candidate international standard for serum amyloid A

[1] In the light of recent research, the qualifier "III" was dropped from the name of the standard.

protein, carried out in seven laboratories in six countries of wide geographical distribution. After considering the report, together with additional data concerning the methodologies used, as well as information from the study coordinator about the need for such a reference material, the Committee established the preparation, coded 92/680, as the International Standard for Serum Amyloid A Protein and assigned a unitage of 150 milli-International Units per ampoule. The Committee requested that information on the methodology and starting materials be added as an addendum to document BS/97.1860.

t-PA antigen in plasma

The Committee noted the results of a collaborative study undertaken by 10 laboratories in nine countries to calibrate a proposed international standard for tissue plasminogen activator (t-PA) antigen in plasma. However, results indicated that the use of the proposed standard did not improve interlaboratory variability in assays of low concentrations of t-PA antigen in plasma. The Committee considered that the data presented were insufficient to allow the establishment of an international standard and asked for an evaluation of the need for such a reference material, as well as an investigation into the possibility of using a more sensitive assay. Information about the stability of the proposed standard was also needed. The additional information would be evaluated at a future meeting of the Committee.

Haemiglobincyanide reference preparation

Following a request made by the Committee at its 1995 meeting, further information had been obtained concerning the haemiglobincyanide reference material established by the Measurement and Testing Programme of the European Commission. The Committee noted an informal report on a "Proposed primary International Standard for measurement of haemoglobin in blood by the haemiglobincyanide method", prepared by the Expert Panel on Haemoglobinometry of the International Council for Standardization in Haematology (ICSH). The recommendation made in the report that an International Standard for haemiglobincyanide should be continued was accepted by the Committee. This decision was made in view of the importance of the standard for global public health.

The ICSH had acquired 1000 ampoules of a candidate reference preparation of bovine haemiglobincyanide, coded CRM 522, calibrated by the Measurement and Testing Programme of the European Commission in 1993. The Committee requested the Secretariat to ensure that all available data were provided for evaluation at the 1998 Committee meeting.

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

The Committee noted a report of a working group to assess the need for reference materials for testing diagnostic kits used for detection of hepatitis B surface antigen (HBsAg), antibodies to hepatitis C virus (anti-HCV) and antibodies to human immunodeficiency virus (anti-HIV) in blood, held 11–12 December 1996 at WHO, Geneva. It noted the progress made in characterizing a number of international reference sera for HBsAg, anti-HCV and anti-HIV. The Committee expressed the need to clarify that such serum reagents are not intended to replace the seroconversion panels used to evaluate the clinical sensitivity of diagnostic kits, but to enable a national control laboratory to characterize a kit and the consistency of its production.

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

The Committee received a report on the sixth meeting of a working group concerned with the standardization of gene amplification methods for the viral safety testing of blood and blood products and noted that the group supported a proposal to establish a reference material for gene-amplification-based assays for detecting hepatitis C virus RNA (see p. 23).

International Society on Thrombosis and Haemostasis

The Committee heard a presentation on the activities of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis. The fruitful collaboration with the WHO Expert Committee on Biological Standardization in the field of standardization of plasma coagulation factors was appreciated.

Cytokines and growth factors

The Committee recalled its decision in 1995 (WHO Technical Report Series, No. 872, 1998, p. 4) to establish a class of interim reference reagents on the basis of limited data in rapidly developing fields in order to allow the use of such materials before full collaborative studies could be undertaken.

The following interim reference reagents for cytokines and growth factors are a result of this decision. However, in each case the Committee was concerned that there should be a more complete record relating to the establishment of these preparations as interim reference reagents. The Committee therefore requested that relevant information relating to each substance mentioned below, which should

include the information mentioned previously on pp. 3–4, be supplied to the Secretariat for inclusion with the documentation already received.

Interleukin-10

The Committee noted the report on a preparation of interleukin-10 (BS/97.1868), and on this basis established the preparation, coded 93/722, as the first interim Reference Reagent for Interleukin-10, with an assigned potency of 5000 units per ampoule.

Bone-morphogenic protein-2 (BMP-2)

The Committee noted the report on a preparation of bone-morphogenic protein-2 (BMP-2) (BS/97.1857), and on this basis established the preparation, coded 93/574, as the first interim Reference Reagent for Bone-Morphogenic Protein-2 with an assigned potency of 5000 units per ampoule.

FMS-like tyrosine kinase-3 ligand

The Committee noted the report on a preparation of FMS-like tyrosine kinase-3 ligand (BS/97.1858), and on this basis established the preparation, coded 96/532, as the first interim Reference Reagent for FMS-like Tyrosine Kinase-3 Ligand with an assigned potency of 1000 units per ampoule.

Stem cell factor

The Committee noted the report on a preparation of stem cell factor (BS/97.1859), and on this basis established the preparation, coded 91/682, as the first interim Reference Reagent for Stem Cell Factor with an assigned potency of 1000 units per ampoule.

Brain-derived neurotropic factor

The Committee noted the report on a preparation of brain-derived neurotropic factor (BS/97.1867), and on this basis established the preparation, coded 96/534, as the first interim Reference Reagent for Brain-derived Neurotropic Factor with an assigned potency of 5000 units per ampoule.

Follicle-stimulating hormone (FSH) for immunoassay

The Committee noted a proposal to establish a preparation of recombinant follicle-stimulating hormone (FSH) as an International Standard for immunoassay (BS/97.1871). Although it recognized that there are a number of issues associated with the standardization of immunoassays, including the appropriateness of using recombinant

proteins as standards, the Committee established the recombinant DNA-derived FSH preparation, coded 92/510, as the International Standard for Follicle-stimulating Hormone, Human, for Immunoassay, and assigned a potency of 60 International Units to the contents of each ampoule.

Platelet-derived growth factor (PDGF)

The results of a collaborative study (BS/97.1864) of a preparation of recombinant-human-sequence platelet-derived growth factor, isoform BB (PDGF-BB), were noted by the Committee. On the basis of this study, carried out in seven laboratories in three countries, the Committee established the preparation, coded 94/728, as the International Standard for Platelet-derived Growth Factor (isoform BB) and assigned a potency of 3000 International Units to each ampoule. The Committee requested that the histograms referred to in the text of BS/97.1864 be attached to that document.

The Committee also considered a proposal to establish a preparation of platelet-derived growth factor, isoform AB, in ampoules coded 94/558, as an interim reference reagent. In view of the limited data available on the preparation and uncertainty about the need for such a reagent, the Committee declined to establish an interim reference reagent but referred the matter to the consultative group on cytokine and growth factors for advice on the need for reagents in this area.

Miscellaneous

MAPREC analysis of poliovirus type 3 (Sabin)

The Committee noted a proposal to establish two reference materials for a method of molecular analysis (mutant analysis by polymerase chain reaction and restriction enzyme cleavage, or MAPREC) for poliovirus type 3 (Sabin) (BS/97.1865). The Committee also noted the results of a collaborative study, performed by 14 laboratories in nine countries, which had shown very reproducible results. The Committee was informed that the two materials are intended for use as validation controls in the MAPREC assay. The Committee also noted that the two liquid preparations display satisfactory stability.

The Committee established one of the preparations, coded 96/572, as the International Reference Reagent for MAPREC Analysis of Poliovirus Type 3 (Sabin) (Low Virus Reference) and assigned a value of 0.7% 472-C nucleotide to the contents of each vial. It established the preparation, coded 96/578, as the International Reference Reagent for MAPREC Analysis of Poliovirus Type 3 (Sabin) (High

Virus Reference) and assigned a value of 1.1% 472-C nucleotide to the contents of each vial.

The Committee encouraged the development of similar reference materials for type 1 and 2 poliovirus (Sabin).

Hepatitis C virus RNA

The Committee noted a proposal to establish a reference material for hepatitis C virus RNA (BS/97.1861). The proposal was based on the results of a collaborative study performed by 22 laboratories in 11 countries. It further noted data showing that the candidate material had adequate stability in accelerated stability studies and was informed that real-time stability studies would also be carried out.

The Committee established the preparation, coded AA, as the International Standard for Hepatitis C Virus RNA, and assigned a potency of 50000 International Units to each vial. It requested that the additional stability data supplied by the study coordinators be appended to document BS/97.1861.

The Committee further noted the high variability of the fill volume (coefficient of variation = 8.39%), which did not meet the strict specification stipulated in the Guidelines for the preparation, characterization and establishment of international and other standards and reference reagents for biological substances (WHO Technical Report Series, No. 800, 1990, Annex 4). As the material was highly infective, it was not possible to process it in the usual facilities at the National Institute for Biological Standards and Control, Potters Bar. As a result of the inherent variability of the gene amplification technology, assays on ampoules with the highest and lowest fill volumes did not, however, show differences.

The Committee also noted the availability of another material, coded BB, as a possible future replacement of AA, and recommended that it should also be tested in the ongoing stability studies.

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Annex 1
Guidelines for the production and quality control of synthetic peptide vaccines

1. Scope

These guidelines are intended to provide a scientifically sound basis for the development, production and control of peptide vaccines for use in humans and to ensure their consistent safety and efficacy. In

addition to providing background information on the development of peptide vaccines, the guidelines cover the following main areas:

- control of starting materials, including background data on the synthesis of the peptide of interest;
- control of the manufacturing process;
- control of the final product.

The guidelines should be read in conjunction with the general manufacturing requirements contained in Good Manufacturing Practices for Pharmaceutical (*1*) and Biological (*2*) Products. Guidelines for Good Clinical Practice for Trials on Pharmaceutical Products (*3*) should also be applied during all stages of the development of synthetic peptide vaccines. Many of the general requirements for the quality control of biological products, such as tests for potency, pyrogenicity, stability and sterility, also apply to synthetic peptide vaccines. The development of synthetic peptide vaccines is at an early stage; no vaccines of this type have yet been licensed, although much research and development is under way and several experimental products are under evaluation. Thus, a flexible approach to the control of these vaccines must be adopted, so that requirements can be modified as experience is gained in their production and use. It is hoped that these guidelines, representing an international consensus view, will be of benefit to those involved in the design and evaluation of peptide vaccines.

Individual countries may wish to use this document to develop their own national guidelines for synthetic peptide vaccines for human use. The guidelines may also, to a considerable extent, be relevant to synthetic peptide vaccines for veterinary use.

These guidelines are intended to facilitate the process of submission and evaluation of a peptide vaccine for licensing. Experience has shown that the process of obtaining a licence is much easier if the considerations set out below are kept in mind from the earliest stages of development.

2. Introduction

Traditional vaccines have been prepared either by using an attenuated version of a pathogen, such as for yellow fever, poliomyelitis and measles vaccines or BCG, or by preparing and inactivating a disease-causing organism (such as for pertussis, influenza and typhoid vaccines) or a suitable part of it, for instance, a toxin, as in the case of diphtheria and tetanus vaccines, and administering it in quantities

sufficient to induce immunity. These approaches have led to many effective vaccines. However, difficulties often remain in the manufacture of vaccines, in maintaining their efficacy and in ensuring their safety on a lot-by-lot basis.

For attenuated vaccines, examples of common difficulties are:

- developing stable attenuated strains of the pathogen;
- the reversion to virulence of the attenuated strains;
- controlling strain properties;
- the presence of adventitious agents;
- the necessity for a cold chain.

For inactivated vaccines, examples of common difficulties are:

- the necessity of achieving total inactivation;
- avoiding reactogenicity while retaining protective immunogenicity.

Common difficulties for both types of vaccine are:

- the culture of pathogenic organisms in the amounts necessary for production;
- the possibility of inducing an inappropriate immune response (cross-reactivity with host antigens, for instance).

In addition to the traditional use of vaccines for protection against infection and toxins, there is great interest in the potential of immunotherapeutic techniques in such areas as stimulating immunity to cancer cells, down-regulating the effects of polypeptide hormones, and controlling inappropriate immune responses (such as anaphylaxis).

2.1 The potential of peptides as vaccines

An alternative approach to immunization would be to identify on immunogens the peptide epitopes that induce the requisite response and to use synthetic versions of these peptides in the production of vaccines. Unlike traditional vaccines, such vaccines, because they are totally synthetic, would not carry the risk of reversion or of incomplete inactivation, and, in principle, epitopes could be selected to avoid components that give rise to unwanted side-effects. In addition, the use of synthetic peptides makes available antigens that may be difficult to prepare in quantity from a natural source, such as parasite antigens, offers the prospect of eliciting immunity to antigens that are not normally recognized, for instance, "self" antigens such as tumour-specific antigens in cancers, and makes it possible to elicit a response to epitopes that remain cryptic during natural infection.

2.2 Peptides and the immune system

For a peptide (or other antigen) to stimulate an effective immune response, it must be efficiently processed and presented to T cells. T cells recognize the antigen or peptide by the different MHC (major histocompatibility complex) molecules on the surface of the antigen-presenting cell, and this is critical in determining the nature of the immune response generated. The type of MHC-antigen complex that results is dependent on the source of the antigen or peptide. Proteins derived from inside a host cell (e.g. viral proteins) are usually processed via the MHC class-I pathway and elicit predominantly cytoxic T-cell responses. Exogenous antigens (e.g. proteins or peptides excreted by bacteria) are taken up by specialist antigen-presenting cells and normally processed via the MHC class-II pathway. This elicits CD4 T-helper (Th) responses that in turn can lead to either a predominantly humoral response resulting in antibody formation or a predominantly cell-mediated response characterized by the activation of macrophages, the expansion of the antigen-reactive T-cell pool and the production of cytokines.

An effective peptide vaccine must be targeted to the appropriate processing pathway to elicit the desired response in the host. This may be achieved by a variety of strategies based on an understanding of the processing of the antigen *in vivo* and known antigenic epitopes. Peptide vaccines may be linked to macromolecular carriers (such as tetanus toxoid), used in combination with lipid micelles or be expressed with specific peptide modifications (such as linking to ubiquitin) that target different processing pathways.

2.3 Potential limitations of the peptide approach

The approach outlined is not without practical and theoretical difficulties. In practice, the choice of epitope to synthesize is restricted to linear epitopes. A major limitation of this approach is that the conformational B-cell epitopes which are involved in the neutralization of, for instance, some viruses and toxins are difficult to mimic by a simple synthetic analogue. One approach that attempts to address this problem is the synthesis of template-assembled synthetic proteins (TASPs), in which assembly of peptides on a suitable "backbone" ensures the formation of the desired three-dimensional structural elements. Another possible problem is that, if immunization is based on a few epitopes, the frequency of antigenic variants that do not cross-react may be relatively high, and the vaccine may serve only to select for a sub-population of the pathogen that evades immune surveillance. There is some evidence for this in vaccinees receiving recombinant hepatitis B surface antigen (HBsAg). Because of the

limited repertoire of Th epitopes available in a vaccine based on a limited number of peptides, the immune response may be MHC restricted, which raises the possibility of a limited and selective response in the target population. However, it may be possible to cover MHC variation in the target population by the use of multiple or promiscuous T epitopes, and to include multiple B-cell epitopes to address antigenic diversity. Antibodies recognizing a peptide immunogen may be readily obtained, but, as with other approaches, this response may be neither sufficient nor appropriate for protection.

Antibodies elicited in response to peptides may have unexpected cross-reactivity to normal tissues, possibly giving rise to an autoimmune reaction. This is a major concern in some areas of immunotherapeutic vaccination, but may arise in more conventional types of vaccine, for instance where an infectious agent has adopted the strategy of host mimicry to evade an immune response.

3. Delivery to the immune system

Small peptides by themselves are poor immunogens, with short physiological lifetimes, and for an efficient immune response it is necessary to take special steps to enhance their stability and delivery to the immune system. These approaches include: the construction of retro-inverso peptides (*4*), where physiological persistence is enhanced by the construction of an analogue of an epitope by synthesizing the inverse sequence using D-amino acids; polymerization of a peptide (for instance by disulfide cross-linking (*5*, *6*)); construction of dendrimers, such as multiple antigen peptides (MAPs) (*7*) and TASPs (*8*); and conjugation to a suitable carrier. One major difficulty with peptides conjugated to a macromolecular carrier is that the immune response can be dominated by immune response to the carrier or, even worse, suppressed by the carrier (usually a protein). Peptides may also be synthesized with lipophilic or glycosidic functional groups that improve delivery and targeting to antigen-presenting cells and may enhance incorporation into vehicles or adjuvants (*9*, *10*). All of these approaches may be used singly or in combination. However, it is clear that one critical factor in the type of immune response elicited is likely to be the choice of adjuvant.

