WHO Expert Committee on Biological Standardization

Thirty-fifth Report

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WHO EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION

Geneva, 12–18 June 1984

Members

Dr D.R. Bangham, Head, Division of Hormones, National Institute for Biological Standards and Control, London, England
Dr C. Guthrie, Operations Director, Commonwealth Serum Laboratories, Parkville, Victoria, Australia (Rapporteur)
Dr H.W. Krijnen, Director, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, Netherlands
Professor B. Lunenfeld, Director, Institute of Endocrinology and Chief of Division of Laboratories, Chaim Sheba Medical Center, Tel Hashomer, Israel
Mr J. Lyng, Head, Laboratory for Biological Standardization, State Serum Institute, Copenhagen, Denmark
Dr F.H. Meskal, Director, Central Laboratory and Research Institute, Addis Ababa, Ethiopia (Vice-Chairman)
Dr J. Petricciani, Director, Blood and Blood Products, Office of Biologics, National Center for Drugs and Biologics, Food and Drug Administration, Bethesda, MD, USA (Chairman)
Dr W.W. Wright, Senior Scientist, Drug Standards Division, The United States Pharmacopelia, The National Formulary, Rockville, MD, USA
Dr Xiang Jian-zhi, Head, Division of Science and Technology, Shanghai Institute of Biological Products, Shanghai, China

Secretariat

Dr J.N. Ashworth, Division Vice-President, Scientific Affairs, Cutter International, Emeryville, CA, USA (Temporary Adviser)
Professor W. Hennessen, Berne, Switzerland (Temporary Adviser)
Dr J.W. Lightbown, London, England (Temporary Adviser)
†Dr F.T. Perkins, Chief, Biologicals, WHO, Geneva, Switzerland (Co-Secretary)
Dr D.P. Thomas, Head, Division of Blood Products, National Institute for Biological Standards and Control, London, England (Temporary Adviser)
Dr A. Shishido, Professor, Department of Microbiology, School of Medicine, Juntendo University, Tokyo, Japan (Temporary Adviser)
Dr P. Sizaret, Scientist, Biologicals, WHO, Geneva, Switzerland (Co-Secretary)

† Unable to attend: Professor S.G. Dzagurov, Director, Tarasevi State Research Institute for the Standardization and Control of Medical Biological Preparations, Ministry of Health of the USSR, Moscow, USSR
†† Deceased 26 August 1984.
WHO EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION

Thirty-fifth Report

The WHO Expert Committee on Biological Standardization met in Geneva from 12 to 18 June 1984. The meeting was opened on behalf of the Director-General by Dr Lu Rushan, Assistant Director-General.

GENERAL

International standards and units for biological substances

The Committee was informed that, in compliance with the Constitution of the World Health Organization, a list of biological standards updated since 1973 and, for the first time, a list of international biological reference preparations had been placed before the Executive Board and the Thirty-seventh World Health Assembly.\(^1\) The Health Assembly recommended that Member States recognize officially the international standards and reference preparations and units for biological substances enumerated in the two lists.\(^2\) The Health Assembly was informed that in many instances the potency, in terms of international units, of each biological standard is now defined on the basis of the contents of the ampoule rather than in terms of a defined weight of the standard.

Biological standards for biological and chemical reference purposes

The Committee considered the need for reference preparations of biological substances for use in control procedures of physico-chemical tests. Such preparations may be needed for tests of purity by liquid chromatography or for tests of identity in which a map of the peptides produced by treating the test material with an

enzyme is compared with that of the reference material treated identically. For such purposes well characterized preparations of purified stable authentic substances are required; these should be free from extraneous materials that might interfere with the analyses. For certain antibiotics, the international standards themselves may be suitable for such tests, as well as serving their original purpose as standards for bioassay. Moreover, when it becomes possible to characterize a substance completely by chemical and physical means and an international standard for bioassay is no longer required, the remaining stock of the standard could be reserved for use for such physicochemical tests. Conversely, if for certain substances the international standard established for bioassay is unsuitable for physicochemical tests, there may be a need for an authentic preparation of the pure substance. Thus, a preparation of human growth hormone (form with relative molecular mass 22,000) is needed for tests of identity in connection with the product made by recombinant DNA (rDNA) methods, since the International Standard for Growth Hormone, for Bioassay, consists of a mixture of the several natural forms of the hormone (see item 25, page 23).

The Committee also considered the question of replacing biologically standardized reference preparations, (e.g., the International Standard for Erythromycin) with authentic chemical reference preparations. The Committee recognized that authentic chemical reference substances could also be used for reference purposes in biological assays or for identification purposes. The technical problems involved in confirming the purity of materials being considered for use as authentic chemical reference substances were discussed. It was recognized that although methods of analysis such as chromatography can reveal the presence of extremely small quantities of certain impurities, they may not detect certain others. The demonstration of a single chromatographic peak does not of itself provide proof of the purity of the preparation. Frequently, other chemical and physical tests, such as phase-solubility analysis, can provide strong confirmatory evidence of purity.

In the past, whenever it was concluded that an international biological reference material should be discontinued, in most instances, it was found convenient and helpful for the WHO Collaborating Centre for Chemical Reference Substances, Solna, Sweden, to establish and provide authentic chemical material for international use. Also, in some such instances, the International Pharmacopoeia specifies that the authentic chemicals may also be
used as standards in biological assays. In other instances, bulk chemical materials meeting all the tests specified in the pharmacopoeial monograph for the substance itself were authorized for use as standards in the biological assays of dosage forms.

The Committee considered a hypothetical situation in which, because of other priorities, WHO decided not to revise the specifications for a biological substance in the International Pharmacopoeia that could be characterized only by chemical and physical tests, or a situation in which the WHO Collaborating Centre for Chemical Reference Substances might be unable to provide an authentic chemical reference substance in place of a discontinued international biological reference material. It was decided that in such circumstances either WHO should continue to make available the international biological reference material or the Expert Committee on Biological Standardization should establish a suitable, chemically defined reference substance.

Matrix standards

Recently, a national group of immunoassay kit manufacturers asked WHO about the possibility of WHO establishing matrix standards. “Matrix standards” are preparations of an analyte in the same kind of biological fluid—matrix—as that present in the clinical test specimens that are assayed against them. A matrix standard for prolactin in serum, for example, would consist of an ampoule of freeze-dried serum containing a defined number of international units of prolactin. The reason for this proposal is that the nature of the solution in which the analyte standard is prepared can strongly influence the potency estimate of a clinical specimen made with different assay kits. Several international standards (for example, of hormones) are freeze-dried in ampoules with a carrier sugar and a small amount of protein as stabilizer. The use by manufacturers of different buffer or serum solutions to dissolve and dilute some international standards of this type has led to clinically unacceptable diversity of assay results. Moreover, it has been claimed that the use of international matrix standards to calibrate manufacturers’ own standards would improve agreement.

The proposal to provide matrix standards, however, raises many scientific, technical, logistic, and organizational problems. The Committee therefore requested the WHO Secretariat to arrange a meeting of manufacturers to investigate the feasibility of this proposal.
Biotechnology

Recent scientific and technological advances have been applied rapidly to the development of a variety of biologicals. For example, recombinant DNA (rDNA) techniques have already been used to produce human insulin and other hormones, and more recent work has shown that hepatitis B surface antigen (HBsAg) can be produced in yeast cells as well as in continuous mammalian cell lines. While requirements for at least some of these products made by traditional techniques already exist, additional requirements will need to be formulated for rDNA products such as HBsAg because the manufacture of biological products by rDNA methods raises special questions concerning their safety and purity.

The production of monoclonal antibodies by hybridoma techniques also gives rise to a wide variety of potentially useful products for which special requirements will be needed. The use of continuous cell lines grown on microcarriers, in mass culture, to produce inactivated vaccines may also result in a class of compounds requiring special provisions, since, in some of these techniques, abnormal mammalian cells are used as substrates and the safety of the biological product is therefore of particular concern. The WHO Secretariat was therefore requested to consider convening an informal group to advise WHO on the possible changes in current requirements and the development of new requirements for biologicals produced in abnormal mammalian cells.

Causes of damage to polypeptides during freeze-drying

Studies at the National Institute for Biological Standards and Control, London, on the freeze-dried highly purified human, porcine, and bovine insulins prepared as candidate international standards had shown that degradation had taken place during freeze-drying. Analyses with reverse-phase liquid chromatography had shown that the degraded insulin products were ill-defined molecular forms that had appeared only after freeze-drying and secondary desiccation of the initially pure insulins that were produced in bulk. These forms were difficult to isolate in a pure state, but their net charge and apparent relative molecular masses were the same as those of the natural insulins. Comparable degradation could be produced by the action, during freeze-drying, of a free-hydroxyl-radical generating system consisting of ferrous iron and peroxide, and also by prolonged secondary desiccation that reduced the
moisture content to very low levels (around 100 mg/kg). Furthermore, it had been shown that freeze-drying of insulin or certain other polypeptides with carrier lactose, mannitol, trehalose, or inositol could also lead to the formation of degraded polypeptide products. Although the exact cause of the degradation that occurred in the three large batches of ampouled insulins has not yet been identified, the studies have shown three possible causes of damage during the freeze-drying and ampouling of polypeptides. Thus, the Committee re-emphasized the need to carry out freeze-drying trials prior to the ampouling of large batches of candidate materials for use as standards.

Information memoranda distributed with standards

In the use of certain international biological standards, only a minimum of information about the standard (e.g., the identity and unitage of antibiotics) is necessary for its effective use. In the case of many other standards, however, in order to be able to use them properly, it is essential to know the nature and content of the components constituting the standards. For example, for a standard used in certain sensitive bioassay or immunoassay systems, it may be essential for the user to know how the material was extracted, the animal or tissue source as well as the carrier, and the type of stabilizer, buffer, and bacteriostatic substances present in it. Guidance may be needed also on how best to dissolve, subdivide, and store solutions of the standard (WHO Technical Report Series, No. 673, 1982, p. 10). In yet other instances, valuable information may be provided to the user on the performance of the standard in various types of assay. Lastly, it may also be necessary to provide the user with reports of the international collaborative studies that are carried out to establish standards and which are specifically designed to investigate certain properties (e.g., to provide additional evidence of its identity) of the standard or of other materials included in the study, or to compare different methods of assay.