Adjuvants are materials administered at the same time as an antigen to enhance the immune response. Different adjuvant components may serve different purposes (*10*, *11*). Some may act simply as vehicles for the immunogen, allowing it to be more efficiently made available to the antigen-presenting cells, while others may act to

target particular types of antigen-presenting cells through interaction with specific cell receptors, to enhance pinocytosis by the antigen-presenting cells, or as immunomodulators, which may stimulate either a general up-regulation of the immune system or direct the development of immunity into either the cellular or the humoral branch. Until very recently, almost the only kinds of adjuvant in use in vaccines for humans have been the mineral gels aluminium hydroxide and calcium or aluminium phosphate, all of which are excellent at directing a humoral response, but are poor stimulators of cellular immunity. However, other adjuvants are now appearing as components of vaccines that have been licensed or are in advanced stages of clinical trial. Many candidate adjuvants consist of mixtures of components with different activities. Antigens may be incorporated into particulate or liposomal preparations or into emulsions which may contain other components to stabilize and modify the activity, as with immune-stimulating complexes (ISCOMs), which contain cholesterol, phospholipids and saponins. Other proposed adjuvants are based on bacterial cell-wall components (muramyl dipeptide), non-ionic block copolymers, saponins, carbohydrate polymers and polysaccharides, cytokines such as granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-1, -2, -4 and -12 or bacterial (lipid A, proteosomes) or viral components. See reference *12* for a compendium of adjuvants and reference *10* for a tentative classification of their modes of action.

Many new adjuvants raise issues of toxicity and safety that will need to be addressed on an individual basis and in conjunction with the antigens with which they are to be used.

4. Peptides and their synthesis

The synthesis of even a moderate-sized peptide involves a very extended sequence of reactions — several for each amino acid added. Before the α-amino group of one amino acid can be combined with the carboxyl group of another to form a peptide bond, one of the two groups must be "activated" by conversion to a highly reactive derivative. The choice invariably falls on the carboxyl group, and synthesis thus proceeds from the C- to the N-terminus. If the carboxyl group of amino acid A is to be combined with the α-amino group of amino acid B to produce the dipeptide A–B, then the amino function of A and the carboxyl function of B have to be protected by conversion into forms that will allow the formation of only the specific peptide bond between A and B. In addition, amino acid side-chain groups that will react with an activated carboxyl must also be protected. The

side-chain-protecting groups used must be capable of withstanding the conditions used to remove the α-amino-protecting group in order to produce a fresh free amino group for continuation of the peptide elongation. At the end of synthesis, all the protecting groups must be removed to reveal the desired peptide. Peptide synthesis thus relies on a series of reactions in which a fresh peptide bond is created during each cycle. Peptide synthesis is most efficient for the synthesis of peptides of lengths up to 50 residues. From about 50 residues upwards, recombinant techniques, in which a synthetic DNA sequence is expressed in a suitable vehicle, become more attractive, though technical issues (such as the incorporation of non-natural amino acids) may still weigh the choice in one direction or another.

There are two main strategies for synthesis: in the classical solution (fragment-condensation technique) a number of small peptides are synthesized first. These are then purified, deprotected and recombined to form larger peptides, and so on, until the final coupling produces the desired sequence. This technique allows the maximum flexibility in the choice of coupling chemistry and the combination of blocking groups, and also offers the opportunity to characterize and purify intermediate peptides as the synthesis proceeds. On the other hand, large protected peptides are difficult to handle because of problems of solubility, and because reaction products and solvents are difficult to remove. With this approach, each synthesis is an optimized "one-off" design.

In the solid-phase ("Merrifield") method of sequential synthesis, problems of handling are resolved by coupling the C-terminal amino acid to a solid resin bead. The peptide is constructed by stepwise addition of amino acids at the N-terminus. Attachment to a solid support allows reagents to be easily removed at each coupling cycle. At the end of synthesis all the protecting groups are removed (deprotection) and the peptide is cleaved from the resin support in a single- or double-stage reaction. Solid-phase methods are conveniently classified into two main types: tBoc and Fmoc, named after the N-terminal protecting groups that determine the type of chemistry to be used. The former (tBoc) exploits an acid-labile N-terminal and side-chain protecting group, with cleavage and deprotection achieved simultaneously by use of very strong acid (hydrogen fluoride or trifluoromethane sulfonic acid). The Fmoc method employs a base-labile N-terminal protecting group in combination with acid-labile side-chain protecting groups. The side-chain protecting groups can be removed and the peptide simultaneously cleaved from the support under less extreme conditions than with the original tBoc method.

Within this broad framework there are many variations, and the field is continually evolving with the introduction of improved protecting groups, new supports, better cleavage reagents, the exploitation of enzymic methods for peptide-bond formation, and so forth. In the field of vaccine synthesis additional complexity is introduced by techniques in which the peptide is attached to or assembled on a backbone or skeleton designed to provide desirable structural (TASPs) and immunogenic properties (TASPs and MAPs), or where peptides are modified with lipid or carbohydrate groups. Both liquid- and solid-phase methods work well in experienced hands, but solid-phase methods are simpler in conception, have fewer variables, are easily automated and are far more rapid. They are thus well suited to the non-specialist producer of synthetic peptides. They are perhaps not so well adapted to large-scale use, but several small-scale solid-phase syntheses can be carried out in the time taken for one synthesis in solution. In practice, solid-phase methods now heavily predominate, particularly in such areas as epitope mapping, and are likely to provide the method of choice for synthetic peptide vaccines.

5. Characterization and control

In the case of an industrial-scale "classical" fragment condensation, the process of synthesis and purification can easily take more than a year. At the end of the synthesis, the product is inevitably contaminated by a wide range of closely related by-products resulting from side-reactions and partial reactions which may occur at each cycle of synthesis. These by-products vary greatly in relative amount, but it is often possible to demonstrate as many as 100 individual species for a large peptide. For a conventional "pharmaceutical" — a peptide hormone, for instance — the product often needs to be extensively purified to reduce the level of impurities. It is often assumed that the purity requirements for peptides for immunological use are much less demanding, but there is evidence that specific minor impurities may significantly interfere with the immunological properties of a peptide preparation (*13*, *14*).

When synthetic peptides first started to replace peptides of natural origin for therapeutic use, control procedures were very much based on those developed for the natural product: definitive tests relied very heavily on bioassays, and though physicochemical tests were included in specifications, these were a comparatively modest part of the whole. It is only recently that the greatly increased resolving power and quantitative abilities conferred by modern analytical methods — notably high-performance liquid chromatography and

mass spectrometry — have allowed the identification, assay and purity testing of pharmaceutical peptides to be based almost entirely on physicochemical techniques. However, full biological characterization retains a critical role in product development. Synthetic pharmaceutical peptides are well established in therapeutic use, with a history going back almost 30 years. Examples of peptides in everyday therapeutic use include oxytocin, vasopressin and analogues, gonadorelin (gonadoliberin) and its analogues, the calcitonins and corticotropin and its analogue tetracosactrin.

Pharmacologically active peptides are administered either alone (usually by injection) or sometimes incorporated into a formulation designed to delay release. None is covalently linked to a complex polymer. In contrast, a peptide antigen selected for vaccine development may be conjugated to a macromolecular carrier or incorporated into a polymeric structure that may be important for its immunogenic properties, but may also lead to undesirable pharmacological properties of either the peptide or its impurities. Evidence of consistency from batch to batch of the peptide is extremely important and should be based on a wide range of physicochemical analytical techniques designed to address complementary properties of the product. These might, as appropriate, include analytical techniques based on the following: distinctions of charge (electrophoretic techniques such as polyacrylamide gel or capillary electrophoresis; cation- or anion-exchange chromatography and isoelectric focusing and chromatofocusing); distinctions of size (size-exclusion chromatography, SDS-polyacrylamide gel electrophoresis — both reducing and non-reducing); distinctions of hydrophobicity (reversed-phase or hydrophobic-interaction chromatography); or distinctions of mass (mass spectrometry based on a range of ionization methods). Also included may be analytical techniques based on determination of composition (amino-acid analysis), sequence (sequential degradation from the N-terminal (Edman degradation) or C-terminal (chemical or enzymic degradation)), mass spectrometry, and peptide mapping following enzyme or chemical (e.g. cyanogen bromide) digestion.

In a similar fashion, it is important to demonstrate the consistency of successive stages of manufacture, as the peptide is incorporated into or combined with other components. The techniques chosen should be appropriate for demonstrating consistency of stoichiometry of combination, and should, as above, be as widely based as possible. In addition to the suggestions mentioned above, additional techniques based on other physicochemical techniques or characteristics (e.g. ultraviolet spectroscopy, circular dichroism, fluorescence spectroscopy, light scattering) may be appropriate, as well as immunologically

based techniques. Information gained from clinical use may also be relevant.

More detailed information relating to the development and use of synthetic peptides as vaccines can be obtained elsewhere (*15–29*).

6. Developmental overview

Because of the range of preparations that can make up a peptide vaccine, it is not possible to give detailed recommendations covering all examples. However, adequate control of the starting materials and manufacturing process is just as important as control of the final product. Considerable emphasis should therefore be put on "in-process" controls, as well as on the comprehensive characterization of the vaccine itself, for ensuring the safety and efficacy of the vaccine. Appropriate attention therefore needs to be given to the quality of all reagents used in production. Although these guidelines set out points which should be considered when developing synthetic peptide vaccines, not all points will be appropriate to all vaccines. Individual vaccines may present particular control problems. The production and quality control of each vaccine must therefore be given careful individual consideration so that any special features can be taken into account. Furthermore, the application of these guidelines to a particular product should reflect its intended clinical use.

A full description of the development of the vaccine should be provided to the national control authority, including the identification of the antigenic determinants, the reasons for choosing these particular determinants, and the rationale behind such aspects as choice of carrier, combination of epitopes, choice of conjugate, form of presentation of the peptides and choice of adjuvant, together with any evidence bearing on these choices.

The different elements of the vaccine should be rigorously characterized, both individually and in combination, to ensure that the final product is safe, effective and of consistently reproducible quality. A wide range of physicochemical techniques designed to address complementary properties of the product should be used. The techniques should provide evidence of correct sequence and purity, together with evidence for the correct and quantitative incorporation of modifications such as glycosylation or lipidation. In addition, a range of immunological techniques should be used to evaluate the antigenic and biological properties of the product and its components, including immunogenicity, antigenicity, specificity and biological potency. Techniques will need to be selected on a case-by-case basis.

Because of the complexity of the possible antigenic constructs and the analytical problems involved in characterizing polymers and conjugates, it is important that a reference preparation of the antigen be established. Such preparations should be representative of, or prepared from, batches of material used for clinical studies. It may be necessary to prepare different reference preparations appropriate to separate stages of manufacture.

7. Control of production

7.1 Peptide synthesis and characterization

The epitopes within each peptide sequence should be described and identified. There should be a description of the source of each epitope and, where there is more than one epitope, the reasons for the sequence, combination, and type of epitope (B, T, etc.) should be specified.

A full description of the synthesis of the peptide and in-process controls should be given. The description should include sources and specifications of starting materials, methodology used, coupling and deprotection conditions, criteria for proceeding to the next step of synthesis, and details of any groups (glycosyl or lipid, for instance) incorporated. If synthesis is performed in solution, a flow chart and specifications of the intermediate peptides should be supplied.

Where a peptide monomer is an intermediate, it should be isolated and characterized. Evidence should be provided to confirm that:

- the principal peptide sequence is the intended structure, i.e. that the correct amino acids have been added in the intended order and that the correct number and type of modifications (e.g. incorporation of lipid or glycosyl moieties) have been carried out;
- major and, where possible, minor impurities in the peptide monomer have been identified and characterized. Techniques used to demonstrate purity should be based on as wide a range of physicochemical properties as possible. It may be necessary to test for materials added during the production or purification processes;
- the peptide is of consistent quality over a number of consecutive batches.

A suitable specification for the monomeric peptide or peptide derivative should be established. This may need to be defined, in part, in terms of a suitably characterized reference preparation (see above).

Limits should be set for peptide purity, for individual major and minor impurities and for total impurities. If full characterization of the peptide monomer is not possible, a detailed explanation should be given. Finally, studies should be carried out to assess the stability of the peptide for proposed periods of storage.

7.2 Conjugates

In addition to recommendations regarding the peptide and other components of the conjugate (see section 7.3), conjugated product should meet the following criteria:

- Proof of conjugation should be established, and the level of conjugation should be consistent and reproducible. Where more than one peptide is conjugated to the same carrier, the proportions of each should be reproducible.
- Side reactions (e.g. cross-linking of carrier) should be minimized, as should the surface concentration of non-productive coupled linker on the carrier and the presence of linker not involved in coupling. Related parameters should be reproducible.
- Evidence should be obtained that conjugation does not alter the antigenic sequences.
- Levels of residual reagents and by-products should be minimized (defined limits should be set).

In addition, the following issues are important:

- A suitable acceptance specification for the conjugated product should be set. A suitable reference preparation may be necessary.
- Potential clinical effects of pre-existing or induced immunity to the carrier protein should be considered.
- Specific epitope suppression should be investigated when a carrier is used for several vaccines or for several different epitopes of one vaccine.

7.3 Carriers

Peptides may be conjugated to or incorporated into a carrier. Care should be taken in choosing a carrier, since an immune response to the carrier may dominate response to the peptide. Any carrier to which pre-existing hypersensitivity in the target population is likely should be avoided, as should carriers that involve the risk of stimulating autoimmunity.

Carriers should have a complete specification and dossier of background information. This information should include characterization, evidence of consistency of production, safety-testing data and other information appropriate to the protein, polysaccharide, polymer, liposome etc., as the case may be. For instance, polymers and polysaccharides should have a reproducible molecular-weight distribution, polysaccharides should have consistent monosaccharide composition, and liposomes a consistent dimension and composition. Components and impurities should be characterized and quantified. For a carrier of biological origin, measures should be undertaken to ensure the absence of infectious agents.

7.4 Polymerized, cyclized or carrier-synthesized peptides

Where a peptide is synthesized on a polymeric backbone (such as in MAP synthesis) or allowed to polymerize or cyclize following synthesis (for instance, through oxidation of terminal cysteine residues), either alone or in a copolymerization with other peptides, evidence should be obtained to ensure that the reaction proceeds to a reproducible endpoint. In particular, the following should be ensured:

- The distribution of relative molecular mass of the product is consistent from batch to batch, so that, for a cyclized product, it is shown to be predominantly and consistently the cyclic monomer (for instance) and polymeric forms are shown to be within the limits of specification; for a polymer, that the level of monomer is within specified limits, and the pattern of polymerization is defined (for example, in terms of molecular-mass distribution and heterogeneity (M_z/M_w, M_w/M_n, etc., where "M" stands for relative molecular mass and "Z", "W" and "N" refer to specific polymers), or in terms of limits set for the proportions of specific polymeric species).
- Where appropriate, the relative proportions of different peptides are consistent.
- The nature of the product(s) is well defined by means of an appropriate range of complementary analytical techniques, which might include techniques exploiting differences in charge, molecular size, hydrophobicity, mass, or spectral or immunological properties (see p. 32).
- Levels of reagents used in polymerization, cyclization or synthesis, together with unwanted by-products, are within the limits that have been set.

Where successive production stages result in polymers of increasing complexity (as, for instance, in MAP synthesis followed by disulfide

dimerization of the product), it may be appropriate to apply the above-mentioned criteria after each production stage.

A suitable acceptance specification for the oligomeric product should be set and a reference preparation established (see pp. 39–40).

7.5 Adjuvants, vehicles and excipients

A wide range of different materials may in principle be added to an immunogen in order to improve handling, stability and persistence and to modify the degree and type of immunogenicity. Such materials vary widely in their characteristics and range from small molecules to bacterial-cell components and synthetic polymers. They may include components acting as general immunostimulants, components selectively activating different parts of the immune system, and components interacting directly with the different cells involved in the immune process. Many adjuvants can present questions of toxicity, and, because of the way in which they can modify the immune process, raise safety concerns when combined with an antigen. Traditional adjuvants have been based on mineral gels (aluminium hydroxide, aluminium or calcium phosphate); few others have so far been licensed.

Established adjuvants based on aluminium or calcium salts should be of the requisite pharmacopoeial grade (i.e. free of heavy metal ions and of consistent quality and binding characteristics). Since operations such as autoclaving may alter an adjuvant's binding characteristics, it is important that batches of adjuvant should be handled in the same well defined manner. If aluminium or calcium compounds are used as adjuvants, their concentrations should not exceed the customary limits of 1.25 mg of aluminium and 1.3 mg of calcium per single human dose.

For additives, a full description of all components should be provided, together with a detailed rationale for their inclusion. Each component should have an appropriate analytical specification, preferably on the basis of a drug master file, and there should be a specification for the mixture of components in the final formulation. In addition, there should be a full assessment of the antigenic and toxic properties of the final formulation, with attention given to the possibility that antigenic properties may be adversely modified through the combination of components.

7.6 Preservatives

If a preservative is added, the preservative content should be determined and the amount used should be shown not to have any delete-

rious effect on individual vaccine components, nor cause any unexpected adverse reactions in humans. Preservatives should not be added to single-dose preparations.

8. Formulated final product

After combination of the antigen with adjuvant and any cofactors, additional characterization should be carried out as indicated below.

8.1 Interaction with adjuvant

The degree and manner in which the different vaccine components interact with the adjuvant should be defined, and reproducible behaviour from batch to batch demonstrated. For instance, the degree to which the different species in a polymeric preparation bind to the adjuvant may depend on molecular mass, hydrophobicity or pH, and the preparation may therefore effectively become fractionated during formulation. Alternatively, components of the adjuvant may associate as micelles, and the different vaccine components may be incorporated to different extents. Desorption of vaccine components from the adjuvant may take place over time, and specifications should be set to ensure consistency.

8.2 Stability

Adequate stability studies form an essential part of vaccine development. The results are used to set a maximum shelf life under appropriate storage conditions. For each component for which a claim of efficacy is made, real-time stability studies should be undertaken to support the immunogenicity and lack of toxicity of the product up to its expiry date. Accelerated stability studies may provide preliminary supporting evidence for the stability of the product, but cannot replace real-time studies for the purpose of licensing.

Guidance on stability studies can be found in the International Conference on Harmonisation's (ICH) guidelines on stability testing.[1]

8.3 Potency

The potency assay need not necessarily reflect the functional activity of the vaccine or its mechanism of action in humans. However, a

[1] *Quality of biotechnological products: stability testing of biotechnological biological products.* Geneva, International Conference on Harmonisation, 1995 (available from ICH Secretariat, International Federation of Pharmaceutical Manufacturers Associations, 30 rue de St-Jean, 1211 Geneva 18, Switzerland).

suitable test of vaccine function should be included where appropriate. The main purpose of the potency assay is to demonstrate batch-to-batch consistency by methods which depend in some measure on biological activity. A suitable *in vitro* or *in vivo* assay for immunogenicity or antigenicity should therefore be considered. Such an assay should compare the preparation with a reference material, and the results should be statistically validated. Since the mode of action of vaccines may vary, details of such assays must be determined on a case-by-case basis. However, confidence limits should be calculated for the mean value of any batch in order to take account of inherent variation in the bioassay. Acceptance criteria should be defined on this basis. A suitably formulated reference preparation should be established for this purpose.

9. Routine control

Routine testing is that which is carried out on every batch, both of the component products and of the final formulation. For each stage of production, intermediate products should be subject to an acceptance specification, and a suitable range of tests carried out to ensure compliance. Such tests are usually a subset of those carried out for developmental characterization. They should test aspects of the intermediate and final product that are critical to safety, efficacy and quality.

The product specification should also include tests to ensure that the final dosage form complies with the usual safety tests, such as pyrogenicity and sterility, appropriate to a parenteral preparation, together with tests of identity, antigen content and general innocuity.

Consistency of production is essential. Demonstration of consistency of production should include the results of tests on at least three satisfactory, consecutive production batches of a size corresponding to that intended for routine manufacture. The data obtained from such studies may be used as the basis for the final vaccine specification.

The final product specification should also include tests to ensure that the content of any preservatives is within specified limits.

10. Reference materials

The studies described in sections 7 and 8 will contribute to a definitive specification for the vaccine.

It may be necessary to establish appropriate reference materials. In addition, a suitable batch of the final formulated vaccine, preferably one that has been clinically evaluated, together with relevant intermediate products, should be fully characterized in terms of its chemical composition, purity and biological activity and retained for use as a chemical and biological reference material. This material should be used as the basis for defining the specifications for production batches.

11. Preclinical safety evaluation

The general aim of preclinical safety evaluation is to determine whether new medicinal products have the potential to cause unexpected and undesired effects. Clinical safety or toxicological testing as recommended for chemical drugs may, however, be of only limited relevance for synthetic peptide vaccines. Toxicity testing in animals poses particular problems, such as those due to species specificity, and the safety evaluation of peptide vaccines will have to take a large number of factors into account.

For these reasons, it is likely that a flexible approach will be necessary for the preclinical safety evaluation of synthetic peptide vaccines. Account should be taken of any possibility of adverse immunopathological reactions arising from the use of a synthetic peptide vaccine.

It is important to check that the peptide sequences used possess no significant unwanted pharmacological activity. The peptide monomer should therefore be screened for intrinsic toxic or pharmacological activity. The potentiation of any unwanted pharmacological activity through conjugation or polymerization should also be considered (*16*). A peptide may well have unanticipated but minor pharmacological effects that could be significantly and harmfully magnified by conjugation or polymerization. The pharmacological activity of both peptide and conjugate should therefore be examined.

Antibodies elicited in response to peptides may have unexpected cross-reactivity with human tissues and elicit an autoimmune reaction. Any undue cross-reactivity of antibodies (induced with the final vaccine formulation) with intrinsic human antigens should be assessed using a panel of human tissues. Conjugation and polymerization can create epitopes that would not be addressed by evaluation of only the peptide monomer.

Although safety testing will be required, the range of tests that need to be carried out should be decided on a case-by-case basis, and in

consultation with the relevant national control authority. For preclinical safety evaluation, a wide range of biological, biochemical, immunological, toxicological and histopathological techniques should be used as appropriate, covering a relevant range of doses and including both acute and chronic exposure.

Authors

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Annex 2
Requirements for tick-borne encephalitis vaccine (inactivated)
(Requirements for Biological Substances No. 51)

Introduction

Tick-borne encephalitis (TBE) is an acute viral illness caused by two closely related viruses of the family *Flaviviridae*: the central European encephalitis (CEE) virus, found in many European countries, and the Russian spring–summer encephalitis (RSSE) virus, found predominantly in the Asian parts of the former Soviet Union. These viruses, which are endemic to forested areas, are transmitted by ticks. In addition to humans, they infect small mammals and, to a lesser extent, birds (*1*, *2*). In Asia, the disease is characterized by abrupt onset of fever, severe headache, nausea and vomiting and severe back pain often associated with focal epilepsy and flaccid paralysis, especially of

the shoulder girdle. Such paralysis may be permanent. The central European form of the disease has a longer course, often with biphasic fever, but severe sequelae are less frequent. The initial febrile stage is normally not associated with disease of the central nervous system, but the second phase, following approximately 4–10 days after apparent recovery, is characterized by fever and meningoencephalitis. The case-fatality rate is approximately 20% for the Asian form of the disease and 1–5% for the European form.

TBE is endemic to most European countries, the Russian Federation and possibly China. It is the most important arthropod-transmitted viral disease in Europe, and in some countries it represents a major public-health problem. The disease has been known by several names, including RSSE, Far Eastern encephalitis and CEE.

CEE virus is found in every European country, with the exception of Belgium, Luxembourg, the Netherlands, Portugal, Spain and the United Kingdom, and is transmitted primarily by the tick *Ixodes ricinus*. RSSE virus is most prevalent in the eastern part of the former Soviet Union and is transmitted by the tick *Ixodes persulcatus* (*3*).

Flaviviruses are a large group of small, enveloped viruses responsible for a number of severe human diseases, including yellow fever, Japanese encephalitis, dengue haemorrhagic fever and TBE. TBE virus particles are roughly spherical in shape, 40–50 nm in diameter, and contain a core, 20–30 nm in diameter. The genome consists of single-stranded positive-sense RNA with a relative molecular mass of about 4×10^6. Three structural proteins and the capsid, membrane and envelope proteins are all encoded by the viral genome (*4*). The envelope glycoprotein induces neutralizing and haemagglutination-inhibition antibodies and is the most important antigen for providing protection from disease.

The first vaccine against TBE was prepared in 1941 in the brains of mice (*5*). Some 20 years later TBE vaccines derived from cell cultures (chicken embryo fibroblast cells) were developed and used for active immunization in humans in the former Soviet Union (*6–8*). Later, a purified, inactivated virus vaccine was developed which proved to be more immunogenic than previous TBE vaccines (*9*, *10*).

The efficacy of these vaccines has been well documented (*11*, *12*). They have also been shown to protect mice from a lethal challenge with several TBE-virus isolates obtained over a period of more than 30 years from all over Europe and the Asian part of the former Soviet Union. In addition, it has been demonstrated that antibodies induced by vaccination of human volunteers neutralized all tested isolates (*13*).

Large outbreaks of TBE, sometimes involving thousands of cases, continue to occur in endemic areas. In addition, certain special groups, such as forest workers, geologists, travellers to endemic areas and laboratory workers, are also at risk. In view of the need to immunize large numbers of people, requirements for inactivated TBE vaccine have been formulated. In drafting these requirements, account has been taken of the regulations and requirements for the manufacture and control of TBE vaccines that have already been established in several countries.

General considerations

Inactivated TBE vaccines for human use are prepared from harvested virus propagated in appropriate cells. Harvest is followed by inactivation, purification and formulation. Inactivation is one of the critical production steps, and the inactivation process should be carefully monitored.

These requirements are scientific and advisory in nature. The parts of each section printed in normal type have been written in the form of requirements so that, should a national control authority so desire, they may be adopted as they stand as national requirements. If changes are made, they should be validated and approved by the national control authority. The parts of each section printed in small type are comments or recommendations for guidance.

To facilitate the international distribution of vaccine made in accordance with these requirements, a summary protocol is available (*14*). The summary protocol indicates the kind of information that should be included for viral vaccines in a routine batch-release document submitted to a national control laboratory, as well as that to be included in a release certificate provided by the national control authority.

Part A. Manufacturing requirements

A.1 Definitions

A.1.1 *International name and proper name*

The international name shall be "*Vaccinum encephalitidis ixodicae advectae inactivatum*". The proper name shall be the equivalent of the international name in the language of the country of origin.

The use of the international name should be limited to vaccines that satisfy the requirements formulated below.

A.1.2 *Descriptive definition*

Tick-borne encephalitis vaccine (inactivated) is a fluid or freeze-dried preparation of a suitable strain of TBE virus grown in cell cultures and inactivated by an appropriate and validated method. The preparation shall satisfy all the requirements formulated below.

A.1.3 *International reference materials*

An International Reference Reagent for Anti-tick-borne Encephalitis Serum (Russian spring–summer encephalitis (Sophyn and Absettarov) virus) is available on request from the National Institute of Biological Standards and Control, Potters Bar, England.

A.1.4 *Terminology*

The following definitions are given for the purposes of these requirements only.

Master cell bank. A quantity of adequately characterized cells stored in liquid nitrogen or the vapour phase of liquid nitrogen in aliquots of uniform composition, one or more of which may be used for the production of a manufacturer's working cell bank.

Manufacturer's working cell bank. A quantity of cells of uniform composition derived from one or more aliquots of the master cell bank. A master cell bank is expanded by serial subculture up to a passage number (or population doubling, as appropriate) selected by the manufacturer, at which point the cells are combined to yield a single pool, distributed into containers and preserved cryogenically to form the manufacturer's working cell bank. All containers are treated identically and, once removed from storage, are not returned to the stock. One or more of the containers from such a working cell bank may be used for inoculating the continuous-cell-line production culture.

Continuous-cell-line production cell culture. A cell culture, derived from one or more containers of the manufacturer's working cell bank, suspended in an appropriate culture medium and used for the production of TBE virus.

Chicken-fibroblast production cell culture. A number of cell cultures derived from a pool of cells and processed together.

Adventitious agents. Microorganisms contaminating the cell substrate or materials used in its culture, including bacteria, fungi, mycoplasmas and endogenous and exogenous viruses.

Master seed lot. A quantity of virus that has been prepared as a single lot and has a uniform composition. It is used for the preparation of working seed lots.

Working seed lot. A quantity of virus of uniform composition derived from the master seed lot by a method and at a passage level approved by the national control authority. The working seed lot is used to inoculate production cell cultures.

Single harvest. A quantity of virus suspension harvested from production cell cultures inoculated with the same working seed lot and incubated and harvested together in a single production run. Multiple harvests from the same production cell culture may be pooled and considered a single harvest.

Bulk material. A purified, inactivated single harvest or a pool of inactivated single harvests that have been purified at the same time.

Final bulk. The finished homogeneous material prepared from one or more batches of bulk material present in the vessel from which the final containers are filled.

Final lot. A collection of sealed final containers of vaccine that are homogeneous with respect to the risk of contamination during the preparation process, including filling and, where applicable, freeze-drying. All the final containers in a final lot must, therefore, have been filled from one container of final bulk in a single working session and freeze-dried together under standardized conditions in one chamber.

A.2 General manufacturing requirements

The general manufacturing requirements contained in Good Manufacturing Practices for Pharmaceutical (*15*) and Biological (*16*) Products shall apply to establishments manufacturing TBE vaccine with the following addition: the staff involved in the production and quality control of TBE vaccine shall be shown to be immune to TBE.

A.3 Source materials

A.3.1 *Cell cultures for virus propagation*

A.3.1.1 Cells used for providing production cell cultures

If chicken fibroblast cells are used for the manufacture of vaccine they shall meet the general requirements set out in Requirements for the Use of Animal Cells as *in vitro* Substrates for the Production of Biologicals (*17*). The tissues of chicken embryos used in the manufacture of vaccine shall be obtained from a healthy flock. Monitoring of the flock or embryos shall include tests for exclusion of infection by at least *Salmonella*, *Mycobacterium avium* and fowl pox virus. If the

flock has to be vaccinated against Newcastle disease virus, then inactivated Newcastle disease virus vaccine must be used.

> Manufacturers are strongly encouraged to use only tissues from closed, specific-pathogen-free flocks of chickens, in particular those free from avian leukosis virus.

If continuous cell lines are used for the propagation of TBE virus, they shall be derived from a master cell bank and approved by and registered with the national control authority. They shall also meet the requirements for continuous-cell-line substrates set out in Requirements for the Use of Animal Cells as *in vitro* Substrates for the Production of Biologicals (*17*).

β-Lactam antibiotics shall not be used at any stage of manufacture.

> Minimal concentrations of suitable antibiotics such as kanamycin may be used when approved by the national control authority.

The cells in any cell bank shall have been characterized with respect to their genealogy, growth characteristics and viability during storage, and shall have been shown to be free from detectable adventitious agents (*17*).

A.3.1.2 Cell-culture medium

Serum used for the propagation of cells for TBE vaccine production shall demonstrate freedom from bacteria, fungi and mycoplasmas as specified in Part A, section 5.2, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (*18*) and the revised Sterility Test for Mycoplasmas (General Requirements for the Sterility of Biological Substances, amendment 1995) (*19*) and shall demonstrate freedom from viruses. Serum of bovine origin should comply with guidance given in Requirements for the Use of Animal Cells as *in vitro* Substrates for the Production of Biologicals (*17*) and *Report of a WHO Consultation on Medicinal and Other Products in Relation to Human and Animal Transmissible Spongiform Encephalopathies* (*20*) and shall be approved by the national control authority.

Suitable tests for detecting viruses in bovine serum are found in Appendix 1 of the revised Requirements for Biological Substances No. 7 (Requirements for Poliomyelitis Vaccine (Oral)) (*21*). Where appropriate, more sensitive methods may be used.

> In some countries, sera are examined for freedom from certain phages.
>
> In other countries, irradiation is used to inactivate potential viruses.

Trypsin. Trypsin used for preparing cell cultures shall be bacteriologically sterile and free from mycoplasmas and viruses, in particular porcine parvoviruses. If bovine trypsin is used, it shall comply with

guidance given in *Report of a WHO Consultation on Medicinal and Other Products in Relation to Human and Animal Transmissible Spongiform Encephalopathies* (*20*) and in Requirements for the Use of Animal Cells as *in vitro* Substrates for the Production of Biologicals (*17*). It shall be approved by the national control authority.