It has been the practice to attach with each standard a memorandum containing all information that may benefit the user. In the case of certain standards, the memorandum has consisted of copies or reprints of the full report of the international collaborative study as submitted to the Committee. In the case of others, a short memorandum has been distributed, summarizing essential information and including references for further details. The
Committee considered that the issuing of such information on international standards should be coordinated by WHO, and requested the WHO Secretariat to collect suggestions from the users in order to define a policy on this issue.

**Veterinary biologicals produced by modern techniques**

In the WHO biologicals programme a close watch has been kept on the rapid developments recently made in the production by modern techniques of biologicals used in human medicine. However, the Committee was concerned that similar developments in the veterinary field (e.g., the production of bovine growth hormone by rDNA techniques) had not been brought to the attention of the biologicals programme. The Committee therefore requested the WHO Secretariat to seek information on such developments in the veterinary field and consider how best to respond to them.

**Human interferons**

Rapid progress has been made in the standardization of interferons and especially those being produced by modern techniques. Such preparations, now available in quantities hitherto unattainable, require new standards for each of the categories of interferon. The Committee established four new standards, one of which—the standard for leukocyte interferon—is to be made widely available to help research studies.

Now that interferons are being used in the treatment of some forms of malignancy, an *in vitro* test to measure the ability of interferons to prevent the proliferation of cells or to cause the regression of tumour cells is urgently needed. Work is in progress on the development of an internationally accepted, rapid, and simplified test for the assay of interferons as well as to investigate appropriate tests to measure the antiproliferative effect of interferons.

**Potency assay of diphtheria and tetanus toxoids**

The potency assay of diphtheria and tetanus in single and combined vaccines is still a cause of problems owing to the lack of a statistically valid test using a small number of animals. A solution may be to carry out a statistically valid test on one final bulk vaccine
made from given concentrates and thereafter to use a simplified test on subsequent final bulk vaccines made similarly from the same concentrates. The Committee recommended that the WHO Secretariat should keep this matter under review.

Varicella vaccine

Although varicella vaccine made from a virus attenuated in human diploid cells may not be used on a wide scale, it has been shown to be safe and effective in children who have malignancies, who are on immunosuppressive therapy, or who are naturally immunocompromised. In such children, the use of the vaccine can help avoid the dangerous complications of chickenpox. The vaccine is also useful in adults who have not had chickenpox in childhood and in those at special risk.

Standard materials are required for the measurement of live virus content of the vaccine in order to ensure that an adequate dose is given, and an immunoglobulin is needed to compare the antibody responses given by different groups of subjects. The preparation of a standard for the virus is being actively pursued, and the varicella zoster immunoglobulin is at present the subject of a collaborative study.

SUBSTANCES

ANTIBIOTICS

1. Erythromycin

The Committee noted that, in accordance with the request in its thirty-fourth report (WHO Technical Report Series, No. 700, 1984 p. 12), the Secretariat had investigated the possibility of establishing an authentic chemical reference substance of erythromycin (WHO/BS/84.1436). The Committee noted also that the WHO Collaborating Centre for Chemical Reference Substances, Solna, Sweden, based on its experience several years ago in analysing preparations of erythromycin, had advised that at that time it was not feasible to establish a chemical reference substance of erythromycin.
The Committee was informed that liquid chromatographic methods for analysing erythromycin now made it possible to reveal extremely small amounts of impurities. The Committee was informed also that erythromycin preparations consisting apparently of only the major erythromycin component had been prepared, and that these could be used as standards in the microbiological assay of erythromycin specified in the International Pharmacopoeia.

The Committee agreed, however, that until international pharmaceutical specifications require the use of an authentic chemical reference substance of erythromycin, the international biological standard of erythromycin should be continued.

2. Netilmicin

The Committee noted that the preparation of netilmicin, referred to in its thirty-fourth report (WHO Technical Report Series, No. 700, 1984, p. 13), had been freeze-dried in ampoules and that no degradation due to the processing could be detected (WHO/BS/84.1445). The Committee was informed that the preparation was stable and that a collaborative study was being arranged. The Committee authorized the National Institute for Biological Standards and Control, London, to establish the material as the International Standard for Netilmicin on the basis of the results of the collaborative study, and, with the agreement of the participants, to define the international unit.

3. Sisomicin

The Committee noted the results of the collaborative study of the proposed international standard for sisomicin (WHO/BS/84.1434). The Committee was informed that, in accordance with the authorization in its thirty-fourth report (WHO Technical Report series, No. 700, 1984, p. 13), the National Institute for Biological Standards and Control, London, on the basis of the results of the collaborative study and with the agreement of the participants, had established the preparation studied as the International Standard for Sisomicin and had defined the activity of the contents of each ampoule as 35 200 International Units of Sisomicin.
4. Tobramycin

The Committee noted that the collaborative study of the proposed international standard for tobramycin, referred to in its thirty-fourth report (WHO Technical Report Series, No. 700, 1984, p. 13), had been completed and that the results were being analysed (WHO/BS/84.1451).

5. Kanamycin

The Committee noted that, as stocks of the International Reference Preparation of Kanamycin were depleted, the National Institute for Biological Standards and Control, London, had obtained suitable replacement material and had freeze-dried it in ampoules (WHO/BS/84.1444). The Committee noted also that a collaborative study had been performed and that the results were being analysed.

The Committee authorized the National Institute for Biological Standards and Control, London, to establish the material studied as the International Standard for Kanamycin on the basis of the results of the collaborative study and, with the agreement of the participants, to define the international unit.

ANTIBODIES

6. Clostridium botulinum Type B Antitoxin, Equine

The Committee noted that the preparation intended to replace the International Standard for Clostridium botulinum Type B Antitoxin, Equine was being subjected to a collaborative study (WHO/BS/84.1435).

7. Cholera Antitoxin, Goat

The Committee noted that there was an urgent need for an international standard for cholera antitoxin for the assay of cholera antitoxin levels in man and for the calibration of cholera enterotoxins and toxoids (WHO/BS/84.1438).
The Committee noted also the generous offer of 400 ampoules by the Office of Biologics, Food and Drug Administration, Bethesda, MD, USA, of the US Standard Lot 1 of cholera antitoxin prepared in goats to serve as an international standard. The Committee accepted and established these ampoules of cholera antitoxin as the International Standard for Cholera Antitoxin and, in order to maintain the continuity of the national unit, assigned a potency of 2200 International Units of Cholera Antitoxin to the contents of each ampoule.

ANTIGENS

8. House Dust Mite (*Dermatophagoides pteronyssinus*) Extract

The Committee noted that, after a preliminary investigation of eight extracts of house dust mites (*D. pteronyssinus*), one preparation was selected as a proposed international reference preparation and that a collaborative study organized by the National Institute for Biological Standards and Control, London, had been completed (WHO/BS/84.1417). The results obtained by using several different assay systems had shown that all 20 participating laboratories agreed with the ranking of the activities of the five preparations included in the study.

The Committee established the proposed reference material (82/518) as the International Standard for House Dust Mite (*D. pteronyssinus*) Extract and defined the activity of the contents of each ampoule as 100 000 International Units of House Dust Mite (*D. pteronyssinus*) Extract.

BLOOD PRODUCTS AND RELATED SUBSTANCES

9. Anti-C Complete and Anti-E Complete Blood Typing Sera

The Committee was informed that, in accordance with the authorization in its thirty-fourth report (WHO Technical Report Series, No. 700, 1984, p. 17), the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, had
established the two preparations studied as, respectively, the International Standard for Anti-C Complete Blood Typing Serum, and the International Standard for Anti-E Complete Blood Typing Serum. The Central Laboratory of the Netherlands Red Cross Blood Transfusion Service had, with the agreement of the participants in the collaborative study, assigned an activity of 100 International Units of Anti-C Complete Blood Typing Serum to the contents of each ampoule of the International Standard for Anti-C Complete Blood Typing Serum, and an activity of 100 International Units of Anti-E Complete Blood Typing Serum to the contents of each ampoule of the International Standard for Anti-E Complete Blood Typing Serum.

10. Anti-D Complete Blood Typing Serum

The Committee was informed that serum had been collected and was being tested by the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, to be used as a standard for anti-D complete blood typing serum. After stability studies had been completed and other tests had also shown that the preparation is satisfactory, a collaborative study would be arranged.

11. Anti-varicella Zoster Immunoglobulin

The Committee was informed that nine laboratories had agreed to take part in the collaborative study of the anti-varicella zoster immunoglobulin referred to in its thirty-fourth report (WHO Technical Report Series, No. 700, 1984, p. 18).

12. Rabies Immunoglobulin

The Committee noted that a preparation of a rabies immunoglobulin, human, needed to replace the International Standard for Anti-Rabies Serum, Equine, had been obtained by the Office of Biologics, Food and Drug Administration, Bethesda, MD, USA, and had been freeze-dried in ampoules by the Royal Institute of Public Health and Environmental Hygiene, Bilthoven. Furthermore, a collaborative study organized by the Office of Biologics had been completed (WHO/BS/84.1433).
The Committee noted also that although the rapid, fluorescent, focus-inhibition test (RFFIT) was used by all laboratories, other in vitro methods yielded good agreement of potency estimates and were acceptable. The Committee established the material studied as the International Standard for Rabies Immunoglobulin and assigned an activity of 59 International Units of Rabies Immunoglobulin to the contents of each ampoule.

13. Prekallikrein Activator (PKA)

The Committee noted that a stable, freeze-dried preparation of prekallikrein activator (PKA) had been obtained and that a collaborative study in 13 laboratories organized by the National Institute for Biological Standards and Control, London, had been completed (WHO/BS/84.1454). The Committee noted also that there was good agreement between laboratories on the potency estimates of preparation 82/530.