Human albumin. If human albumin is used in the cell-culture medium, it shall meet the requirements set out in Parts C and D of the revised Requirements for Biological Substances No. 27 (Requirements for the Collection, Processing and Quality Control of Blood, Blood Components and Plasma Derivatives) (*22*), as well as the guidance given in *Report of a WHO Consultation on Medicinal and Other Products in Relation to Human and Animal Transmissible Spongiform Encephalopathies* (*20*).

A.3.2 *Virus seed*

A.3.2.1 Virus strain

The virus strain used in the production of virus seed lots shall be approved by the national control authority and shall yield safe and immunogenic vaccines when the virus has been inactivated. Each strain shall be identified by historical records that include information on virus origin, infectivity tests, serological tests and animal responses to inoculation.

A.3.2.2 Seed lot system

The preparation of TBE vaccine shall be based on the use of a master and working seed lot system. The national control authority shall determine the maximum acceptable number of passages in mice between the master seed lot and any working seed lot. Vaccine shall be made from a working seed lot without additional passages in mice. Virus seed lots shall be maintained either in dried or in frozen form. The dried seed shall be kept at a temperature below –20 °C, and the frozen seed at a temperature below –60 °C.

Virus seed lots shall have been shown to the satisfaction of the national control authority to be capable of yielding vaccine that meets all the present requirements.

> Mice used for production of master seed lots should ideally be specific-pathogen-free. In addition, suckling mice are preferred.
>
> In some countries the national control authority distributes the master virus seed to manufacturers.

A.3.2.3 Tests on master and working seed lots

Identity test. Each virus seed lot shall be identified as TBE virus by an appropriate method.

Suitable serological methods include enzyme immunoassay or a TBE-virus-neutralization assay using a reference serum or a monoclonal antibody known to neutralize TBE virus. Sera should be characterized using the International Reference Reagent of Anti-tick-borne Encephalitis Serum.

Freedom from bacteria, fungi and mycoplasmas. Each virus seed lot shall be tested for bacterial, mycotic and mycoplasmal contamination by appropriate tests as specified in Part A, section 5.2, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (*18*, pp. 49–52) and the revised Sterility Test for Mycoplasmas (General Requirements for the Sterility of Biological Substances, amendment 1995) (*19*).

Tests for adventitious viruses. Each virus seed lot shall be tested for adventitious viruses. For the tests, the virus shall be neutralized by a specific anti-tick-borne-encephalitis-virus serum, which shall itself have been shown to be free from adventitious viruses.

The individual tests on the virus seed lots should be designed so that they satisfy the requirements of the national control authority.

A.4 Production control

A.4.1 *Cell cultures*

At least 5% or 500 ml but no more than 1000 ml of the cell suspension employed to prepare the production cell cultures shall be used to prepare control cultures of uninfected cells.

The treatment of control and production cell cultures shall be similar. The control cultures shall be maintained for at least 2 weeks or until the time of final viral harvest from the production cultures if this is longer.

At the time of virus harvest, control cell cultures shall be examined for degeneration caused by adventitious viruses. In addition, samples of fluid collected from control cell cultures shall be pooled and tested for the presence of adventitious viruses as described below in sections A.4.1.2 and A.4.1.3. Samples not tested immediately shall be stored at –60 °C or below.

If multiple harvests are made from the same production cell culture, the samples of fluid collected from control cell cultures at the time of each harvest shall be frozen and stored at –60 °C or below until the last harvest has been completed. The samples shall then be pooled in proportion to the respective amounts of each harvest and submitted to the required tests.

If any test shows evidence of the presence of any adventitious viruses in a control cell culture, the TBE virus grown in the corresponding production cultures shall not be used for vaccine production.

For a test to be valid, no more than 20% of the control vessels shall have been discarded for non-specific reasons by the end of the test period.

In some countries, the national control authority may permit reduced testing for adventitious viruses where the manufacturer has demonstrated that:

- all materials of animal origin used in the cell culture process, the manufacturer's working cell bank and the virus seed lots are free from detectable adventitious viruses;
- at the end of the cell culture process, the cell culture system is free from bacterial, fungal and mycoplasmal contamination as specified in Part A, section 5.2, of the revised Requirements for Biological Substances No. 6 (General Requirements for Sterility of Biological Substances) (*18*) and the revised Sterility Test for Mycoplasmas (General Requirements for the Sterility of Biological Substances, amendment 1995) (*19*);
- the manufacturing process is consistent and has been validated to remove or inactivate a panel of representative adventitious agents.

A.4.1.1 Identity test

If a continuous cell line is used in TBE vaccine production, an identity test shall be performed on the control cell cultures by a method approved by the national control authority.

Methods for identity testing include, but are not limited to, biochemical (e.g. isoenzyme analysis), immunological (e.g. histocompatibility antigen assays), cytogenic (e.g. chromosomal) marker and genetic marker (DNA-fingerprinting) tests.

If continuous cell lines are used in TBE vaccine production the cells shall be documented as being derived from the characterized manufacturer's working cell bank.

A.4.1.2 Test for haemadsorbing adventitious viruses

At the end of the observation period, an amount equivalent to 25% of the control cell-culture suspension shall be tested for the presence of haemadsorbing viruses. If the erythrocytes have been stored, the duration of storage shall not have exceeded 7 days, and the temperature of storage shall have been in the range of 2–8 °C.

Some national control authorities require production cell cultures to be tested for the presence of haemadsorbing viruses at the end of the incubation period(s) as described in section A.4.1.3. If so, the test on control cell cultures described here may not be required.

The test has usually been performed using guinea-pig erythrocytes. In some countries, the national control authority requires that alternative or additional tests for haemadsorbing viruses are made on control cultures between 3–5 days and 12 days after inoculation of the production cultures, and that other types of erythrocytes, including those from humans (blood group O), monkeys and chickens (or other avian species), should be used. In all tests, readings should be taken after incubation for 30 minutes at 0–4 °C, and again after a further incubation for 30 minutes at 20–25 °C. For

the test using monkey erythrocytes, readings should also be taken after a final incubation for 30 minutes at 34–37 °C.

For a test to be valid, no more than 20% of the control culture vessels shall have been discarded for non-specific reasons by the end of the test period.

A.4.1.3 Tests for non-haemadsorbing adventitious viruses

At 14 days after the day of inoculation of the production cultures or at the time of final virus harvest, a sample of cell-culture fluid shall be taken from each control cell culture and pooled. A sample of 10 ml of the pool shall be tested in the same cell culture, but not the same batch, as that used for the production cell culture. Additional 10-ml samples of the pool shall be tested in both human and monkey cells.

Each sample shall be inoculated into bottles of cell culture in such a way that the dilution of the pooled fluid in the nutrient medium does not exceed 1:4. The area of the cell sheet shall be at least 3 cm^2 per ml of pooled fluid. At least one bottle of the cell culture shall remain uninoculated as a control.

The inoculated cultures shall be incubated at a temperature of 35–37 °C for a period of at least 14 days, after which the cells shall be examined for abnormal morphology.

The tests are satisfactory if no cytopathic changes attributable to adventitious viruses are detected.

Some national control authorities require that, at the end of the observation period, a subculture is made in the same culture system and observed for at least 7 days. Furthermore, some national control authorities require that the subcultures are tested for the presence of haemadsorbing viruses.

For the tests to be valid, no more than 20% of the control culture vessels shall have been discarded for non-specific reasons by the end of the test.

A.4.2 ***Control of single virus harvests***

A.4.2.1 Cells used for vaccine production

On the day of inoculation with the seed virus, each production cell culture and control cell culture shall be examined for degeneration caused by infective agents. If such examination shows evidence of the presence in a cell culture of any adventitious agent, the whole group of cultures concerned shall not be used for vaccine production.

After virus inoculation, cell cultures for vaccine production shall be incubated under controlled temperature conditions approved by the national control authority.

If animal serum is used in the growth medium for cell cultures, the serum shall be removed from the cell cultures either before or after inoculation with seed virus. Before the virus is harvested, the cell cultures shall be rinsed and the growth medium replaced with serum-free maintenance medium.

A.4.2.2 Sterility tests

A sample removed from each single harvest shall be tested for bacterial, fungal and mycoplasmal contamination by appropriate tests as specified in Part A, section 5.2, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (*18*) and in the revised Sterility Test for Mycoplasmas (General Requirements for the Sterility of Biological Substances, amendment 1995) (*19*). Any single virus harvest in which contamination is detected shall be discarded.

A.4.2.3 Virus content

A sample removed from each single harvest shall be tested for virus content by intracerebral inoculation of mice or by plaque assay.

> Experience suggests that a virus content of not less than $10^{7.0}$ LD_{50}/ml (median lethal dose for mice per ml) results in satisfactory vaccine.

If a plaque assay is used, a laboratory shall establish a correlation between the plaque assay and the mouse-LD_{50} assay.

A.4.2.4 Inactivation of virus

The virus in each single harvest shall be inactivated by a validated method. The kinetics of inactivation shall be suitably monitored and demonstrated by the manufacturer to be consistently effective. Each harvest shall be processed to remove virus aggregates immediately before inactivation.

The method and agents used for inactivation shall be approved by the national control authority.

> In some countries, the virus is inactivated by the addition of formaldehyde to a final concentration of 0.05% followed by continuous stirring for 5 days at a temperature of 22 °C.

A.4.2.5 Test for effective inactivation

Each single harvest shall be tested for virus inactivation. The test shall be performed prior to pooling with a sample of undiluted virus suspension by a method approved by the national control authority.

> The test sample should correspond to at least 20 human doses of the final bulk. The total volume of the test sample should be inoculated into a primary chicken fibroblast cell culture, or a culture of any other cells with no less susceptibility to the virus than chicken embryo cells, and incubated at a temperature of 37 ± 1 °C for a period of 14 days. A cell culture sheet of

not less than 3 cm^2 should be used for each 1 ml of the test material. No cytopathic change should be detected at the end of the incubation period.

At the completion of the observation period, the culture fluid should be collected and inoculated intracerebrally at a dose of 0.03 ml into at least 10 mice of about 4 weeks of age. The animals should be observed for 14 days. The single harvest passes the test if the product has been shown to be free from residual live virus.

Other methods to assess inactivation may be approved by the national control authority.

A.4.3 *Preparation and control of purified bulk material*

A.4.3.1 Pooling of inactivated single harvests

Only harvests satisfying the requirements of section A.4.2 shall be pooled.

In some countries the pooled material is retested for inactivation of virus content (see section A.4.2.4). If so, the test should be approved by the national control authority.

A.4.3.2 Purification procedures

Each pool of inactivated virus shall be purified by suitable methods approved by the national control authority.

The inactivated TBE virus can be purified and concentrated by continuous-flow zonal ultracentrifugation or by ultrafiltration.

Each manufacturer shall demonstrate, by testing of each lot, or by validation of the purification process, that all residual contaminants are consistently reduced to a level acceptable to the national control authority.

The protein content per human dose and the minimum purity shall be approved by the national control authority. The upper limit for residual DNA for vaccine produced in continuous cells is 10 ng/dose (*17*).

Serum albumin is an appropriate marker substance which can be monitored, though other components may be more appropriate depending on the specific manufacturing process employed.

A.4.3.3 Potency test

A potency test shall be carried out on each batch of purified bulk material. The method used and specification set shall be approved by the national control authority.

A potency test in mice may be used for this purpose. Groups of 10 mice are vaccinated subcutaneously with 0.2 ml of serial dilutions of TBE vaccine containing 0.2% aluminium hydroxide.

Animals are revaccinated 14 or 21 days later. They are challenged 7 or 14 days following revaccination by intraperitoneal inoculation of 100–1000 mouse LD_{50} of a suitable TBE virus strain. The median mouse protective dose is calculated after observation for 20 days. In some countries different immunization schedules are used.

A.4.3.4 Test for avian leukosis virus

If production is based on chicken fibroblast cells obtained from chicken flocks which are not demonstrated to be free from avian leukosis virus, each batch of bulk material shall be tested for infectious avian leukosis virus by a method approved by the national control authority.

A.4.4 ***Preparation and control of the final bulk***

A.4.4.1 Preservatives and other added substances

In the preparation of the final bulk only preservatives or other substances approved by the national control authority shall be added. Such substances shall have been shown by appropriate tests not to impair the safety or effectiveness of the product in the amounts used.

No antibiotics shall be added to TBE vaccine intended for human use after the virus has been harvested.

A.4.4.2 Addition of adjuvant

If the final bulk contains adjuvant, the adjuvant and its concentration shall be approved by the national control authority.

If aluminium compounds are used, the concentration of aluminium shall not exceed 1.25 mg per single human dose.

A.4.4.3 Test for completeness of adsorption to adjuvant

If an aluminium compound is used as adjuvant, tests shall be carried out to confirm that TBE antigen is adsorbed to the adjuvant. The tests and acceptance limits shall be approved by the national control authority. Consistency of adsorption is important, and the degree of adsorption of production lots shall be within the specifications of lots shown to be clinically effective.

A.4.4.4 Sterility tests

Each final bulk shall be tested for bacterial and fungal contamination as specified in Part A, section 5.2, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (*18*) or by a method approved by the national control authority.

A.4.4.5 Potency test

A potency test may be performed on the formulated final bulk instead of on the purified bulk material (section A.4.3.3), if approved by the national control authority. The acceptance limits for potency of the vaccine and the assay method used shall be based on evidence submit-

ted to prove efficacy in clinical trials and shall be approved by the national control authority.

The test shall be performed in parallel with a test on a local reference vaccine. The challenge strain, the reference vaccine, and the test procedure used shall be approved by the national control authority.

A potency test in mice may be used for this purpose. Groups of 10 mice are vaccinated subcutaneously with 0.2ml of serial dilutions of TBE vaccine containing 0.2% aluminium hydroxide.

Animals are revaccinated 14 or 21 days later. They are challenged 7 or 14 days following revaccination by intraperitoneal inoculation of 100–1000 mouse LD_{50} of a suitable TBE virus strain. The median mouse protective dose is calculated after observation for 20 days. In some countries different immunization schedules are used.

A.4.4.6 Tests for chemicals used in production

The concentration in the final vaccine of any organic solvent or inactivating agent added during production shall be determined by methods approved by the national control authority. The concentration shall not exceed the upper limits specified by the national control authority.

If formaldehyde has been used for inactivation, the concentration in the final bulk shall be no greater than 0.02%. The test method used shall be approved by the national control authority.

A.5 Filling and containers

The requirements concerning filling and containers given in Good Manufacturing Practices for Biological Products (*16*) shall apply.

A.6 Control tests on final lot

The national control authority may permit tests for pyrogenic substances, protein content, preservatives and adjuvants to be performed on the final bulk rather than on the final lot.

A.6.1 *Identity*

An identity test shall be performed by an appropriate method to identify the virus in at least one labelled container from each final lot.

The potency test described in section A.6.2 may serve as an identity test.

A.6.2 *Potency*

A potency test on the vaccine in the final containers shall be performed if it has not already been performed on the formulated final bulk (see section A.4.4.5).

A.6.3 *Sterility*

Each final lot shall be tested for sterility as specified in Part A, sections 5.1 and 5.2, of the revised General Requirements for the Sterility of Biological Substances (*18*).

A.6.4 *General safety*

Each final lot shall be tested for abnormal toxicity. The test procedures shall be approved by the national control authority.

A.6.5 *Pyrogenic substances*

Each final lot shall be tested for pyrogenic substances. The test shall be approved by the national control authority.

A.6.6 *Preservatives*

Each final lot shall be tested for the presence of preservatives, if used. The test used and the permitted concentrations shall be approved by the national control authority.

A.6.7 *Protein content*

Limits shall be defined for the protein content of the finished product. Each final lot shall be tested for protein content by a method approved by the national control authority.

A.6.8 *Adjuvant content*

Each final lot shall be assayed for the content of adjuvant. The method used and the permitted concentration shall be approved by the national control authority.

The formulation shall be such that a homogeneous suspension is administered to the recipient.

A.6.9 *Residual moisture in freeze-dried vaccine*

The residual moisture in a representative sample of each freeze-dried lot shall be determined by a method approved by the national control authority.

Moisture levels of less than 3% are usually considered satisfactory.