The Committee established preparation 82/530 as the International Standard for Prekallikrein Activator (PKA) on the basis of the results of the collaborative study and, with the agreement of the participants, assigned an activity of 85 International Units of Prekallikrein Activator (PKA) to the contents of each ampoule.

14. Beta-Thromboglobulin (β-TG) and Platelet Factor 4 (PF₄)

The Committee noted that international standards for the platelet-specific proteins beta-thromboglobulin and platelet factor 4 (β-TG and PF₄) would serve a useful purpose in the comparison of data from different laboratories on the measurement of these two antigens (WHO/BS/84.1455). The Committee noted also that a collaborative study organized by the National Institute for Biological Standards and Control, London, in which purified materials and human plasma had been studied by 9 laboratories, had been completed.

The Committee established preparation 83/501 as the International Standard for Beta-Thromboglobulin (β-TG) on the basis of the results of the collaborative study and, with the agreement of the participants, assigned an activity of
500 International Units of Beta-Thromboglobulin (β-TG) to the contents of each ampoule. The Committee also established preparation 83/505 as the International Standard for Platelet Factor 4 (PF₄) on the basis of the results of the collaborative study and, with the agreement of the participants, assigned an activity of 400 International Units of Platelet Factor 4 (PF₄) to the contents of each ampoule.

15. Human Tissue Plasminogen Activator (t-PA)

The Committee noted the urgent need for a reference material for tissue plasminogen activator (t-PA) to standardize human tissue plasminogen activator produced by continuous cell lines (WHO/BS/84.1456). The Committee noted also that 7 laboratories had used a fibrin clot lysis assay for comparing the activities of three preparations, one of which (83/517) had been shown to be suitable as a reference material. The National Institute for Biological Standards and Control, London, had coordinated the study and selected the most suitable preparation.

The Committee established preparation 83/517 as the International Standard for Human Tissue Plasminogen Activator (t-PA) on the basis of the results of the collaborative study and, with the agreement of the participants, assigned an activity of 1000 International Units of Human Tissue Plasminogen Activator (t-PA) to the contents of each ampoule. The Committee stressed that the activity of this unit was not related to that of the International Unit of Urokinase.

16. Beta₂ Microglobulin (β₂m)

The Committee was informed that a reference material for the assay of beta₂ microglobulin (β₂m)—a serum protein of interest for the diagnosis and monitoring of various pathological conditions, such as diseases of the kidney and certain malignant diseases of the lymphoid system—had been prepared by the National Institute for Health and Medical Research (INSERM), Hôpital Edouard Herriot, Lyons, France, and subjected to a collaborative study. The Committee was informed also that another collaborative study was being carried out in order to characterize the material further and
to show whether its use improves the comparability of results using different assay methods.

ENDOCRINOLOGICAL AND RELATED SUBSTANCES

17. Insulins

The Committee noted that the collaborative study of the preparations of purified human, porcine, and bovine insulins ampouled in the form of hydrated insulin crystals, referred to in its thirty-fourth report (WHO Technical Report Series, No. 700, 1984, p. 19), was in progress (WHO/BS/84.1446).

18. Proinsulins and Human Insulin C-Peptide

The Committee noted that the preparation of human proinsulin and human insulin C-peptide, referred to in its thirty-fourth report (WHO Technical Report Series, No. 700, 1984, p. 20), had now been freeze-dried in ampoules with carrier lactose and bovine albumin, without degradation, and that a collaborative study was being arranged by the National Institute for Biological Standards and Control, London (WHO/BS/84.1447).

The Committee noted also that, in accordance with the request in its thirty-fourth report, quantities of purified porcine and bovine proinsulins had been obtained and freeze-dried in ampoules with carrier lactose and human albumin, without degradation, and that a collaborative study was being arranged.

19. Prolactin, Human, for Immunoassay

The Committee noted that, in accordance with the request made in its thirty-fourth report (WHO Technical Report Series, No. 700, 1984, p. 20), two additional candidate preparations of human prolactin had been obtained and freeze-dried in ampoules at the National Institute for Biological Standards and Control, London,
and that a collaborative study had been arranged (WHO/BS/84.1442). The Committee noted also that, in addition to these candidate materials, the collaborative study included the preparation (in ampoules coded 81/541) that was being issued as an interim measure because the supply of the first International Reference Preparation of Prolactin, Human, for Immunoassay, was depleted.

20. Calcitonins

The Committee noted that at the National Institute for Biological Standards and Control, London, analysis of the International Reference Preparation of Calcitonin, Salmon, for Bioassay, and of the International Reference Preparation of Calcitonin, Human, for Bioassay, had shown the presence of significant quantities of peptide impurities in both of these preparations, which had been ampouled more than 12 years ago. Quantities of highly purified preparations of each of the two calcitonins had therefore been obtained as replacements and freeze-dried in ampoules and would be subjected to a collaborative study (WHO/BS/84.1439).

The Committee noted also that there was a need for an international standard for a synthetic analogue (ASU 17) of eel calcitonin and requested the National Institute for Biological Standards and Control, London, to obtain suitable material.

21. Growth Hormone Releasing Factor

The Committee noted that there might be a need for an international standard for one of the two human growth hormone releasing factors for use in immunoassays in the investigation of growth disorders (WHO/BS/84.1441).

The Committee noted also that various fragments and analogue forms of this peptide, rather than the 40 or 44 residue forms, were being studied for their use in clinical treatment. The Committee therefore requested the National Institute for Biological Standards and Control, London, to investigate whether there is a need for an international standard and if necessary to obtain suitable material and arrange a collaborative study.
22. Alpha and Beta Subunits of Human Pituitary
Luteinizing Hormone

In 1974 the Committee had noted the need for reference materials of the alpha and beta subunits of human pituitary luteinizing hormone for use in the assessment of specificity of immunoassay kits. The Committee noted also that, in accordance with the request made in its twenty-sixth report (WHO Technical Report Series, No. 565, 1975, p. 16), preparations of these subunits had been obtained, ampouled, and characterized at the National Institute for Biological Standards and Control, London (WHO/BS/84.1443). For the past five years these ampouled preparations had been distributed on request as research reagents. Inquiries made recently to laboratories that had used them had revealed that the two preparations were useful and suitable as international reference materials. There had also been general agreement with the recommendation of the Committee that the international unit assigned to these preparations should be made equivalent to the amount of each of these two preparations considered to contain one microgram of each subunit.

The Committee therefore established one of the preparations, in ampoules coded 78/554, as the International Standard for Alpha Subunit of Human Pituitary Luteinizing Hormone, and assigned an activity of 10 International Units of Alpha Subunit of Human Pituitary Luteinizing Hormone to the contents of each ampoule. The Committee also established the other preparation, in ampoules coded 78/556, as the International Standard for Beta Subunit of Human Pituitary Luteinizing Hormone, and assigned an activity of 10 International Units of Beta Subunit of Human Pituitary Luteinizing Hormone to the contents of each ampoule.

23. Alpha and Beta Subunits of Thyroid Stimulating Hormone (TSH)

The Committee noted that there was a need for international standards for the alpha and beta subunits of human thyroid stimulating hormone for the assessment of specificity of immunoassay kits and monoclonal antibodies (WHO/BS/84.1449). The Committee was informed that suitable materials derived from
a well characterized preparation of thyroid stimulating hormone could be made available. The Committee therefore requested the National Institute for Biological Standards and Control, London, to obtain these materials and to arrange a collaborative study.

24. Follicle Stimulating Hormone (FSH)

The Committee noted that three batches of highly purified human pituitary follicle stimulating hormone had been obtained and had been shown, at the National Institute for Biological Standards and Control, London, to be similar to each other (WHO/BS/84.1450). The Committee noted also that these materials had been pooled, distributed in two large batches of ampoules, and freeze-dried without loss of biological activity, and that a collaborative study was being arranged.

25. Growth Hormone and Growth Factors

The Committee noted that there was a need for an international standard of the single molecular form of human growth hormone (relative molecular mass 22,000) for use in comparison tests of potency and of physicochemical identity of preparations of it made by recombinant DNA methods; the International Standard for Growth Hormone, for Bioassay, is unsuitable for certain physicochemical tests since it consists of a mixture of the several natural forms extractable from pituitary glands (WHO/BS/84.1453).

The Committee noted also that there was a need for an international standard for somatomedin C—the insulin-like growth factor whose secretion into the blood is stimulated by growth hormone—for use in tests to investigate metabolic disorders of growth and diabetes.

The Committee therefore authorized the National Institute for Biological Standards and Control, London, to arrange collaborative studies of the 22,000 relative molecular mass form of human growth hormone and somatomedin C.
26. Endotoxin

The Committee was informed that, in accordance with the request in its thirty-fourth report (WHO Technical Report Series, No. 700, 1984, p. 21), a quantity of bulk *Escherichia coli* endotoxin, from which the USA national standard for endotoxin for use in the *Limulus* amoebocyte lysate test was prepared, had been obtained by the National Institute for Biological Standards and Control, London. The Committee was informed also that preliminary trials were being carried out to study optimum ampouling conditions in order to ensure stability and reproducible solubility of the material.

27. Interferons

The Committee noted that an informal consultation on the standardization of interferons had taken place in Geneva to discuss the results of collaborative studies of four highly purified preparations of human interferons prepared by more modern techniques than those available when the International Reference Preparation of Interferon, Human, Leukocyte had been made (WHO/BS/84.1432).

On the basis of these collaborative assays, carried out under the aegis of the National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA, the Committee established the following standards.

1. Preparation Ga23-901-532 was established as the International Standard for Interferon, Human, Lymphoblastoid (Namalwa) (HuIFN-α (Ly)), and the contents of each ampoule were assigned an activity of 25,000 International Units of Interferon, Human, Lymphoblastoid (HuIFN-α (Ly)).

2. Preparation Gxa01-901-535 was established as the International Standard for Interferon, Human, rDNA (HuIFN-α₂ (αA)), and the contents of each ampoule were assigned an activity of 9000 International Units of Interferon, Human, rDNA (HuIFN-α₂ (αA)).

3. Preparation Gg23-901-530 was established as the International Standard for Interferon, Human (HuIFN-γ), and the
contents of each ampoule were assigned an activity of 4000 International Units of Interferon, Human (HuIFN-γ).

(4) Preparation Ga23-902-530 was established as the International Working Standard for Interferon, Human, Leukocyte (HuIFN-α (Le)), and the contents of each ampoule were assigned an activity of 12,000 International Units of Interferon, Human, Leukocyte (HuIFN-α (Le)).

The Committee noted also that there was a need for a working reference preparation of human interferon prepared in leukocytes and agreed that this standard should be made widely available for this purpose.

The Committee stressed that the units assigned to these preparations relate only to their in vitro antiviral activity and may not correlate with clinical activity.

The Committee agreed that the report of the informal consultation that gave a description of the results of the collaborative study on the standardization of interferons should be annexed to this report (Annex 1).

28. Rubella Antiserum, Rabbit

The Committee noted that, in accordance with the request made in its thirty-fourth report (WHO Technical Report Series, No. 700, 1984, p. 22), the Secretariat had made inquiries about the suitability of the International Reference Reagent of Rubella Antiserum, Rabbit, prepared against the M-33 rubella virus strain, for use in a virus neutralization test (WHO/BS/84.1452).

The Committee noted also that further data had been obtained from the National Institute of Health, Tokyo, which supplied the material, showing that the International Reference Reagent of Rubella Antiserum, Rabbit was suitable for use in a virus neutralization test.

29. Human Liver Ferritin Protein

The Committee noted the detailed description, by the International Committee for Standardization in Haematology, of the preparation of ferritin from human liver (WHO/BS/84.1457). The Committee noted also that the most suitable preparation, coded
80/602, had been freeze-dried in ampoules and subjected to a collaborative study.

On the basis of the results of the collaborative study and since the preparation had been shown to have adequate stability, the Committee established preparation 80/602 as the International Standard for Human Liver Ferritin Protein and assigned a quantity of 9.7 µg of human liver ferritin protein to the contents of each ampoule.

**REQUIREMENTS FOR BIOLOGICAL SUBSTANCES**

30. Requirements for Diphtheria Toxoid, Pertussis Vaccine, Tetanus Toxoid, and Combined Vaccines

The Committee noted that an informal consultation had been held in Geneva to discuss the potency assay of diphtheria and tetanus toxoids in single or combined vaccines (WHO/BS/84.1437 and WHO/BLG/UNDP/84.1). The Committee noted also that at the informal consultation the opportunity had been taken to revise some parts of the requirements.

The Committee agreed that further discussion was needed on the potency testing of diphtheria and tetanus toxoids and therefore deleted these sections from the proposed addendum. However, the other amendments were considered satisfactory. After this change, the Committee adopted the proposed addendum to the Requirements for Diphtheria Toxoid, Pertussis Vaccine, Tetanus Toxoid, and Combined Vaccines and agreed that it should be annexed to this report (Annex 2).

31. Requirements for Hepatitis B Vaccine Prepared from Plasma

The Committee was informed that since the Requirements for Hepatitis B Vaccine were first formulated (WHO Technical Report Series, No. 658, 1981, Annex 4) there had been a number of technical developments that necessitated amendments. Accordingly, the requirements had been revised, distributed for comment, and further
amended in the light of comments received (WHO/BS/83.1391 Rev. 2).

The Committee was informed that since the requirements were revised reports had been received that the production of the immunogen of the hepatitis B surface antigen had been successfully achieved in yeast by rDNA techniques and that the revised requirements were applicable only to hepatitis B vaccine prepared from the hepatitis B surface antigen obtained from HBsAg-positive human plasma. This had been made clear in the document.

The Committee adopted the revised requirements as the Requirements for Hepatitis B Vaccine (Revised) Prepared from Plasma and agreed that they should be annexed to this report (Annex 3).

32. Requirements for Varicella Vaccine (Live)

The Committee noted that varicella (chickenpox) vaccine would be useful for: (a) immunosuppressed persons, especially persons with malignancies and those who are receiving immunosuppressive therapy; (b) non-immune adults; (c) susceptible patients in hospital at risk of exposure; (d) susceptible health care professionals; (e) non-pregnant teenage females; and (f) susceptible children (WHO/BS/ 83.1394 Rev. 1). The Committee was informed, however, that at present it was unlikely that such a vaccine would be offered for routine immunization in children. The Committee noted also that a strain of varicella virus had been successfully attenuated and tested in specific groups of children, and that requirements had been formulated for this vaccine.

The Committee adopted the requirements as the Requirements for Varicella Vaccine (Live) and agreed that they should be annexed to this report (Annex 4).

ACKNOWLEDGEMENTS

The Committee thanks Dr. O. Sobeslavsky, Virus Diseases, WHO, Geneva for his special contribution to its deliberations.
STANDARDIZATION OF INTERFERONS

Report of a WHO Informal Consultation

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1. INTRODUCTION

A WHO informal consultation on the standardization of interferons took place in Geneva from 7 to 9 November 1983. The meeting was opened on behalf of the Director-General by Dr F. T. Perkins, Chief, Biologicals, who noted that, since the 1982 WHO informal consultation on the standardization of interferons, further progress had been made, particularly in the testing of new interferons.

1 Held in Geneva from 7 to 9 November 1983.
2. RESULTS OF THE 1983 INTERNATIONAL COLLABORATIVE ASSAY STUDY

Eight laboratories in five countries measured the activity of one proposed standard for human gamma-interferon (HuIFN-\(\gamma\)) and compared the activity of three types of human alpha-interferon (HuIFN-\(\alpha\)) preparations with the International Reference Preparation of Interferon, Human, Leukocyte (HuIFN-\(\alpha\)) (IRP 69/19). It was noted that this study represented the first international collaborative effort to measure interferon standards using a single bioassay with cells and virus obtained from a single source. All four preparations had been shown to be suitable as international reference materials and a recommendation was made that three of them be established as international standards. It was further recommended that the fourth, a preparation of human leukocyte HuIFN-\(\alpha\), should be recognized as an international working standard. Since the three proposed international standards had been hitherto unavailable, it was important that they be established without delay in order that laboratories throughout the world could compare their results. The report of the findings of the participating laboratories is attached (Appendix 2). It was anticipated that more studies investigating additional proposed standards would be carried out in the future.

3. REFERENCE BIOASSAY

The participants agreed that a reference bioassay suitable for the three types of human interferon was desirable for two main reasons. First, such an assay would be most useful for the standardization of interferon preparations containing mixtures of the various subtypes, i.e., mainly for preparations derived from fresh white blood cells or lymphoblastoid cells. Second, the availability of an assay that was reliable, accurate, and relatively easy to perform would be of value especially to laboratories that did not specialize in interferon research. However, it was important to realize that no single bioassay would be suitable for all kinds of interferon studies. Furthermore, not all problems inherent in the evaluation of multicomponent interferon preparations would be solved by the use of a standard assay procedure. Attempts to develop a reference bioassay suitable for all types of interferon have already been made. In the international
collaborative study, reduction in infectivity-yield of a single-cycle encephalomyocarditis virus in a human carcinoma cell line (A549) was measured to calibrate several human interferon reference preparations (Appendix 2, Part I). Although satisfactory results were obtained, most investigators who participated in the international collaborative study felt that this assay was too cumbersome to serve as a permanent reference assay.

The participants felt that another reference bioassay should be selected and suggested that the US National Institutes of Health should consider convening a meeting of experts devoted to a more thorough analysis of this subject.

4. IMMUNOASSAYS

Interferons can be quantified by immunoassay in a variety of ways as outlined in the 1982 report of the WHO informal consultation on the standardization of interferons.¹ These assays require either pure interferon, highly specific polyclonal antibody, or monoclonal antibody to interferon. One of these reagents is usually labelled, either with a radioisotope (e.g., ¹²⁵I) or conjugated to an enzyme, (e.g., horseradish peroxidase).

Although calibration of the antigen content in relation to units of biological activity remains a problem,² available evidence indicates that a high degree of agreement can be achieved. Two of the available immunoassays have proved very useful in quantifying interferon in the sera of patients treated with HuIFN-α, and indications are that these assays may replace bioassays for this particular application.² It is important to note that these two immunoassays do not detect all HuIFN-α subtypes and are therefore not suitable for quantifying multicomponent HuIFN-α preparations.

In addition to these current methods, new immunoassays designed to detect HuIFN-γ are being developed. One sandwich-type assay, in which two different monoclonal antibodies to HuIFN-γ are used, can detect interferon concentrations as low as 0.3 IU/ml.

Recent developments in immunoassays should allow the standardization of interferons by weight of pure interferon (e.g., individual HuIFN-α subtypes). This will require the availability of international standards with interferon contents defined by weight;

these standards could serve to calibrate other immunoassays. However, an assay for antiviral activity will still be required for preparations intended for clinical use.

5. MEASUREMENT OF ANTIBODIES TO INTERFERON IN SERA OF PATIENTS

When assaying for the presence of antibodies to interferon in serum samples from patients, it is desirable to use the most sensitive procedure available. One procedure for neutralization assays is to use a constant dilution of interferon and different dilutions of antiserum; this method is described in the 1982 report of the WHO informal consultation on the standardization of interferons.¹

In order to allow for interlaboratory comparisons of assay sensitivities and antibody content of serum specimens, the following details of the assay used should be reported: final concentration of interferon and serum in the reaction mixture; final volume of reaction mixture; and lowest final dilution of serum tested. It should be noted that when a mixture of interferon subtypes is used in a neutralization assay, complete neutralization will occur only if the patient’s serum contains neutralizing antibodies against all the subtypes present.