A.6.10 *Inspection of final containers*

Every container in each final lot shall be inspected, and those showing any abnormalities shall be discarded.

A.7 Records

The requirements in section 8 of Good Manufacturing Practices for Biological Products (*16*) shall apply.

A.8 Samples

The requirements in section 9 of Good Manufacturing Practices for Biological Products (*16*) shall apply.

A.9 Labelling

The requirements in section 7 of Good Manufacturing Practices for Biological Products (*16*) shall apply, with the addition of the following:

The leaflet accompanying the package shall:

- specify the cell substrate(s) used for production;
- state the name of the virus strain used;
- state the method used for inactivating the virus;
- specify the nature and amount of adjuvant and preservative present, if used;
- if the vaccine is in freeze-dried form, state that, after its reconstitution, it shall be used immediately (unless data have been provided to the licensing authority that it may be stored for a limited time without loss of potency).

A.10 Distribution and shipping

The requirements in section 8 of Good Manufacturing Practices for Biological Products (*16*) shall apply.

A.11 Storage and expiry date

The requirements in section 8 of Good Manufacturing Practices for Biological Products (*16*) shall apply.

A.11.1 *Storage conditions*

Inactivated TBE vaccine in liquid form shall be stored at all times at a temperature of 2–8 °C, without freezing.

Inactivated TBE vaccine in freeze-dried form shall be stored below 10 °C.

> Alternative storage temperatures must be justified to and approved by the national control authority.

A.11.2 *Expiry date*

The expiry date shall be based on data submitted by the manufacturer. Adequate stability studies form an essential part of vaccine development studies. The stability of the vaccine in its final form, stored at the recommended storage temperatures, shall be demonstrated to the satisfaction of the national control authority

with final containers from at least three consecutive lots of final product.

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

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

Part B. National control requirements

B.1 General

The general requirements for control laboratories contained in the Guidelines for National Authorities on Quality Assurance for Biological Products (*23*) shall apply, with the following additions:

- The national control authority shall approve the virus strain and the cell substrate used in vaccine production, and shall specify potency requirements.
- The national control authority shall be satisfied that the results of all tests, including those done on pools during the manufacturing process, are satisfactory and that consistency has been established.

B.2 Release and certification

A vaccine lot shall be released only if it fulfils the national requirements and/or part A of these requirements.

A certificate signed by the responsible official of the national control authority shall be provided at the request of the manufacturing establishment and shall certify whether the lot of vaccine in question meets all national requirements and/or Part A of the present requirements. The certificate shall state the date of the last satisfactory potency test by the manufacturer and the final lot number, which must also appear on the labels of the containers.

> The purpose of the certificate is to facilitate the exchange of TBE vaccine between countries. An example of a suitable certificate is given in the forty-sixth report of the Expert Committee on Biological Standardization (*14*).

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WHO Technical Report Series, No. 889, 1999

Annex 3
Guidelines for thromboplastins and plasma used to control oral anticoagulant therapy[1]

Introduction

Oral anticoagulant drugs derived from coumarin (and sometimes from indandiones) are widely used in the treatment and prophylaxis of thrombotic disorders. Coumarin drugs inhibit the biosynthesis of vitamin-K-dependent coagulation factors by the liver. For each patient, the dose of these drugs must be adjusted periodically to ensure that an adequate, but not excessive, degree of anticoagulation is achieved. The adjustments are made on the basis of the results of the prothrombin-time or a similar test on the patient's blood. The test, which requires reagents called thromboplastins, is controlled by the use of calibrated thromboplastins and plasmas.

Various types of thromboplastin are prepared commercially and, in order to be able to interpret the results of the prothrombin-time test, it is essential that each reagent is correctly calibrated. This will ensure that the results of tests with different products and batches are reproducible and can be compared. A procedure for the calibration of thromboplastins using a logarithmic plot of prothrombin times has been developed (*1*) and was described in the thirty-third report of the WHO Expert Committee on Biological Standardization (*2*). With this procedure, the definition of a calibration parameter called the International Sensitivity Index (ISI) became feasible. It is possible to express prothrombin-time results on a common scale, i.e. the International Normalized Ratio (INR), if the ISI of the thromboplastin used is known.

There is now substantial evidence that coagulometers can have unpredictable and marked effects on the ISI of thromboplastins (*3–6*). Because of these effects, some manufacturers provide a "system ISI" for a particular thromboplastin/coagulometer combination. However, this procedure appears to have limitations since variations in the

[1] These guidelines replace Requirements for Thromboplastins and Plasma Used to Control Oral Anticoagulant Therapy (Requirements for Biological Substances No. 30), which are discontinued (see WHO Technical Report Series, No. 889, 1999:7–8).

system ISI with the same reagent and coagulometer at different centres have been demonstrated in collaborative studies (*7*, *8*).

In general, the calibration of a given thromboplastin is more precise if performed against an international reference preparation[1] of similar composition and from the same species (*9–11*). A system of coexisting international reference preparations has been established in which each of these materials is related to the first primary international reference preparation, the first International Reference Preparation of Thromboplastin (Human, Combined), coded 67/40 (see Fig. 1, p. 69). Three international reference preparations of thromboplastin are currently available from the relevant WHO International Laboratory for Biological Standards: the second International Reference Preparation of Thromboplastin, Bovine, Combined (coded OBT/79) (*9*); the third International Reference Reagent for Thromboplastin, Rabbit, Plain (coded RBT/90) (*11*); and the third International Standard for Thromboplastin, Human, Recombinant, Plain (coded rTF/95) (*12*). Other international reference preparations have been discontinued. The development of these preparations is described in section 2 (pp. 68–70).

In theory, the ISI/INR system should ensure that the ISI value calculated for a given reagent is independent of the species from which the international reference preparation is derived, because all have been directly or indirectly calibrated against the first International Reference Preparation of Thromboplastin, Human, Combined (coded 67/40). However, this is not always the case; several recent observations have demonstrated that reagents calibrated against the second International Reference Preparation of Thromboplastin, Human, Plain, a material coded BCT/253 (the predecessor of rTF/95) (*13*), provide lower INR values than those calibrated against RBT/79 (the predecessor of RBT/90) or OBT/79 (*9*, *11*, *14*). The extent of these differences in INR is not usually large enough to cause serious concerns from a practical point of view. The discrepancy is due to minor calibration errors that persist because the different international reference preparations were not checked against each other in the original studies. A new procedure has now been agreed upon: international thromboplastin reference preparations of whatsoever origin

[1] International reference materials established by the WHO Expert Committee on Biological Standardization have been denoted, variously, as International Reference Preparations, International Reference Reagents and International Standards. These guidelines refer to all thromboplastin reference materials established by the WHO Expert Committee, independent of the nomenclature. International reference materials so established are by definition "primary" reference preparations, secondary reference preparations being calibrated in relation to them.

and composition will be calibrated against all existing international reference preparations in order to ensure consistency of results between different routes of calibration (*15*).

It is recommended that the international reference preparation of the same species or composition should be used for calibration of secondary standards, e.g. working standards, by manufacturers and national reference laboratories. Thus, plain rabbit thromboplastins should be calibrated against RBT/90; plain human thromboplastins against the human recombinant material rTF/95; and bovine or combined thromboplastins (of whatever species) against OBT/79.

The calibration of prothrombin-time systems is not an easy task. Furthermore, there is considerable variation in results from different laboratories performing the same procedures, as shown by published multicentre calibration studies (*9–13*, *16*, *17*). In these studies, interlaboratory variation in ISI, expressed as a coefficient of variation, ranged from approximately 1.7% to 8.1%. The purpose of these guidelines, which replace the requirements published in the thirty-third report of the WHO Expert Committee on Biological Standardization (*2*), now discontinued, is to take account of the above-mentioned observations and to describe in detail the technical methods currently in use. These guidelines represent the state of the art. Modifications to the methodology may give comparable results, but must be validated against the methodology described in the guidelines.

1. Definitions

Tissue factor. An integral transmembrane protein functioning as a cofactor enhancing the proteolytic activity of factor VIIa towards factor X and factor IX in the blood. Tissue factor needs to be associated with coagulant phospholipids for the full expression of its cofactor function.

Thromboplastin. A reagent containing tissue factor and coagulant phospholipids. Many commercial thromboplastins are crude extracts prepared from mammalian tissues, in which tissue factor is only a minor component on a weight basis, and which also contain phospholipids. A preparation of a thromboplastin consisting of a tissue extract alone, either with or without added calcium chloride, is termed "plain". When the preparation contains adsorbed bovine plasma as a source of additional factor V and fibrinogen it is termed "combined". Thromboplastins may also be grouped into types, according to the

tissue source from which they are derived, e.g. human, bovine, rabbit brain or lung, or human placenta. The tissue-factor component of recombinant human thromboplastin reagents is produced in *Escherichia coli* or insect cells by recombinant DNA techniques and then lipidated *in vitro*.

Prothrombin time (PT) (tissue-factor-induced coagulation time). The clotting time of a plasma (or whole blood) sample in the presence of a preparation of thromboplastin and the appropriate amount of calcium ions. The time is reported in seconds (*18*).

Prothrombin-time system. A procedure by which the prothrombin time is determined using a specific thromboplastin reagent and a particular method, which may be manual, e.g. a tilt-tube method, or involve the use of an instrument that records the coagulation endpoint automatically. The method should be described and the description should include all procedures and equipment used, e.g. the pipettes and test-tubes.

Mean normal prothrombin time (MNPT). The geometric mean of the prothrombin times of the healthy adult population. For practical purposes, the geometric mean of the prothrombin time calculated from at least 20 fresh samples from healthy individuals, including those of both sexes, is a reliable approximation of MNPT. It is not necessary to collect and test all the individual samples in one session. It is recommended that each laboratory should determine MNPT using its own prothrombin-time system. Pooled normal plasma (either deep-frozen or freeze-dried) may be suitable if the clotting time obtained is related to the MNPT value and its storage stability is acceptable (see p. 78).

Prothrombin-time ratio (tissue-factor-induced coagulation relative time). The prothrombin time obtained with a test plasma or whole blood divided by the MNPT, all times having been determined using the same prothrombin-time system.

International Sensitivity Index (ISI). A quantitative measure, in terms of the first International Reference Preparation of Thromboplastin, Human, Combined, coded 67/40, of the responsiveness of a prothrombin-time system to the defect induced by oral anticoagulants (see Appendix 1, p. 86).

International Normalized Ratio (INR). For a given plasma or whole blood specimen from a patient on long-term oral anticoagulant therapy, a value calculated from the prothrombin-time ratio using a prothrombin-time system with a known ISI according to the formula $INR = (PT/MNPT)^{ISI}$.

2. International reference preparations of thromboplastins

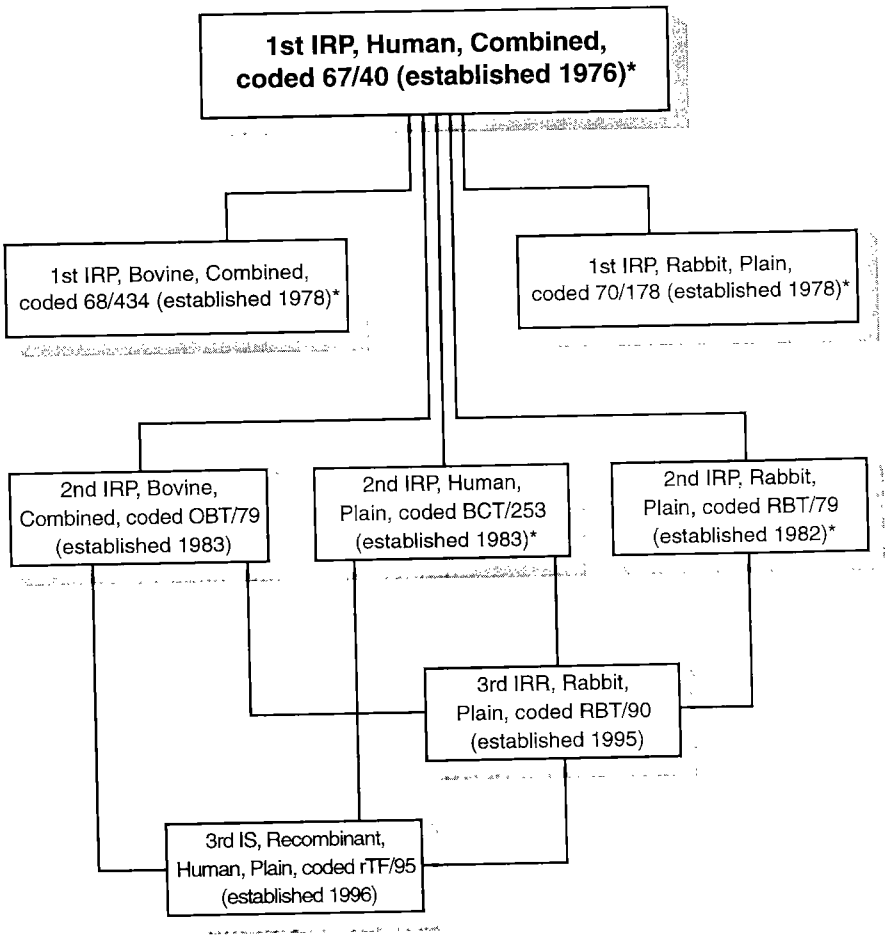
International Reference Preparations, International Standards and International Reference Reagents are intended to serve throughout the world as sources of defined biological activity quantitatively expressed in International Units or in terms of a suitable property or characteristic defining the biological activity. These preparations are used to calibrate secondary standards, which include regional, national and manufacturers' working standards. Normally, working standards are used for routine calibration of individual batches of thromboplastin, and working standards should have been calibrated with the appropriate international reference preparation. If secondary standards are developed using procedures that involve multiple calibration steps, there is a risk that unnecessary variability and discontinuity will occur in relation to the primary international reference preparation because of cumulative serial calibration errors.

Current prothrombin-time systems are based on the use of three different species of thromboplastin reagents: human, bovine and rabbit. The standardization of these thromboplastin reagents likewise involves three different reference preparations, one for each of the three species of plain thromboplastin reagents in use (Fig. 1).

The first International Reference Preparation of Thromboplastin, Human, Combined (coded 67/40), was established by the WHO Expert Committee on Biological Standardization in 1976 (*19*). It was a freeze-dried preparation, filled in sealed glass ampoules, and contained a human brain extract to which adsorbed bovine plasma had been added to optimize the content of non-vitamin-K-dependent coagulation factors (i.e. factor V and fibrinogen). Its ISI value was set at 1.0 by definition. In 1983, this preparation was discontinued and replaced by the second International Reference Preparation of Thromboplastin, Human, Plain (coded BCT/253), a human brain extract with no added coagulation factors and an assigned ISI value of 1.1 (*20*). When stocks of BCT/253 became exhausted, a new preparation of human recombinant thromboplastin (coded rTF/95) was established in 1996 as the third International Standard for Thromboplastin, Human, Recombinant, Plain, with an assigned ISI value of 0.94 (*12*, *21*).

The first International Reference Preparation of Thromboplastin, Bovine, Combined (coded 68/434) was established by the WHO Expert Committee on Biological Standardization in 1978 (*22*). It was calibrated using the first International Reference Preparation of Thromboplastin, Human, Combined (67/40). Another material, also

Figure 1
International reference preparations for thromboplastins and their calibration relationships



IRP = International Reference Preparation.
IRR = International Reference Reagent.
IS = International Standard.
* Now discontinued.

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calibrated against 67/40, was established as the second International Reference Preparation of Thromboplastin, Bovine, Combined (coded OBT/79), in 1983 with an assigned ISI of 1.0 (*23*), in accordance with the revised Requirements for Thromboplastins and Plasma used to Control Oral Anticoagulant Therapy (*2*). This material (OBT/79), which was derived from bovine brain and combined with factor V and fibrinogen, should be used to calibrate thrombo-

plastin materials of bovine origin and combined thromboplastins of whatever origin.

Finally, for the calibration of thromboplastins of rabbit origin, a first International Reference Preparation of Thromboplastin, Rabbit, Plain (coded 70/178), was established in 1978. This material was calibrated against the first International Reference Preparation of Thromboplastin, Human, Combined (coded 67/40), in an international collaborative study which also included bovine thromboplastin, combined (*22*). When stocks of 70/178 became exhausted, the second International Reference Preparation of Thromboplastin, Rabbit, Plain (coded RBT/79), was established in 1982 with an ISI value of 1.4; this was also calibrated against 67/40 (*24*). The third International Reference Reagent for Thromboplastin, Rabbit, Plain (coded RBT/90), obtained from rabbit brain with no added factors, was calibrated against each of the three species of thromboplastins and established by the WHO Expert Committee on Biological Standardization in 1995 with an ISI of 1.0 (*25*).