When a highly purified radiolabelled interferon is available, an additional procedure that detects non-neutralizing as well as neutralizing antibodies may be used. Aliquots of serum are incubated with a defined quantity of labelled interferon, and putative interferon–antibody complexes are precipitated by the use of a second antibody (e.g., goat anti-human IgG). The second antibody may be immobilized on an inert substance, such as agarose, if appropriate. If alternative tests are used, such as an immunoradiometric assay of interferon not neutralized by serum, they must be properly validated.

6. ASSAYS OF ANTIPROLIFERATIVE AND OTHER CELLULAR EFFECTS OF INTERFERONS

In addition to their antiviral effect, interferons have various other biological actions, all of which can be assayed by one or more procedures. Comparisons of experimental results between

laboratories are often difficult because different methods are used and some form of standardization would seem advisable.

6.1 Inhibitory effect of interferons on cell growth

Interferons can inhibit the proliferation of cells. While it is assumed that this effect plays some role in the anticancer potential of interferons, its importance relative to other biological actions is not certain. Therefore, assays of the antiproliferative effect of interferon preparations cannot at present be said to have a predictive value with regard to their clinical antitumour potential. With this reservation, it would be desirable that laboratories include a reference antiproliferative bioassay in their panel of assays. Thus, studies to develop an antiproliferative assay applicable to all available types and subtypes of human interferon are encouraged. Currently, the most widely used assay utilizes the lymphoblastoid cell line (Daudi), which is among the most sensitive to the antiproliferative effects of HuIFN-α and HuIFN-β. However, this cell line has poor sensitivity to the antiproliferative effect of HuIFN-γ.

6.2 Activation of mononuclear cells

Interferons activate the cytoidal effect of lymphocytes in various assay systems (natural killer activity, antibody-dependent-cellular cytotoxicity, cytotoxicity of macrophages or adherent mononuclear cells). The predictive value of these assays for the clinical antitumour potential of a given interferon preparation is uncertain. Since results obtained in these assays are highly variable and depend on the origin and physiological state of the mononuclear cells as well as the target cells, it is not possible to recommend a reference bioassay at present.

6.3 Other assay systems

In the future, assays relying on various other effects of interferons may become of increasing interest to laboratory workers or clinicians—for example, induction of cellular enzymes (2',5'-oligoadenylate synthetase, protein kinase (EC 2.7.1.37)), enhanced expression of Fc receptors and various cell-surface antigens, and modulation of cellular differentiation. Some form of standardization of assays for these effects may be useful.
7. NEW INTERFERON STANDARDS

7.1 Human standards

In addition to the proposed standards for the three HuIFN-α and one HuIFN-γ, proposed standards for HuIFN-α₁ and HuIFN-β have also been developed and are awaiting evaluation.

Further research standards for a number of human interferons are likely to be required. In this regard it should be noted, for example, that since there are several preparations of HuIFN-β (glycosylated molecules derived from human cells, non-glycosylated molecules derived from *E. coli*, and modified molecules with serine instead of cysteine in position 17 also derived from *E. coli*), and since there are preliminary indications that different relative potencies may be obtained for *E. coli*-derived HuIFN-β and the International Reference Preparation of Interferon, Human, Fibroblast (β) depending on assay conditions, it may be necessary to develop homologous standards for individual recombinant interferons. In the meantime, units can be assigned to preparations of such interferons by comparison with the international reference preparations. However, such an assignment of units should not be taken to imply biological equivalence in all systems.

7.2 Animal standards

Since the 1960s, chicken, hamster, mouse, rabbit, and rat interferons have been used in antiviral or antitumour studies in the homologous animal host. It is recognized that further animal studies are required to show how best to use interferons clinically. Appropriate standards will enable data from different laboratories to be compared in a meaningful way. International standards for some animal interferons are already available, and for others purified mouse α-, β-, and γ-interferons are being prepared. Standards for other animal interferons are likely to be needed, but the impetus for their provision must come from the workers directly involved. The participants in the consultation suggested that those interested in a particular standard material should contact the Antiviral Substances Program, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health,
8. SUMMARY AND CONCLUSIONS

With the continuing increase in clinical and experimental studies on interferons, greater emphasis must be placed on the precise measurement of the activities of these substances, especially in terms of appropriate international standards. To this end, additional proposed standard preparations have been prepared, characterized, and tested: three purified HuIFN-α preparations and one HuIFN-γ preparation were titrated for potency and were compared with the HuIFN-α International Reference Preparation of Interferon, Human Leukocyte (69/19) by eight laboratories in five countries. The materials were tested by means of a reference bioassay as well as by the routine assays used in the individual laboratories. Since the data summarized herein established their suitability, it is recommended that the following be accepted as WHO international standards for interferons:

(a) Preparation Ga23-901-532\textsuperscript{1} as the international standard for interferon, human, lymphoblastoid (Namalwa) (HuIFN-α (Ly)), having an activity of 25 000 (4.40 log_{10}) IU per ampoule;

(b) Preparation Gxa01-901-535\textsuperscript{1} as the international standard for interferon, human, recombinant (HuIFN-α₂ (αA)), having an activity of 9000 (3.95 log_{10}) IU per ampoule; and

(c) Preparation Gg23-901-530\textsuperscript{1} as the international standard for interferon, human (HuIFN-γ), having an activity of 4000 (3.6 log_{10}) IU per ampoule.

\textsuperscript{1} The description of the preparation and the results of testing these materials are attached as Appendix 2, Part 2.

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In view of the urgent need for these standards, the participants requested the WHO Secretariat to take the necessary steps to establish them as soon as possible.

The participants further recommended that WHO should adopt as an international working standard the preparation Ga23-902-530\(^1\) for interferon, human, leukocyte (HuIFN-α (Le)), having an activity of 12,000 (4.08 log\(_{10}\)) IU per ampoule.

Other purified interferon preparations suitable for possible future use as international standards should be available soon for international collaborative testing. These include HuIFN-α₁, a replacement HuIFN-β, and mouse MuIFN-α, -β, and -γ preparations.

Additional standard preparations for both human and animal interferons are likely to be needed.

The recommendations made in the previous report,\(^2\) emphasizing the need for details of assay methods and results (either for publication or for submission to regulatory agencies) are strongly reaffirmed.

It was agreed that a reference antiviral bioassay suitable for measuring all three types of human interferon is desirable. A virus infectivity yield-reduction method, the previously proposed reference bioassay employed in the recent international collaborative assay, gave satisfactory results but was found to be cumbersome and uneconomical. Another reference bioassay should be selected as soon as a meeting of experts can be arranged to analyse suitable alternative methods.

Progress continues to be made in the development of radiometric and enzyme-linked interferon immunoassays, as well as of monoclonal antibodies and other suitable reagents. Further work needs to be done on these rapid and reproducible assays, particularly to establish whether a suitable correlation exists between biological potency and the specific antigenic content of interferon preparations and clinical samples.

The need to employ the most sensitive procedures for the detection of antibodies to interferon in the sera of patients was stressed. Although interferons are known to inhibit cell proliferation, induce the production of certain cellular enzymes, alter the expression of cellular surface antigens, and activate mononuclear cells, the correlation of such effects with the course of

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\(^1\) See footnote on page 34.

a disease remains to be established. There is a need to develop tests to measure the non-antiviral, biological activities of interferons that can have predictive or clinical value.

Appendix 1

LIST OF PARTICIPANTS

Professor A. Billiau, Catholic University of Louvain, Raga Institute, Louvain, Belgium
Mr P. Dennis, Celltech Ltd., Slough, England
Dr E. De Maeyer, Biology Department, Curie Institute, Orsay, France
Dr N. Finter, Wellcome Research Laboratories, Beckenham, England
Dr G.J. Galasso, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA (Chairman)
Professor S.F. Grossberg, Department of Microbiology, Medical College of Wisconsin, Milwaukee, WI, USA (Rapporteur)
Dr J. Hilfenhaus, Behringwerke AG, Marburg, Federal Republic of Germany
Dr M. de J. Limonta, Director, Biological Research Center, Havana, Cuba
Dr A. Meager, National Institute for Biological Standards and Control, London, England
Mr E.M. Oden, Microbiological Research, Schering Corporation, Bloomfield, NJ, USA
Dr G. Petranyi, National Institute of Haematology and Blood Transfusion, Budapest, Hungary
Dr H. Surprenant, Schering Corporation, Bloomfield, NJ, USA
Dr D. Testa, Interferon Sciences, Inc., New Brunswick, NJ, USA
Dr P.W. Trow, Director, Department of Experimental and Applied Biology, Hoffmann-La Roche, Nutley, NJ, USA
Professor J. Vilcek, Department of Microbiology, New York University Medical Center, New York, NY, USA
Dr S. Yamazaki, Director, Central Virus Diagnostic Laboratory, National Institute of Health, Tokyo, Japan

WHO Secretariat

Dr F.T. Perkins, Chief, Biologicals, World Health Organization, Geneva, Switzerland (Secretary)
Dr P. Sizaret, Biologicals, World Health Organization, Geneva, Switzerland
Dr V. Bektimirov, Chief, Virus Diseases, World Health Organization, Geneva, Switzerland
Appendix 2

PART I
RESULTS OF THE INTERNATIONAL COLLABORATIVE ASSAY STUDY—1983

1. HUMAN INTERFERON REFERENCE PREPARATIONS

An international collaborative study to test the potency of one proposed standard for HuIFN-γ and three proposed standards for HuIFN-α was completed in 1983. Each participating laboratory was asked to use its routine bioassay, as well as a proposed reference bioassay to titrate these preparations and the International Reference Preparation of Interferon, Human, Leukocyte (IRP 69/19), on five different occasions. The reference bioassay was an infectivity yield-reduction method employing encephalomyocarditis (EMC) virus in the human lung carcinoma cell line A549; infectivity yields were measured in mouse L cells by a cytopathic effect endpoint. The virus and cell lines were sent by Professor S.E. Grossberg, Department of Microbiology, Medical College of Wisconsin, Milwaukee, WI, USA, to each participating laboratory along with details of the appropriate procedures. Among those who analysed the raw data obtained from each laboratory was the biostatistician involved in all previous collaborative testing of freeze-dried interferon standards prepared at the Medical College of Wisconsin for the National Institutes of Health. This study represents the first international collaborative effort to measure interferon standards using a single bioassay with cells and virus obtained from a single source.