The wide use of these international reference preparations for calibrating secondary standards reflects the value placed on them by the scientific community responsible for the control of thromboplastins. An independent control of a manufacturer's ISI assignments by a national reference laboratory is also recommended. National control authorities should consider designating an expert laboratory in the country for testing thromboplastin reagents and plasmas used by clinical laboratories to control oral anticoagulant therapy to ensure that they are in accordance with guidelines published by WHO.

The international reference materials for thromboplastins are in the custody of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam. Samples of these materials are distributed free of charge, on request, to national reference laboratories or national control laboratories for biological products and, with small handling charges, to other organizations such as manufacturers, universities, research institutes and hospital laboratories. The principle that international reference preparations are distributed free of charge to national control authorities for the purpose of the calibration of national standards has been adhered to since the establishment of international biological standardization activities (*26*).

3. Preparation of thromboplastins

The method of preparation of thromboplastins should be such that there is consistency from batch to batch and that the preparations are

suitable for use in the control of oral anticoagulant treatment. The thromboplastins shall comply with the specifications outlined in section 4, below.

All attempts should be made to use the least contaminated source material possible and to use a manufacturing procedure that prevents further contamination and the growth of organisms during manufacture. Thromboplastins of animal origin shall be prepared only from healthy animals. Thromboplastins prepared from bovine brain shall be derived only from cattle from countries that have not reported indigenous cases of bovine spongiform encephalopathy (BSE) and which have a compulsory BSE notification system, compulsory clinical and laboratory verification of suspected cases and a surveillance programme in place (*27*).

Human brain tissue should not be used because of the risk of transmission of Creutzfeldt-Jakob disease. Thromboplastins derived from human placenta shall be prepared from donors in whom there is no evidence of systemic microbiological infection or localized infection and who have been shown to be free from hepatitis B surface antigen, antibodies to human immunodeficiency viruses (HIV-1 and HIV-2) and antibodies to hepatitis C virus.

4. Tests on thromboplastins

Each batch of thromboplastin shall satisfy the following criteria.

4.1 Response to coumarin-induced coagulation defect

The response to the coumarin-induced coagulation defect shall be measured by the prothrombin time obtained using normal and coumarin plasmas. Thromboplastins with a manual ISI between 0.9 and 1.7 are acceptable. However, ISIs towards the lower end of this scale are desirable, since some studies have shown that interlaboratory variation in ISI is greater for high than for low ISI systems (*17*). It has been suggested that the INR is less accurate when prothrombin time is determined with insensitive thromboplastins that have high ISI values (*28*).

4.2 Content of haemoglobin and serum

To prevent contamination of the product with (activated) clotting factors, the thromboplastin preparation should be free from serum and show no detectable haemoglobin.

4.3 Opacity and sediment volume

The method of manufacture, particularly the method of breaking up the tissue, has a marked effect on the activity, opacity and sediment

volume of the thromboplastin. The opacity of preparations intended for use in photoelectric instruments should be suitably low.

4.4 Containers

International reference preparations for thromboplastins are freeze-dried in sealed glass ampoules (*29*), but secondary standards may be freeze dried in ampoules or vials.

4.5 Stability

The method of manufacture shall be such that the thromboplastin preparations are stable. All reagents eventually lose activity when stored at elevated temperatures, and stability shall be checked by an accelerated degradation test. When a thromboplastin reagent is exposed to accelerated degradation at 37 °C, the initial change in the prothrombin time of normal plasma should not exceed 0.05% per day (*30*). With regard to the response to the effect of coumarin, the reagent when stored at 37 °C should maintain activity as measured by the prothrombin-time ratio of coumarin plasma for 6 months (*31*).

Accelerated degradation studies are considered to be only a useful rather than an absolute guide to the stability of thromboplastins maintained at the storage temperatures recommended by the manufacturer. Therefore, the stability of the thromboplastins must also be determined for the conditions under which they are stored, i.e. in a real-time stability study (*32*, *33*).

5. Calibration of prothrombin-time systems

Four types of calibration should be distinguished:

(a) calibration of international reference preparations,
(b) calibration of secondary standards, e.g. national reference preparations and manufacturers' working standards;
(c) calibration of manufacturers' commercial preparations against the corresponding working standard ("lot-to-lot" calibration);
(d) local-system calibration.

In general, the results of calibrations are used by laboratories other than the calibrating laboratories. The clinical laboratories should therefore be aware of the interlaboratory variation in ISI values for the thromboplastin reagent. The possibility of correcting for local-thromboplastin/instrument-combination effects (i.e. prothrombin-time system) by means of type (d) calibration is currently under study (*34*, *35*). Type (d) calibration involves the use of freeze-dried plasmas

with assigned INR or prothrombin-time values which are not described in these guidelines. Type (a) and (b) calibrations should be carried out with a large number of fresh plasma or whole blood samples. Several studies suggest that, under certain circumstances, fresh plasmas for type (c) calibrations can be reliably replaced by frozen, freeze-dried, pooled plasma or plasmas artificially depleted of vitamin-K-dependent coagulation factors (*36–38*). Manufacturers should validate this procedure by means of fresh plasmas.

Prothrombin-time systems shall be calibrated in terms of the appropriate international reference preparation of thromboplastin, and the response to the coumarin-induced coagulation defect shall be defined by the ISI obtained in the calibration procedure. Supplies of international reference preparations are limited, and it is not possible to use these materials in routine tests to calibrate each batch of the many thromboplastins produced by different manufacturers. Calibration of individual batches of thromboplastin shall be carried out by comparison with a secondary standard, which shall be a batch of the same or a similar thromboplastin calibrated against the appropriate international reference preparation.

The basis of the thromboplastin calibration model is necessarily an empirical one. While there is good evidence that the calibration relationship defined in a double-logarithmic plot of prothrombin times is usually linear, and that the same line represents data points for both patients and healthy subjects, the possibility of departure from these assumptions cannot be ruled out. Statistical methods to test deviations from the latter assumption have been described (*39*, *40*). In the case of marked deviation, the assignment of an ISI would not be meaningful. For practical purposes, the assignment of an ISI is acceptable if INRs calculated with the ISI derived from the overall regression line (i.e. for patients plus healthy subjects) do not differ by more than 10%, in the INR range 2–4.5, from INRs calculated with the equation describing the regression line for patients only (see Appendix 1, p. 86).

5.1 The calibration of international reference preparations

The calibration of the international reference preparations for thromboplastins, and their future replacements, should be carried out in international multicentre collaborative studies using fresh coumarin, normal plasma and manual techniques. Each collaborative study for replacement of an international reference preparation should include the testing of all existing international reference preparations. The ISI assigned to the replacement material should be the mean of the

ISIs obtained by calibration with all existing international reference preparations (*15*).

5.2 The calibration of secondary standards

Secondary standards of human origin should be calibrated against the current International Standard, i.e. the third International Standard for Thromboplastin, Human, Recombinant, Plain (coded rTF/95); plain thromboplastins of rabbit brain and rabbit lung should be calibrated against the third International Reference Reagent for Thromboplastin, Rabbit, Plain (coded RBT/90); and thromboplastins of bovine origin should be calibrated against the second International Reference Preparation of Thromboplastin, Bovine, Combined (coded OBT/79). Thromboplastins of rabbit brain combined with adsorbed bovine plasma should also be calibrated against OBT/79.

In view of the interlaboratory variation observed in multicentre calibration studies, it is recommended that calibration of national reference materials or manufacturers' working standards should be performed by at least two laboratories.

5.3 The calibration of individual batches of thromboplastins

The precision of calibration is greatest when similar materials and methods are compared. For this reason, a national reference preparation or manufacturer's working standard used for the calibration of individual batches of thromboplastin should be a thromboplastin of similar characteristics to these batches (i.e. derived from the same tissue of the same species, using a similar manufacturing process). Batch-to-batch calibration should be performed by the manufacturer before release of the reagent and consistency of ISI values should be shown.

6. The calibration procedure

The calibration procedure entails the determination of a series of prothrombin times, using normal and abnormal plasmas or whole blood samples, with both the reference and the test thromboplastin. The tests are performed using either fresh samples from individual subjects (procedure 1) or freeze-dried or frozen plasmas (procedure 2). Abnormal plasmas for procedure 1 are obtained from patients undergoing long-term oral anticoagulant treatment. Freeze-dried or frozen plasmas for procedure 2 may be pooled plasmas from healthy subjects and from patients undergoing long-term anticoagulant treatment.

Procedure 1 is recommended for the calibration of secondary standards against the appropriate international reference preparation and for the calibration of whole-blood coagulometers. Procedure 1 can also be used for the calibration of individual batches of thromboplastin against the corresponding secondary standard (i.e. lot-to-lot calibration), but may be replaced by procedure 2 if the same results are obtained.

The precision of the calibration relationship depends on the number of plasmas and on a balanced distribution of normal and abnormal plasmas over the "therapeutic" range of INR values. The recommended number of abnormal plasmas is three times the number of normal plasmas.

6.1 Procedure 1. Calibration of a secondary standard using individual fresh plasma or blood samples

This procedure consists of a set of tests using freshly opened or reconstituted thromboplastins and different individual samples of fresh plasma or whole blood. The procedure should be repeated on at least five separate occasions using fresh reagents on each occasion (see section 6.1.4, p. 77). The procedure need not be repeated on consecutive days but should be completed as soon as possible. The tests in any one laboratory on any one day should be performed by the same person.

6.1.1 *Blood samples*

Blood samples from healthy subjects and patients who have been on oral anticoagulants for at least 6 weeks should be selected. Samples from patients treated with heparin should not be used. It is recommended that patients' samples with INR values in the range 1.5–4.5 should be selected.

Blood should be obtained by venepuncture, avoiding haemolysis and contamination with tissue fluids. It should be drawn either with a plastic syringe and transferred to a plastic tube, or with other non-contact activation equipment. Nine volumes of blood should be decalcified with one volume of 109 mmol/l trisodium citrate solution (*18*). A mixture of trisodium citrate and citric acid is also acceptable if the total citrate plus citric acid concentration is 109 mmol/l and the pH is no lower than 5. The same procedure and materials should be used for all the samples in a given calibration.

If evacuated tubes are used for blood collection, their lot number should be noted, as there may be lot-to-lot variation. If evacuated tubes are made of glass, they must be properly siliconized internally

and the pH of the trisodium citrate plus citric acid solution must be in the range 5–6 (*41*). The sample should be centrifuged as soon as received but no later than 2 hours after blood collection. The centrifugation should be such that the plasma is rendered poor in platelets (i.e. at least 2500 *g* for 10 minutes at a controlled room temperature). The plasma should be taken off the red-cell layer with a plastic pipette, stored undisturbed in a narrow, stoppered, non-contact tube at room temperature and tested within 5 hours after blood collection.

Some techniques or instruments require the use of non-citrated capillary blood (*42*). Capillary blood can be obtained by finger or heel puncture. The capillary blood should be obtained without squeezing and tested immediately with the technique or instrument to be calibrated. Venous blood should be obtained from the same subjects (healthy subjects and patients) within 5 minutes of taking the capillary sample, for preparation of citrated plasma as described above and testing with the most appropriate international reference preparation.

6.1.2 *Reference thromboplastins*

The appropriate international reference preparation of thromboplastin (human, rabbit or bovine) should be reconstituted as instructed and the contents of the ampoules transferred to a container in sufficient volume for all tests to be performed in a single calibration session. Specific instructions for use should be supplied by the custodian of these materials.

6.1.3 *The prothrombin-time test*

The prothrombin-time test is performed either by mixing equal volumes of citrated plasma, thromboplastin and calcium chloride solution (25 mmol/l), or by adding a volume of plasma to the required volume of thromboplastin premixed with calcium, and therefore available as a single reagent. The time (in seconds) taken for the mixture to clot when maintained at a temperature of between 36.5 °C and 37.5 °C is recorded. Test instructions for commercial thromboplastins shall be provided by the manufacturers.

The coagulation end-point for international reference preparations of thromboplastin should be detected by a manual (tilt-tube) technique. The coagulation end-point for other thromboplastins should be detected by a manual technique or with the aid of an automatic end-point recorder. The same technique should be used throughout the series of tests with a given thromboplastin.

Each laboratory should have a system for internal quality control. Records should be maintained of the lot number of all reagents and

disposable equipment used. Periodic checks of the temperature of incubation baths or heating blocks and of the volumes of pipettes or pumps should be made and recorded.

A suggested protocol for reporting the procedure is shown in Appendix 1 (p. 86).

6.1.4 *Statistical evaluation*

Samples with INRs outside the 1.5–4.5 range shall be excluded. An orthogonal regression line should be calculated on the basis of the natural logarithm[1] of the prothrombin time (PT) of 20 healthy subjects plus 60 patients stabilized on long-term anticoagulant therapy. LnPT for the international reference preparation is plotted on the vertical axis and lnPT for the working reference material on the horizontal. Orthogonal regression analysis provides an estimate of the standard deviation of data points about the regression line. Any samples with a perpendicular distance greater than 3 residual standard deviations from the regression line should be excluded. After removal of such samples, the final orthogonal regression line should be calculated. The suggested procedure for calculation of the ISI is given in Appendix 1.

To define the ISI of the working reference material, a sufficient number of separate tests should be carried out to obtain a within-laboratory coefficient of variation for the slope of the orthogonal regression line of 3% or less.

6.2 Procedure 2. Calibration of individual batches of thromboplastin

Calibration of individual batches of thromboplastin may be carried out with pooled normal plasmas and pooled coumarin plasmas or plasmas artificially depleted of vitamin-K-dependent coagulation factors (*36*, *37*). The number of plasma pools required for precise calibration is, in general, much smaller than the number of fresh individual plasma samples required for procedure 1. The scatter of data points about the regression line is relatively small because the batch to be calibrated is very similar to the working reference preparation and/or because the biological variation caused by individual samples is reduced by the pooling of plasmas. It has been reported that lot-to-lot calibration of bovine and rabbit thromboplastins could be performed with as few as three plasma pools (*36*, *37*), but the accuracy of such a simplified procedure may depend on the quality of the pooled

[1] The abbreviation "ln" is used henceforth for the natural, also known as the Napierian, logarithm.

plasmas and the thromboplastin being calibrated. It is recommended that any procedure using pooled or artificially depleted plasmas be validated against the fresh plasma procedure (procedure 1).

6.2.1 *Pooled plasma*

Properties of pooled normal plasma

Plasma should be obtained from healthy adults and should comply with the appropriate section of Requirements for the Collection, Processing and Quality Control of Blood, Blood Components and Plasma Derivatives (*43*). The normal plasmas for pooling should be obtained from at least 20 different donors. Nine volumes of blood should be decalcified with one volume of 109 mmol/l trisodium citrate solution. The packed-cell volume-fraction should be between 0.35 and 0.45.

The final preparation should be platelet-poor plasma, which has been freeze-dried or frozen (at –40 °C or below) in suitable containers. The stability of deep-frozen plasma should be monitored regularly by testing the prothrombin time. After reconstitution or thawing, the pH should not be lower than 7.3 and should not exceed 7.9, and the plasma should not show any shortening or prolongation of clotting times for at least 2 hours when held at ambient temperature (*44*). The stability of freeze-dried normal plasma should be checked by accelerated degradation tests. Such plasma should not show a prolongation of prothrombin time of over 5% after storage for 4 weeks at 37 °C. The factor V content should be between 60% and 140% of the average content of fresh normal plasma (*45*).

Properties of pooled coumarin plasma

Pooled coumarin plasma is obtained from patients who have been on oral anticoagulant therapy for at least 6 weeks. Coumarin plasmas for pooling should be obtained from at least 20 different donors.

Plasma should not be obtained from donors with a history of jaundice or from those with plasma-lipid abnormalities. The collection of plasma, the properties of the final preparation and the stability of the freeze-dried pools are the same as for pooled normal plasma described above.