The following four human samples were supplied and distributed by the National Institutes of Health, Bethesda, MD, USA:

(a) Ga23-901-532, a preparation produced in Namalwa lymphoblastoid cells, highly purified and characterized by Dr N. Finter, Wellcome Research Laboratories, Beckenham, Kent, England and freeze-dried at the Medical College of Wisconsin;

(b) Gxa01-901-535 HuIFN-α (αA) produced in Escherichia coli by a recombinant DNA technique and highly purified and freeze-dried at Hoffmann-La Roche, Nutley, NJ, USA;

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(c) The HuIFN-γ preparation, Gg23-901-530, produced in peripheral blood lymphocytes, partially purified, freeze-dried, and characterized at the Medical College of Wisconsin.

(d) Ga23-902-530, a preparation produced in leukocytes and partially purified by Professor K. Cantell, Department of Virology, National Public Health Institute, Helsinki, Finland and subsequently diluted, freeze-dried, and characterized at the Medical College of Wisconsin; and

(e) The International Reference Preparation of Interferon, Human, Leukocyte (IRP 69/19).

More detailed information on each of the four proposed standards is given in Part 2 of this appendix.

1.1 HuIFN-α standards

Data were analysed from the eight laboratories in five countries that submitted results of at least two titrations performed with the reference bioassay method. Dose–response curves were constructed by linear regression analysis of the data from each reference bioassay titration performed by each laboratory. The end-point was taken to be the 0.5 log₁₀ reduction in virus yield. The slopes of the response curves for a given sample were much less variable within a laboratory than among laboratories, and no statistically significant differences in slopes were obtained among the different HuIFN-α preparations. The results are presented in Tables 1–3 and 5.

Tables 1, 2, and 3 summarize the observed, uncorrected titres obtained by the reference bioassay. On the basis of these data, a biological activity expressed in International Units per ampoule was assigned to the three NIH (HuIFN-α) reference preparations by comparison with the IRP 69/19 (Table 5). It is recognized that this first assignment of units to the lymphoblastoid α₂ recombinant interferon preparations is arbitrary, since they are not homologous with the IRP 69/19. However, the assigned values are likely to be in the range obtained by most workers in their bioassay.

1.2 HuIFN-γ preparation

Table 4 summarizes the test results obtained with the HuIFN-γ proposed standard, Gg23-901-530. In this first assignment of units to a HuIFN-γ proposed standard, a potency of 4000 (3.6 log₁₀) reference units per ampoule was chosen on the basis of the results
obtained with the reference bioassay, and it was proposed that this unitage be adopted as the potency in international units.

2. REFERENCE BIOASSAY

The EMC virus yield-reduction assay was selected largely on the recommendations of the Woodstock Conference on Standards,1 which were endorsed with minor amendments at the 1980 informal consultation on the standardization of interferons.2 EMC virus was selected because it can be easily cultured, it is stable, it has a wide host range, its use is acceptable throughout the world, and it is relatively non-pathogenic to man. The assay method (reduction in yield of infectious virus) was selected because it gives unequivocal end-points (as do other yield-reduction assays), it is considered to be relatively sensitive, and it satisfies the requirement that interferon action be shown in terms of reduction in virus infectivity. The A549 cell line was selected in preference to other cell lines or strains because it is sensitive to α-, β-, and γ-interferons, it has not changed in its sensitivity to interferons during a large number of passages over a period of several years, and it is easier to handle and grows faster than diploid cell strains. Although originally used with mouse interferon, the proposed reference bioassay for human interferons using an EMC/A549 yield-reduction method was demonstrated by collaborative testing to be feasible and to have relatively high sensitivity in that it measured a greater number of units than that assigned to the IRP 69/19. However, this infectivity yield-reduction assay was shown to be relatively slow and uneconomical.

Together with the reference assay, the participating laboratories also employed their routine assays for testing the interferons. The data suggest that some of the assays used were of sufficient sensitivity and reproducibility and could be considered for use in future collaborative studies of standards. Such assays include the dye-uptake method using FL cells, Sindbis virus with an objectively determined end-point (Lab No. 6), and the haemagglutinin yield-reduction assay (Lab No. 5) using A549 cells and EMC virus. In the selection of components for an assay, consideration should be given to the fact that different sub-lines of continuous cell lines can vary considerably in their sensitivity to interferon. The results from

laboratories 1 and 6 obtained by the same routine assay method illustrate this point.

3. ESTABLISHMENT OF THE HuIFN-α AND HuIFN-γ PREPARATIONS AS INTERNATIONAL INTERFERON STANDARDS

The participants in the WHO informal consultation on the standardization of interferons agreed that it was urgent for the WHO Secretariat to take the necessary steps to obtain the approval of the WHO Expert Committee on Biological Standardization for the establishment of the following proposed standards as WHO international standards for interferons:

(a) Preparation Ga23-901-532 as the international standard for interferon, human, lymphoblastoid (Namalwa) (HuIFN-α (Ly)), having an activity of 25 000 (4.40 log_{10} IU per ampoule;

(b) Preparation Gxa01-901-535 as the international standard for interferon, human, recombinant (HuIFN-α_2 (aA)), having an activity of 9000 (3.95 log_{10}) IU per ampoule;

(c) Preparation Gg23-901-530 as the international standard for interferon, human (HuIFN-γ), having an activity of 4000 (3.6 log_{10}) IU per ampoule.

The participants further requested WHO to adopt the preparation Ga23-902-530 as international working standard for interferon, human, leukocyte (HuIFN-α (Le)), having an activity of 12 000 (4.08 log_{10}) IU per ampoule.
PART 2
DESCRIPTION OF PREPARATION OF PROPOSED INTERNATIONAL STANDARDS FOR INTERFERONS

1. FREEZE-DRIED HUMAN LYMPHOBLASTOID (NAMALWA) INTERFERON ALPHA REFERENCE (Ga23-901-532)

1.1 Preparation

Human lymphoblastoid alpha-interferon (HuIFN-α (Ly)) was prepared by the Wellcome Research Laboratories, Wellcome Foundation Ltd., Beckenham, Kent, England. Cultures of the human lymphoblastoid Namalwa cell line were infected with Sendai virus (1). After one day of incubation, the supernatant fluids were collected following centrifugation, and the interferon was purified by a series of differential precipitation (2) and chromatographic steps to obtain a purity of 87.4% and specific activity of 10⁶ IU/mg in a preparation composed of at least eight different alpha-interferons as distinguished by physicochemical characterization (1). After shipment to the Medical College of Wisconsin, Milwaukee, WI, USA, the material was stored at −70°C. Subsequently, the sterile interferon preparation, containing 44.56 μg of HuIFN-α (Ly), was aseptically diluted into ice-cold, sterile buffer solution composed of 0.1 mol/l sodium phosphate buffer (pH 7) supplemented with 5 mg/ml human serum albumin (Travenol “Buminate”). The vessel was packed in wet ice to keep the solution chilled during the process of filling of ampoules; 1.00-ml portions, containing 13.5 ng of HuIFN-α (Ly), were dispensed into borosilicate glass ampoules using a high-precision Hamilton dispenser. The reproducibility of the fill, as measured by the weight of liquid dispensed into 25 preweighed vials (distributed throughout the fill), was 0.61 (coefficient of variation). Ampoules were filled in groups of 19, and held on ice until five groups were filled, and were then placed in the refrigerated chamber of the freeze-dryer. After all ampoules were filled they were frozen at −30°C, and the material was dried to a residual moisture of about 3%. The ampoules were then backfilled with argon and heat-sealed at atmospheric pressure. The last ampoule filled in each group of 19 was marked for testing of sterility and antiviral activity after
freeze-drying. Ampoules are stored at $-70^\circ$C, but can be shipped at ambient temperatures.

1.2 Recommendations for reconstitution

Add 1.0 ml of sterile distilled water to the lyophilized powder, taking care that no material is lost in the neck or stem of the ampoules. Small portions of the reconstituted interferon may be stored at $-70^\circ$C for dilution at another time. However, a suitable amount of an appropriate dilution, based on the known sensitivity of the assay being used, should be made in the freeze-drying buffer (see above) supplemented with 5 mg of human serum albumin per ml or in serum-containing culture medium used in the biological assay. Aliquots of the diluted interferon should preferably be stored at $-70^\circ$C, and the volume of each aliquot should be sufficient for a single titration. It may be possible to store enough material in a single container at $-70^\circ$C for use in as many as 3 titrations: more extensive repeated thawing and freezing can result in loss of activity. All liquid samples should be stored at $-70^\circ$C or lower.