The INR of the pooled plasma should be stated, as should the thromboplastins used for its assignment. It should be noted that the INR value of a freeze-dried plasma usually depends on the thromboplastin used for its assignment (*46–48*). At least two different plasma pools, having an INR between 1.5 and 4.5 and with a difference of at least 1.0 in their INRs, in combination with one normal plasma pool are necessary for the calibration procedure.

The factor V content, opacity and citrate concentration for blood decalcification should comply with the requirements for normal plasma (see above).

Freedom from infectious agents

The plasma should be shown to be free from hepatitis B surface antigen, antibodies to human immunodeficiency viruses (HIV-1 and HIV-2) and antibodies to hepatitis C virus.

6.2.2 *The test*

The test should be carried out by the same procedure as described for procedure 1 (see section 6.1.3, p. 76). An example of the protocol for the recording of the results is given in Appendix 2 (p. 92). The procedure should be repeated on at least four separate occasions (*38*), with fresh reagents used on each occasion. At least three plasma pools should be used to permit the testing of linearity.

Freeze-dried plasma pools should be reconstituted at least 15 minutes before the actual test. Plasma that has been frozen and subsequently thawed, or reconstituted freeze-dried plasma, should not be centrifuged, and unused reconstituted or thawed material should be discarded after 2 hours.

6.2.3 *Statistical evaluation*

An orthogonal regression line should be calculated on the basis of the ln PT value of the pooled plasmas. Individual determinations shall be entered when multiple determinations for each plasma pool are available. Ln PT for the working reference thromboplastin system is plotted on the vertical axis and ln PT for the test batch of thromboplastin on the horizontal. Any samples with a perpendicular distance greater than 3 residual standard deviations from the regression line should be removed. After removal of such samples, the final orthogonal regression line is calculated.

To define the ISI of a batch of thromboplastin, a sufficient number of tests should be carried out to obtain a within-laboratory coefficient of variation for the slope of the orthogonal regression line of 3% or less. The recommended procedure for calculation of the ISI is given in Appendix 2, p. 92.

7. The use of calibrated thromboplastins in clinical practice

It is possible to express prothrombin-time results on a common scale, i.e. the International Normalized Ratio (INR), provided that the ISI

of the thromboplastin and the method used are known. The following formula is used:

$$INR = (PT/MNPT)^{ISI},$$

where PT is the patient's prothrombin time and MNPT is the mean normal prothrombin time determined with the same thromboplastin and method. The use of the INR enables comparisons to be made between results obtained using different thromboplastins and methods. It is a misconception, however, that for an individual patient's plasma the INR will always be identical with different thromboplastins and methods (*40*, *49*). Different thromboplastins vary greatly in their responsiveness to individual vitamin-K-dependent clotting factors, i.e. factors II, VII and X, as well as to some non-vitamin-K-dependent factors, e.g. factor V. Discrepancies between INRs determined with different thromboplastins arising from these biological variations and from additional technical errors are therefore not unexpected.

All medical staff and health auxiliaries involved in controlling oral anticoagulant treatment should be encouraged to use the INR system. It should be appreciated, however, that this system can be accurate only in the INR range explored by the calibration procedure, i.e. 1.5–4.5.

Manufacturers of commercial reagents should state on the package insert the ISI of the relevant batch of thromboplastin together with the reference preparation against which it has been determined and the instrument for which it is valid.

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Appendix 1

Example of the use of the suggested method for reporting the data for the calibration of a secondary standard of thromboplastin against an international reference preparation

Thromboplastins:
1. Rabbit brain thromboplastin secondary standard
2. Third International Reference Reagent for Thromboplastin, Rabbit, Plain (RBT/90)

End-point recording:
1. Automated photoelectric coagulometer for secondary standard
2. Manual (tilt-tube) technique for RBT/90

The tests were conducted on 5 different days. On each day, fresh samples from 4 healthy subjects and 12 patients were tested (plasma samples from healthy subjects are referred to as "normal"). On each day, different subjects were selected. The automated coagulometer and manual determinations were performed more-or-less simultaneously.

Table 1
Prothrombin times for the calibration of a secondary standard of rabbit thromboplastin

Date	Plasma	RBT/90	Secondary standard
10 March 1997	Normal 1	16.9	12.2
	Normal 2	17.5	12.4
	Patient 1	62.6	33.4
	Patient 2	59.9	34.7
	Patient 3	58.9	32.7
	Patient 4	51.8	28.2
	Patient 5	44.7	25.0
	Patient 6	52.9	30.3
	Patient 7	49.9	28.9
	Patient 8	60.8	31.6
	Patient 9	55.5	32.4
	Patient 10	49.9	28.0
	Patient 11	40.4	24.7
	Patient 12	56.8	29.8
	Normal 3	18.9	13.1
	Normal 4	19.0	13.0
11 March 1997	Normal 5	18.3	13.0
	Normal 6	17.4	12.6
	Patient 13	33.3	21.9
	Patient 14	59.3	31.7

continued

Table 1
Continued

Date	Plasma	RBT/90	Secondary standard
	Patient 15	78.3	37.5
	Patient 16	56.3	29.4
	Patient 17	66.0	36.6
	Patient 18	55.5	31.3
	Patient 19	72.4	36.0
	Patient 20	39.1	25.3
	Patient 21	83.4	37.8
	Patient 22	63.6	33.0
	Patient 23	54.8	32.1
	Patient 24	48.5	28.4
	Normal 7	19.0	13.7
	Normal 8	16.5	12.3
12 March 1997	Normal 9	22.2	14.9
	Normal 10	17.5	12.4
	Patient 25	54.2	30.7
	Patient 26	62.8	33.9
	Patient 27	43.9	23.4
	Patient 28	72.4	34.7
	Patient 29	58.5	33.1
	Patient 30	66.7	37.4
	Patient 31	61.5	34.5
	Patient 32	50.7	29.2
	Patient 33	87.0	41.4
	Patient 34	37.8	23.6
	Patient 35	75.4	39.8
	Patient 36	72.6	38.7
	Normal 11	19.5	13.1
	Normal 12	18.4	12.8
13 March 1997	Normal 13	17.3	13.0
	Normal 14	18.4	13.1
	Patient 37	94.3	39.4
	Patient 38	70.5	32.8
	Patient 39	35.2	22.6
	Patient 40	50.6	28.9
	Patient 41	66.0	32.6
	Patient 42	57.9	32.1
	Patient 43	66.2	36.2
	Patient 44	57.1	30.2
	Patient 45	50.4	27.3
	Patient 46	62.9	32.3
	Patient 47	68.4	35.4
	Patient 48	52.2	28.4
	Normal 15	14.4	11.5
	Normal 16	15.9	11.9
14 March 1997	Normal 17	17.6	12.5
	Normal 18	16.2	12.3
	Patient 49	64.8	34.8

continued

Table 1
Continued

Date	Plasma	RBT/90	Secondary standard
	Patient 50	59.9	34.1
	Patient 51	53.3	31.9
	Patient 52	66.0	34.3
	Patient 53	55.1	28.9
	Patient 54	61.2	33.9
	Patient 55	67.9	33.9
	Patient 56	68.8	34.4
	Patient 57	75.6	35.4
	Patient 58	61.9	33.5
	Patient 59	69.6	37.2
	Patient 60	38.8	23.3
	Normal 19	16.9	12.4
	Normal 20	17.9	12.8

Calculations

The International Sensitivity Index of the secondary standard (ISI_w) is obtained by plotting the prothrombin times of the two thromboplastins on logarithmic axes as shown in Fig. 2 (p. 90), fitting a straight line of the form

$$Y = A + BX \tag{1}$$

and estimating the slope B. The recommended method involves estimation of a linear structural relation (also called an "orthogonal regression equation"). With this technique, the slope B can be estimated as follows.

Consider a set of N independent observations (x_i, y_i), where $i = 1, 2, 3, \ldots, N$; for N paired tests, y_i represents the natural logarithm of the measured prothrombin time of the international reference preparation, and x_i that of the secondary standard. Write x_0, y_0, for the arithmetic means of the N values of x_i, y_i, respectively. Write Q_1, Q_2 for the sums of the squares of $(x_i - x_0)$ and $(y_i - y_0)$, respectively, and P for the sum of their products $\Sigma(x_i - x_0)(y_i - y_0)$. These quantities are all that is necessary for computing a and b, the least-squares estimators for the parameters A and B of equation (1). Now define:

$$E = (Q_2 - Q_1)^2 + 4P^2. \tag{2}$$

Then

$$b = \frac{Q_2 - Q_1 + E^{1/2}}{2P} \tag{3}$$

and

$$a = y_0 - bx_0 \tag{4}$$

are the estimators that minimize the sum of the squares of the perpendicular distances of the N points from the line represented by equation (1). The variance of b is given by:

$$\text{Var(b)} = \frac{\{(1+\text{b}^2)P + N\text{b}V\}\text{b}V}{P^2}, \tag{5}$$

where V is defined as

$$V = \frac{Q_2 - \text{b}P}{N-2} \tag{6}$$

The standard error of b (s_b) is the square root of Var(b). If t is a deviate from the t-distribution, with $(N-2)$ degrees of freedom and at a chosen probability, approximate confidence limits for B can be obtained by setting an interval $t \times s_\text{b}$ on either side of b. The residual standard deviation is the square root of V. Outlying points should be rejected if their vertical distance from the calibration line is greater than $3\sqrt{V}$.

The ISI_w is calculated as follows:

$$\text{ISI}_\text{w} = \text{ISI}_\text{IRP} \times \text{b}, \tag{7}$$

where ISI_IRP is the ISI of the international reference preparation.

The coefficient of variation (%) of b is $100 \times (s_\text{b}/\text{b})$.

An ingenious alternative has been suggested for calculating confidence limits for the slope B, appropriate here if the frequency distribution of the random errors of x_i and y_i can be assumed to be approximately normal (*1*). In practice, these confidence limits are likely to be trustworthy unless N is so small as to introduce problems due to non-normality (*2*). With t as above, define:

$$D = \frac{4t^2(Q.Q_2 - P^2)}{E(N-2)} \tag{8}$$

and calculate

$$\text{b}_0 = \tan^{-1}(\text{b}) \tag{9}$$

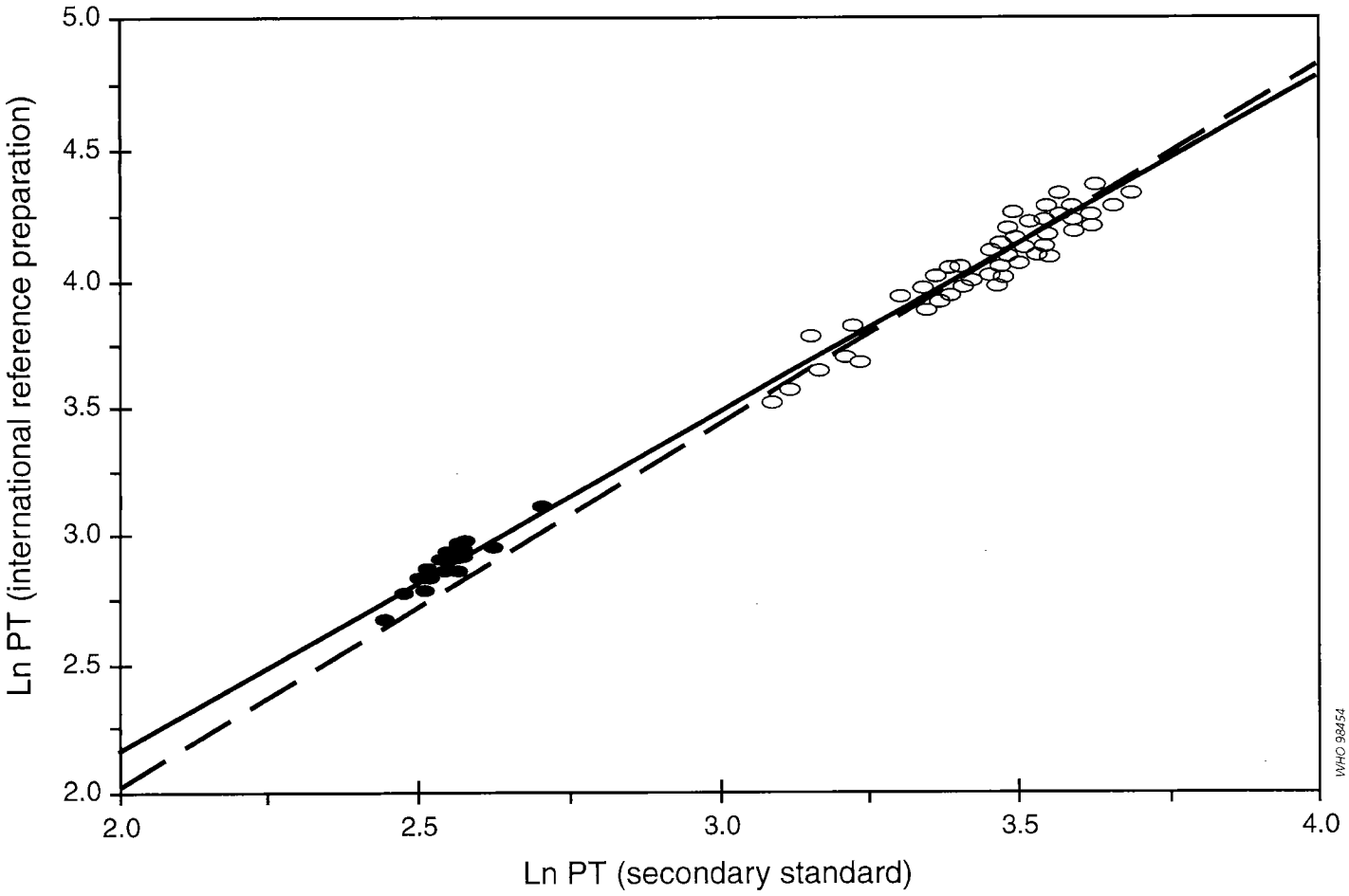
$$d_0 = \frac{\tan^{-1}\{D/(1-D)\}^{1/2}}{2}. \tag{10}$$

Confidence limits then are:

$$\tan(\text{b}_0 - d_\text{c}) \text{ and } \tan(\text{b}_0 + d_0). \tag{11}$$

In most cases, when t is chosen for probability = 0.95, $D < 1.0$. However, at higher probabilities an unusual conjunction of data can make the calculation of confidence limits impossible if D exceeds

Figure 2
Log-log plot of prothrombin times for determination of ISI[1]



[1] The continuous line represents the structural relation for both normal samples (dots) and samples from patients (circles). The dashed line represents the relation for samples from patients only.

1.0, since the square root of a negative number is not defined. There is no simple way of detecting this problem until D has been calculated.

Example

For the data from Table 1, the calculated MNPT value for RBT/90 is 17.7 seconds. The ISI of RBT/90 is 1.0. There were three patients with INRs beyond the 1.5–4.5 range (Patient 21, 33 and 37). These values were excluded from the calculation of b. The calculated value for b, based on the remaining 77 samples, is 1.3138. Thus, the ISI for the secondary standard is estimated as $1.0 \times 1.3138 = 1.3138$.

The standard error for b is calculated as 0.0151. The coefficient of variation for b is $100 \times (0.0151 \div 1.3138) = 1.1\%$. Confidence limits for b are calculated as in reference (*1*) at probability = 0.95, i.e. 1.2841 (lower limit) and 1.3444 (upper limit).

In this example, there was a deviation from linearity. This can be shown by calculating the structural relation for patients' samples only. The latter does not pass through the mean of the normal samples (see Fig. 2). The equation for the line for patients only is characterized by b = 1.4083 and a = –0.8028. For this equation, a clotting time of 35.4 seconds with RBT/90 (i.e. INR = 2.0) corresponds to a clotting time of 22.26 seconds with the rabbit-brain secondary standard. With the ISI (i.e. 1.3138) and the MNPT (12.73 seconds) for this secondary standard, a clotting time of 22.26 seconds can be transformed to an INR of 2.08 (see formula, p. 80). In this example, the assignment of an ISI based on all samples is acceptable because INRs calculated with the ISI do not differ by more than 10% from INRs calculated with the equation describing the line for patients only.

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Appendix 2

Example of the use of the suggested method for reporting the data on the calibration of individual batches of thromboplastin

Thromboplastins: 1. Rabbit brain thromboplastin secondary standard
2. Batch of rabbit brain thromboplastin

End-point recording: automated photoelectric coagulometer

Pooled coumarin plasmas: lot 960606, 1–5 (deep-frozen)

Pooled normal plasma: lot 900423 (deep-frozen)

The ISI and MNPT of the rabbit brain thromboplastin secondary standard used with this automated photoelectric coagulometer are 1.31 and 12.7 seconds, respectively.