1.3 Stability

The freeze-dried reference preparation was tested twice by the linear non-isothermal accelerated degradation test (3) in which the material is progressively heated from 50 to 90 $^\circ$C over a 28-hour period. In further titrations there was a range of inactivation from 0 to 90% between 50 and 80 $^\circ$C; but the observation that some of the degradation curves showed no loss of activity gave evidence that in spite of variability of results, the interferon was sufficiently stable. From the results of the predictive multiple isothermal accelerated degradation test (3) involving storage of ampoules at 52 $^\circ$C, 60 $^\circ$C, 68 $^\circ$C, and 76 $^\circ$C, with samples being removed at appropriate intervals over 11 months, the product was estimated to be stable at $-70^\circ$C for several decades. The length of time during which the preparation was predicted to lose 1 log of activity at higher temperatures was estimated from these data to be: 0.34 years at 56 $^\circ$C, 1.58 years at 37 $^\circ$C, 6.79 years at 20 $^\circ$C, 11.16 years at 4 $^\circ$C, 24.42 years at $-20^\circ$C, and 233 years at $-70^\circ$C.
1.4 Test results

No mycoplasma, bacteria, or fungi were detected in 43 samples tested from the 162 different groups of ampoule comprising the reference lot. The interferon used for freeze-drying was diluted to contain 1 mg of human serum albumin per ml and characterized as follows: it was non-sedimentable at 100,000 g, more than 99% inactivated by trypsin in 1 h, inactivated about 50% during heating at 56°C for up to 3 h, and not inactivated during 48 h of pH 2 dialysis at 4°C. The product was not neutralized by two antisera to HuIFN-γ (one provided by Dr I. Braude, Meloy Labs, Springfield, VA, USA, and the other prepared at the Medical College of Wisconsin against purified HuIFN-γ) or by anti-HuIFN-β serum (NIH GO28-501-568). However, it was neutralized completely by anti-HuIFN-α serum (NIH GO26-502-568). The interferon was found to be of one molecular size (relative molecular mass 15,500) as estimated by sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis in phosphate buffer by the method of Weber & Osborne. Analysis of HuIFN-α by isoelectric focusing revealed one major peak of activity at pH 5.8, with a shoulder at pH 6.2.

The potency was determined from the data provided by the eight international laboratories that had performed two or more titrations of the preparation using a microtitration modification of the proposed reference bioassay technique (Table 1) (4, 5). The reference bioassay involves the reduction in yield of infectious EMC virus in the A549 line of human lung carcinoma cells; EMC virus yields were measured in L cells. The mean of the geometric mean titre (GMT) calculated from the GMT values reported by each laboratory (total number of titrations = 44) was 4.59 log units/ampoule, with a standard deviation, SD of 0.38 log units corresponding to about 2.4-fold variation. The titration of the HuIFN-α (Ly) by routinely used bioassays of different types with various cell–virus combinations (mostly dye-uptake measurements of cytolysis) gave GMT values ranging from 3.69 to 4.95 log units/ampoule, with a mean of 4.20 log units/ampoule (SD 0.53). Additional information is provided in Table 1 (page 60).

There was considerable activity on the cells of heterologous species (which is characteristic of this type of interferon), with the following observed unadjusted titres obtained by the EMC virus haemagglutination yield-reduction method (6): 1.2 × 10^5 laboratory units (LU)/ampoule in human A549 cells; 1.9 × 10^5 LU/ampoule in
bovine EBTr cells; $7 \times 10^4$ LU/ampoule in feline FEA cells; and 320 LU/ampoule in murine L cells.

1.5 Titre assignment

The potency was calculated in relation to that of the International Reference Preparation 69/19. To calculate the unitage of HuIFN-α (Ly) in terms of the IU established for IRP 69/19 HuIFN-α (Le), the slopes of dose–response curves for HuIFN-α (Ly) and IRP 69/19 were analysed. There was no significant difference in the slopes of the two interferons, whether calculated from the 44 individual titrations (1.165 (SD 0.18) and 1.137 (SD 0.18), respectively) or from the average slopes observed in each of the eight laboratories (1.142 (SD 0.24) and 1.074 (SD 0.26), respectively). Hence, the preparation Ga23-901-532 was assigned a potency of 25 000 International Units or 4.40 log IU/ampoule (see Table 5, page 64).

1.6 Use of the proposed international standard for interferon, human, lymphoblastoid (Namalwa)

This standard is intended for the comparison of the sensitivities of the bioassays used in different laboratories for measuring the antiviral activity of HuIFN-α (Ly). This preparation should be used only for the calibration of those laboratory preparations of HuIFN-α (Ly) that have dose–response curves parallel to the dose–response curves of this preparation (4, 5, 7–9). It should be noted that if the number or proportion of different IFN-α subtypes in a given lymphoblastoid interferon preparation under test are known to differ significantly from that in this reference preparation, then the use of this reference preparation may not be appropriate. It is recommended by WHO (4, 5, 7–9) that each laboratory should measure the HuIFN-α (Ly) reference reagent simultaneously with an internal laboratory standard in five or more titrations done on separate occasions, and should report the observed logarithm of the geometric mean titre (GMT) or laboratory unit (LU) and its standard deviation along with the assigned titre (as the logarithm) of the reference reagent interferon in published works.

The potency of the laboratory standard in International Units per ml is calculated in relation to the proposed international standard as follows:
assigned potency
(in IU) of the
international standard
potency (in LU) of the
international standard observed from GMT

\times

potency (in IU)

\frac{\text{potency (in IU)} \times \text{potency (in LU) of the}}{\text{potency (in LU) of the laboratory standard observed from GMT}} = \text{potency (in IU) of the laboratory standard observed from GMT}})

Similarly, the laboratory standard may be used to determine the titre of a test sample (in IU) as follows:

\frac{\text{potency (in LU) of the laboratory standard observed from GMT}}{\text{potency (in LU) of the test sample observed from GMT}} = \text{potency (in IU) of the test sample.}

REFERENCES


2. FREEZE-DRIED HUMAN RECOMBINANT INTERFERON-α₂ (αA) (Gxa01-901-535)

2.1 Preparation

Recombinant human alpha₂ (alpha A) interferon (HuIFN-α₂(αA)) was prepared at the Roche Research Center in Nutley, NJ, USA. E. coli cells transformed with a plasmid derived from pLeIFA25 (1) were grown in a nutrient medium under conditions that permitted the organism to produce HuIFN-α₂ (αA). The cells containing HuIFN-α₂ (αA) were killed by treatment at low pH (about 1.8), harvested, passed through a mechanical grinder, and suspended in extraction buffer. The resultant cell debris and nucleic acids were flocculated and removed by centrifugation. The HuIFN-α₂ (αA) in the supernatant fluid was purified by a series of procedures involving affinity chromatography on immobilized anti-interferon monoclonal antibodies, cation-exchange chromatography (2), and molecular-exclusion chromatography.

The highly purified HuIFN-α₂ (αA) was diluted to the required concentration with a solution containing sodium chloride (9 mg/ml) and human serum albumin (5 mg/ml). The pH was adjusted to 6.9 with sodium hydroxide and the solution was filter-sterilized. One-ml aliquots were aseptically dispensed into sterile ampoules. The contents of the ampoules were lyophilized and the ampoules were sealed under nitrogen. The reproducibility of the fill, as measured by the determination of the protein content of 20 ampoules, was ± 8.8% (coefficient of variation).

2.2 Recommendations for reconstitution

Add 1.0 ml of sterile distilled water to the lyophilized powder, taking care that no loss of material occurs in the neck or stem of the ampoule. Portions of this reconstituted material can be stored at −70°C. The material may also be stored (at −70°C) after appropriate (e.g., 1 : 10) dilution, preferably in 0.1 M solution of sodium phosphate buffer (pH 7) containing 5 mg of human serum albumin per ml; Hanks’ salt solution with 5 mg of human serum albumin per ml or serum-containing culture medium may be substituted. For optimum, long-term preservation of stability, samples of the liquid material should be stored at −70°C.
2.3 Stability

The freeze-dried reference preparation did not lose any activity in the linear non-isothermal accelerated degradation test (2), in which the material is progressively heated from 50 °C to 90 °C over a 28-hour period. The results of the predictive multiple isothermal accelerated degradation test (3), involving storage at 52 °C, 60 °C, 68 °C, and 76 °C for periods up to 1 year, show that the product is estimated to have unlimited stability at −20 °C and −70 °C. The length of time during which the preparation was predicted to lose 1 log of activity at temperatures above freezing was estimated from these data to be: 2.46 years at 56 °C, 16 years at 37 °C, 94.5 years at 20 °C, and 679 years at 4 °C.

2.4 Test results

Forty containers were tested for sterility according to the Direct Plant Method (USA Patent). The vials were reconstituted with sterile water, and 20 vials were tested in fluid thioglycollate medium and 20 in trypticase soy broth. No evidence of microbial growth was observed during the incubation period.

The amino acid sequence of the HuIFN-α₂ (αA) used to prepare this standard differs from that predicted from the DNA sequence reported by Streuli et al (4) in that the amino acid at position 23 is lysine instead of arginine. The purity was 99% as determined by photometric scanning of gels following non-reducing sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis. The relative molecular mass of the HuIFN-α₂ (αA) was estimated to be 18 500. It was non-sedimentable at 100 000 g for 90 minutes, stable at pH 2, and inactivated by trypsin. The specific activity of the HuIFN-α₂ (αA) used was 2 \times 10^8 International Units/mg of protein as determined in a cytopathic effect-reduction assay (5) using WISH cells and vesicular stomatitis virus (VSV) as challenge.

The potency was determined from the data provided by the eight international laboratories that had performed two or more titrations of the preparation using a microtitration modification of the proposed reference bioassay technique (Table 2) (6, 7). The reference bioassay involves the reduction in yield of infectious EMC virus in the A549 line of human lung carcinoma cells; EMC virus yields were measured in L cells. The mean of the geometric mean titre (GMT) values calculated from data reported by each laboratory (total number of titrations = 43) was 4.13 log units/ampoule, with a
standard deviation (SD) of 0.31 log, corresponding to about 2.0-fold variation. Titration of the HuIFN-α₂ (αA) by routinely used bioassays of different types with various virus–cell combinations, mostly dye-uptake measurements of cytolysis, gave GMT values ranging from 2.92 to 4.46 log units/ampoule, with a mean GMT of 3.71 log units/ampoule and an interlaboratory standard deviation of 0.59 log. Additional information is provided in Table 2 (page 61).

Antiviral activity (expressed as a percentage of that observed in human WISH amnion cells) was observed in the following cell lines: monkey, Vero 9%; bovine, MDBK 150%; guinea-pig transformed, 30%; and feline, Felung 130%. Negligible activity (< 1% of that in human WISH cells) was observed in cell lines of the following species: mouse, rat, rabbit, hamster, and horse.

2.5 Titre assignment

The potency was calculated in relation to that of the International Reference Preparation 69/19. On the basis of data from the eight participating laboratories, the preparation was assigned a potency of 9000 IU/ampoule (3.95 log₁₀/ampoule).