The tests were conducted in four separate runs. For each run, thromboplastins were freshly reconstituted and deep-frozen plasmas were freshly thawed. Since the secondary standard and the test batch were both timed with the same photoelectric coagulometer, the order in which the two preparations were tested was alternated from one run to the next. This was done to avoid any bias due to possible instability of the thromboplastins and pooled plasmas.

Calculation

The ISI of the batch (ISI_b) is calculated as $ISI_b = ISI_w \times b$, where b is the slope of the straight line fitted to a double-logarithmic plot (similar to Fig. 2; see Appendix 1, p. 90) of the prothrombin times in Table 2, with the prothrombin times for the secondary standard and the test batch being shown on the vertical and horizontal axes, respectively. The formula for b is given by equation (*3*) in Appendix 1, p. 88. The standard error of b is obtained from equation (*5*) in Appendix 1, p. 89. The coefficient of variation (%) of b is $100 \times (s_b/b)$.

Example

For the data from Table 2, the calculated residual standard deviation is 0.02482. One pair of determinations for plasma lot no. 960606-5 (run no. 3) has a perpendicular distance from the line greater than three residual standard deviations. When this pair is excluded, the calculated value for b is 0.9538. The ISI for the secondary standard is

Table 2
Prothrombin times for the calibration of an individual batch of rabbit thromboplastin

		Secondary standard		Rabbit brain thromboplastin	
Run no.	Plasma lot no.	Order of testing (within-run)	Prothrombin time	Order of testing (within-run)	Prothrombin time
1	900423	1	14.0	7	15.1
	960606-1	2	20.5	8	21.5
	960606-2	3	29.1	9	31.5
	960606-3	4	32.9	10	36.4
	960606-4	5	36.2	11	41.0
	960606-5	6	39.7	12	44.6
2	900423	7	14.1	1	15.4
	960606-1	8	20.3	2	22.6
	960606-2	9	29.5	3	31.2
	960606-3	10	32.8	4	37.6
	960606-4	11	37.3	5	40.8
	960606-5	12	39.8	6	44.5
3	900423	1	14.0	7	15.0
	960606-1	2	20.0	8	21.5
	960606-2	3	28.1	9	32.1
	960606-3	4	31.8	10	34.2
	960606-4	5	35.9	11	40.7
	960606-5	6	37.2	12	47.7
4	900423	7	13.9	1	15.0
	960606-1	8	20.0	2	21.9
	960606-2	9	27.9	3	30.9
	960606-3	10	31.5	4	35.7
	960606-4	11	34.6	5	39.2
	960606-5	12	37.6	6	44.4

given as 1.31. Thus, the ISI for the test batch is estimated as $1.31 \times 0.9538 = 1.25$. The standard error for b is calculated as 0.0130. The coefficient of variation for b is $100 \times (0.0130/0.9538) = 1.36\%$. The confidence limits for b at probability $p = 0.95$ are calculated as in Appendix 1, p. 89, i.e. 0.9271 (lower limit) and 0.9812 (upper limit).

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Annex 4

Requirements for hepatitis B vaccines made by recombinant DNA techniques

(Requirements for Biological Substances No. 45, amendment 1997)

Introduction

Requirements for hepatitis B vaccines made by recombinant DNA techniques were published in 1989 (*1*). The requirement (in section 11.6) for potency tests on the final lot states that "an appropriate quantitative test for antigen content and an immunogenicity assay shall be performed on samples representative of the final filling lots". The test for potency performed by all manufacturers at the time those requirements were published was an immunogenicity test in mice or guinea-pigs, and a suitable test in mice is described in the requirements.

Since then, at least two manufacturers have developed *in vitro* potency tests based on an ELISA assay of the vaccine (after addition of the adjuvant). These assays are product specific and have been validated by demonstrating a correlation with the results of mouse immunogenicity tests. Each manufacturer has set a specification for the *in vitro* test which ensures that vaccines that pass this test would also pass the mouse immunogenicity test. These assays have been approved by the national control authority in the country of manufacture and have been incorporated in the relevant product licences.

However, these vaccines would no longer meet the requirements published by WHO, since mouse immunogenicity tests are no longer being performed on every final lot. Since the vaccines in question are well established and have been used in millions of individuals, the requirements for hepatitis B vaccine published by WHO have now been modified to permit the use of an *in vitro* test that has been validated by correlation with immune response in humans, or with results obtained in mouse immunogenicity tests for which an appropriate specification has been set by a manufacturer.

Because of diversity in the reactivity of vaccines containing hepatitis B surface antigen (HBsAg) produced by different manufacturing pro-

cesses and to which adjuvants have been added by different methods, it is unlikely that a single International Standard will be suitable for the standardization of *in vitro* assays.

It is thus appropriate to amend, as follows, the Requirements for Hepatitis B Vaccines Made by Recombinant DNA Techniques (WHO Technical Report Series, No. 786, 1989, Annex 2).

Replace the text of section 11.6, *Potency and identity tests* (p. 56), by the following:

"The vaccine shall be identified as envelope antigens of hepatitis B virus by appropriate methods. An appropriate quantitative test for potency by an *in vivo* or *in vitro* method shall be performed on samples representative of the final filling lots. Any *in vitro* methods used shall be appropriately validated and the test method approved by the national control authority. The vaccine shall be compared with a reference vaccine, and the national control authority shall establish limits of potency.

> At least two manufacturers have validated *in vitro* potency tests based on the detection of HBsAg in dilutions of the vaccine (plus adjuvant) by an HBsAg detection kit. These tests have been validated against the immune response obtained in mouse immunogenicity tests.
>
> *In vitro* potency tests should be able to distinguish vaccine of low potency, which may induce an inadequate immune response in humans. Several factors must be considered when validating an assay for individual vaccines since they may contain different forms of adjuvant and antigen manufactured by different processes. These factors include the nature of the HBsAg test kit used, the diluent in which the vaccine is prepared, the reference preparation used and the possible need for a pretreatment step to disaggregate the antigen (e.g. with detergent) to ensure consistent responses in vaccinees of all ages.
>
> Test and reference vaccine should be tested concurrently in an assay designed to produce dose–response curves suitable for quantitative analysis by an appropriate statistical method (e.g. parallel-line analysis). Product-specific reference vaccines may be necessary. The statistical validity of the assay should be assessed, the potency of the test relative to the reference vaccine should be estimated and the assay's precision (i.e. confidence interval) should be calculated."

(The requirements' *in vivo* immunogenicity test remains unchanged.)

Reference

Requirements for hepatitis B vaccines made by recombinant DNA techniques (Requirements for Biological Substances No. 45). In: *WHO Expert Committee on Biological Standardization. Thirty-ninth report.* Geneva, World Health Organization, 1989, Annex 2 (WHO Technical Report Series, No. 786).

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Annex 5
Report on the standardization and calibration of cytokine immunoassays

1. Introduction

It has become increasingly clear that available immunoassays for cytokines have some inherent problems. For example, assays can often result in incorrect or invalid estimations of cytokine levels, results obtained by different laboratories are not comparable and discrepancies can be found in the results of tests performed on different occasions. Cytokine assays were discussed at the third annual conference of the International Cytokine Society at Harrogate, England, in 1995 as well as at other meetings mentioned below. Action to resolve these problems was considered an urgent need by both users and producers of commercial immunoassay reagents and kits. At the second WHO Informal Consultation on Cytokine Standards (held during June 1996 in Strasbourg, France) it was proposed that a meeting be organized to attempt to resolve at least some of the problems noted. This proposal was supported at the meeting of the WHO Expert Committee on Biological Standardization in Geneva in October 1996. The following is the report of the meeting that was organized to address issues concerning the standardization and calibration of cytokine immunoassays (held November 1996).

2. General

An international meeting cosponsored by WHO was held at the National Institute for Biological Standards and Control, Potters Bar, Herts., England, 14–15 November 1996 to address issues concerning the standardization and calibration of cytokine immunoassays. Sixty delegates including representatives from immunoassay reagent and kit producers, academia and manufacturers of therapeutic cytokine products attended. The main areas of discussion focused on concerns identified with cytokine immunoassays which included problems due to:

- the use of multiple standards;
- differential epitope recognition by the antibodies used in immunoassays;

- preferential recognition of different cytokine isoforms by different immunoassays;
- matrix effects (i.e. changes in results due to the complex nature of the test environment);
- the presence of soluble receptors, cytokine binding proteins, "auto-antibodies" etc. in the samples under investigation.

Other issues relating to cytokine immunoassays were discussed, for example, the validation of immunoassays and the expression of assay sensitivity (*1–4*). The following questions were identified as being of particular importance:

- Should international standards or other reference materials for cytokines established by the WHO Expert Committee on Biological Standardization be used as immunoassay standards?
- How should results be expressed? (Most current cytokine standards are calibrated in International Units.)
- Are additional standards and reference materials necessary?
- Are guidelines on cytokine immunoassay validation necessary?

On the basis of the data presented, strategies that could be employed to improve immunoassays and/or resolve the various problems were suggested. Results from international collaborative studies had clearly demonstrated that the use of a single reference material drastically reduced interassay and interlaboratory variations. Although variations between different immunoassay kits were still apparent, these were at least partially due to the unique recognition profile of particular antibody pairs used in immunoassays and could only be addressed by a thorough, valid characterization of the antibodies. Characterization should include assessment of reactivity with different forms of a given cytokine, for example, naturally occurring proteins and those produced using different expression systems.

3. Recommendations

A final version of recommendations was approved by the participants at the meeting in November 1996 and was also considered and endorsed by the participants at the third WHO Informal Consultation on Cytokine Standards, held in May 1997 at the National Institute for Biological Standards and Control, Potters Bar, Herts., England. They are as follows.

3.1 Calibration of cytokine immunoassays

For any cytokine, it is difficult or impossible to establish a single standard suitable for all immunoassays in all applications because of

heterogeneity of the test or reference material, the assay matrix and the specificity of the various antibodies used. However, the need for a common primary reference material with well characterized and stable antigenic activity has been clearly identified. The use of a single material as a primary calibrator has been shown to reduce problems associated with the use of multiple reference materials. Standardization of cytokine immunoassays can therefore be achieved only by establishing a primary calibrant and validating assay performance for each application.

3.2 Standardization

Primary calibrant

It is recommended that the International Standard or Reference Reagents established by the WHO Expert Committee on Biological Standardization for the cytokine to be measured should be used to facilitate the comparability of results obtained using different immunoassay procedures or kits. These primary reference materials should be used for the calibration of all secondary and working standards, but not for routine use. The memorandum distributed with the material contains a statement describing the ampoule content to be used for immunoassays. Because of possible differences in the assignment of mass to protein preparations as a result of using different methods or standards, the mass units assigned to cytokine reference materials will not be "absolute" mass values, but experimental values related to a specific, stated method of determination.

Working standard

The structure, the method of production and the biophysical characteristics of the working standard (e.g. the DNA sequence of the construct, the pI (isoelectric point) of the product) should be provided. The biological potency of the material should be stated.

The calibration of the working standard in relation to the international reference material should include information on the confidence intervals of the procedure and the statistical methods used.

3.3 Validation

A dilution series rather than a single-point determination should be used for validation studies. The concentration range for which the assay is valid should be stated. In all cases, the methods used for assay validation should be described.

The ability of an immunoassay to recognize different forms of the cytokine (including precursors where appropriate) should be demon-

strated. A detailed assessment of the immunoreactivity of the antibodies used, i.e. the recognition (or lack of it) of different forms of the cytokine, is essential for this. Wherever possible, data demonstrating recognition of "naturally occurring" forms of the cytokine should be included.

Assay performance indicators, i.e. preparations of a cytokine produced with different recombinant-DNA expression systems, possibly resulting in different primary amino acid sequences or different clipped forms or spliced variants, are useful for demonstrating the ability of immunoassays to discriminate among different forms of the cytokine.

The specificity of the assay should be documented, including immunoreactivity of the antibody with all homologous molecules.

Stability of reagents and antibodies, as well as shelf life and batch-to-batch consistency of the assay, should be documented. Changes on storage in sensitivity and assay background should be noted. Plate consistency should be monitored (e.g. coating efficiency, well-to-well variation).

Problems relating to the presence of soluble receptors, cytokine binding proteins, cytokine antagonists, heterophilic antibodies and autoantibodies should be assessed. Procedures used for detecting such interference should be described.

Possible matrix effects need to be assessed using an appropriate dilution series in matrices representative of the samples to be assayed (e.g. serum, plasma with anticoagulants).

Recommendations for appropriate sample collection, processing and storage should be provided by the manufacturer of the immunoassay.

"Spiking" and recovery studies should be carried out over an appropriate range of temperatures and time points with an appropriate procedure. Spiking studies should encompass the whole working-concentration range of the assay. Details of spiking studies should be documented.

Linearity and parallelism need to be established in all studies relating to assay validation and calibration.

The procedure used to calculate assay sensitivity should be clearly stated. Sensitivity should be defined as limits of detection or limits of quantification. Estimates below the lowest recorded non-zero standard on the dose–response curve are not valid.

The precision of the assay should be documented. Intra- and interassay variation should be assessed and described.

Variability associated with different lots of reagent or with different equipment should be assessed and described.

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Annex 6
Biological substances: International Standards and Reference Reagents

A list of International Biological Standards, International Biological Reference Preparations and International Biological Reference Reagents is issued as a separate publication.[1]

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

Additions

Antibodies

Anti-human platelet antigen 1a	No unitage assigned	First interim Reference Reagent 1997
Anti-hepatitis E serum, human	50 units per ampoule	First interim Reference Reagent 1997

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

Blood products

Antithrombin concentrate	4.7 IU per ampoule (functional activity) 5.1 IU per ampoule (antigenic activity)	Second International Standard 1997
Serum amyloid A protein	150 milli-IU per ampoule	First International Standard 1997

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

Cytokines

Interleukin-10	5000 units per ampoule	First interim Reference Reagent 1997

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

Bone-morphogenic protein-2 (BMP-2)	5000 units per ampoule	First interim Reference Reagent 1997
Brain-derived neurotropic factor	5000 units per ampoule	First interim Reference Reagent 1997
Stem cell factor	1000 units per ampoule	First interim Reference Reagent 1997
FMS-like tyrosine kinase-3 ligand	1000 units per ampoule	First interim Reference Reagent 1997

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

Endocrinological substances

Follicle-stimulating hormone, human, for immunoassay	60 IU per ampoule	First International Standard 1997
Platelet-derived growth factor isoform BB	3000 IU per ampoule	First International Standard 1997

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

Miscellaneous

Hepatitis C virus RNA	50000 IU per vial	First International Standard 1997
MAPREC analysis of poliovirus type 3 (Sabin) (low virus reference)	0.7% 472-C nucleotide per vial	First International Reference Reagent 1997
MAPREC analysis of poliovirus type 3 (Sabin) (high virus reference)	1.1% 472-C nucleotide per vial	First International Reference Reagent 1997

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

Discontinuation

Cardiolipin	Fourth International Reference Preparation 1967

Diphtheria toxoid, plain	Second International Standard 1975
Lecithin (egg)	Fourth International Reference Preparation 1970
Poliomyelitis vaccine (inactivated)	First International Reference Preparation 1962
Tetanus toxoid	First International Standard 1951
Swine erysipelas vaccine	First International Standard 1959

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Annex 7
Requirements for Biological Substances and other sets of recommendations

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

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

Requirements

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

[1] Abbreviated here as TRS.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

30. Requirements for Thromboplastins and Plasma Used to Control Oral Anticoagulant Therapy
    Revised 1982, TRS **687** (1983)
    Discontinued 1997, TRS **889** (1999)

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

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

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

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

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

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

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

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

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

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

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

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

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

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

45. Requirements for Hepatitis B Vaccines Made by Recombinant DNA Techniques
    Adopted 1988, TRS **786** (1989)
    Amendment 1997, TRS **889** (1999)

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

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

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

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

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

51. Requirements for tick-borne encephalitis vaccine (inactivated)
    Adopted 1997, TRS **889** (1999)

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

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

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

Guidelines for assuring the quality of pharmaceutical and biological products prepared by recombinant DNA technology
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