2.6 Use of the proposed international standard for interferon, human, rDNA

This standard is intended for comparison of the sensitivities of the bioassays used in different laboratories for the measurement of the antiviral activity of HuIFN-α₂ (αA). It should be noted that it should be used for the calibration of only those laboratory preparations of HuIFN-α₂ (αA) that have dose–response curves parallel to the dose–response curve of this preparation (6–10).

Each laboratory should measure the potency of the proposed standard simultaneously with an internal laboratory standard in five or more titrations done on separate occasions. It is recommended that the name and assigned unitage of the standard (in international units) should be stated along with the logarithm of the observed geometric mean titre (GMT), its standard deviation, and the number of titrations done to obtain this result, thereby indicating the sensitivity of the assay (6–10).

For the sake of convenience, a large number of aliquots of the calibrated standard material should be kept frozen at –70 °C and titrated every time an assay of HuIFN-α₂ (αA) sample is run.
The potency of the laboratory standard in International Units per ml is calculated in relation to the proposed international standard as follows:

\[
\frac{\text{assigned potency (in IU) of the international standard}}{\text{potency (in LU) of the international standard observed from GMT}} \times \frac{\text{potency (in LU) of the laboratory standard}}{\text{potency (in IU) of the laboratory standard observed from GMT}} = \text{potency (in IU) of the laboratory standard}.
\]

Similarly, the laboratory standard may be used to determine the titre of a test sample (in IU) as follows:

\[
\frac{\text{potency (in LU) of the laboratory standard}}{\text{potency (in LU) of the laboratory standard observed from GMT}} \times \frac{\text{potency (in IU) of the test sample}}{\text{potency (in IU) of the test sample observed from GMT}} = \text{potency (in IU) of the test sample}.
\]

REFERENCES


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3. FREEZE-DRIED HUMAN LEUKOCYTE ALPHA-INTERFERON REFERENCE
(Ga23-902-530)

3.1 Preparation

Human leukocyte alpha-interferon (HuIFN-α Le) was prepared by Professor K. Cantell, Department of Virology, National Public Health Institute, Helsinki, Finland, by Sendai virus infection of human leukocytes suspended in tricine-buffered minimum essential medium supplemented with human serum (5 g/100 ml) (I). After one day of incubation, the supernatant fluids were collected following centrifugation, and the interferon was purified using a method of differential precipitation to obtain fractions P-IF A and P-IF B (2). Equal parts of these two fractions were mixed and sent to the Medical College of Wisconsin. The mixture, having a specific activity of $3.6 \times 10^7$ IU/mg, was stored at $-70^\circ$C. Subsequently, the sterile interferon preparation was aseptically diluted into ice-cold, sterile buffer solution composed of 0.1 mol/litre solution of sodium phosphate buffer, pH 7, supplemented with 5 mg of human serum albumin per ml (Travenol “Buminate”). The vessel was packed in wet ice to keep the solution chilled during the process of filling the ampoules; 1.00-ml portions were dispensed into borosilicate glass ampoules using a high-precision Hamilton dispenser. The reproducibility of the filling, as measured by the weight of liquid dispensed into 24 preweighed vials (distributed throughout the operation), was 0.12% (coefficient of variation). Ampoules were filled in groups of 24, and held on ice until 5 groups were filled and were then placed in the refrigerated chamber of the freeze-dryer. After all ampoules were filled, they were frozen at $-30^\circ$C, and the material was dried to a residual moisture of about 3%. The ampoules were then filled with argon and heat-sealed at atmospheric pressure. The last ampoule filled in each group of 24 was marked for testing of sterility and antiviral activity after freeze-drying. The ampoules are stored at $-70^\circ$C, but can be shipped at ambient temperatures.

3.2 Recommendations for reconstitution

Add 1.0 ml of sterile distilled water to the lyophilized powder, taking care that no material is lost in the neck or stem of the
ampoule. Small portions of the reconstituted interferon may be stored at $-70^\circ$C for dilution at another time. However, a suitable amount of an appropriate dilution, based on the known sensitivity of the assay being used, should be made in the freeze-drying buffer (see above) supplemented with human serum albumin (5 mg/ml) or in serum-containing culture medium used in the biological assay. Aliquots of the diluted interferon should preferably be stored at $-70^\circ$C and the volume of each aliquot should be sufficient for a single titration. It may be possible to store enough material in a single container at $-70^\circ$C for use in as many as 3 titrations; more extensive repeated thawing and freezing can result in loss of activity. All liquid samples should be stored at $-70^\circ$C or lower.

3.3 Stability

The freeze-dried reference preparation did not lose any activity in the linear non-isothermal accelerated degradation test (3) in which the material is progressively heated from 50 $^\circ$C to 90 $^\circ$C over a 28-hour period. From the results of the predictive multiple isothermal accelerated degradation test (3), involving storage at 52 $^\circ$C, 60 $^\circ$C, 68 $^\circ$C, and 76 $^\circ$C for periods up to 1 year, the product is estimated to have unlimited stability at $-20^\circ$C and $-70^\circ$C. The length of time during which the preparation was predicted to lose 1 log of activity at temperatures above freezing was estimated from these data to be: 0.38 years at 56 $^\circ$C, 2.8 years at 37 $^\circ$C, 18.1 years at 20 $^\circ$C, and 144 years at 4 $^\circ$C.

3.4 Test results

No mycoplasma, bacteria, or fungi were detected in 94 samples tested from the 113 different groups of ampoules comprising the reference lot. The preparation contained no detectable viruses, as indicated by the absence of cytopathic effect during blind passage in cultures of human heteroploid A549 cells or mouse L cells, and of interference with EMC virus replication in the same cell cultures. The interferon used for freeze-drying was diluted to contain 1 mg of human serum albumin per ml and characterized as follows: it was non-sedimentable at 100 000 g, more than 99% of the interferon was inactivated by trypsin in 1 h, it was stable during heating at 56 $^\circ$C for up to 3 h, and 38% of it was inactivated during 6 h of dialysis at pH 2 at 4 $^\circ$C (with no further loss of activity through a total of
48 h at pH 2). The product was not neutralized by antisera to HuIFN-γ (neither by the antisera provided by Dr I. Braude, Meloy Labs, Springfield, VA, nor by that prepared at the Medical College of Wisconsin against purified HuIFN-γ), or by anti-HuIFN-β serum (NIH G028-501-568). However, it was neutralized completely by anti-HuIFN-α serum (NIH G026-502-568). The interferon was composed of forms with two relative molecular masses (of 15,000 and 20,000), as estimated by sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis in phosphate buffer by the method of Weber & Osborne. Analysis of HuIFN-α by isoelectric focusing revealed two approximately equal peaks of activity with isoelectric points of 5.6 and 6.1.

The potency was determined from the data provided by the eight laboratories that had performed two or more titrations of the preparation using a microtitration modification of the proposed reference bioassay technique (Table 3) (4, 5). The reference bioassay involved the reduction in yield of infectious EMC virus in the A549 line of human lung carcinoma cells; EMC virus yields were measured in L cells. The mean of the GMT values reported by each laboratory (total number of titrations = 44) was 4.28 log units/ampoule with a standard deviation (SD) of 0.47 log, corresponding to about 3.0-fold variation. Titration of the HuIFN-α (Le) by routinely used bioassays of different types with various cell–virus combinations (mostly dye-uptake measurements of cytolysis) gave GMT values ranging from 3.08 to 4.29 log units/ampoule, with a mean of 3.66 log units/ampoule (SD 0.49). Additional information is provided in Table 3 (page 62). There was considerable activity on the cells of heterologous species (which is a characteristic of this type of IFN), with the following observed unadjusted titres obtained by the EMC virus haemagglutination yield-reduction method (6): 5.5 × 10^4 laboratory units (LU)/ml in human A549 cells, 3.7 × 10^4 LU/ml in bovine EBT cells, 6.2 × 10^4 LU/ml in feline FEA cells, and 420 LU/ml in murine L cells.

3.5 Titre assignment

The potency was calculated in relation to that of the International Reference Preparation 69/19. On the basis of the data provided by the eight participating laboratories, this preparation was assigned a potency of 12,000 IU/ampoule (4.08 log10 per ampoule).
3.6 Use of the proposed international working standard for interferon, human, leukocyte

This working standard is intended for comparison of the sensitivities of bioassays used in different laboratories for the measurement of the antiviral activity of huIFN-α (Le). It should be used for the calibration of only those laboratory preparations of huIFN-α (Le) that have dose-response curves parallel to the dose-response curve of this preparation (4, 5, 7–9). It should be noted that if the number or proportion of different huIFN-α subtypes in a given leukocyte interferon preparation under test is known to differ significantly from that in this proposed standard, then the use of this standard may not be appropriate.

Each laboratory should measure the potency of the working standard simultaneously with an internal laboratory standard in five or more titrations done on separate occasions. It is recommended that the name and assigned unitage of the working standard (in international units) should be stated along with the logarithm of the observed geometric mean titre (GMT), its standard deviation, and the number of titrations done to obtain this result, thereby indicating the sensitivity of the assay (4, 5, 7–9).

The potency of the laboratory standard in international units per ml is calculated in relation to the proposed international working standard as follows:

\[
\frac{\text{assigned potency} \times \text{potency (in LU) of the laboratory standard}}{\text{potency (in LU) of the international working standard observed from GMT}} = \text{potency of the laboratory standard observed from GMT (in IU)}
\]

Similarly, the laboratory standard may be used to determine the titre of the test sample in IU as follows:

\[
\frac{\text{assigned potency of the laboratory standard (from (1))} \times \text{potency (in LU) of the test sample observed from GMT}}{\text{potency (in LU) of the laboratory standard observed from GMT}} = \text{potency of the test sample (in IU)}
\]
REFERENCES


4. FREEZE-DRIED HUMAN GAMMA-INTERFERON REFERENCE (Gg23-901-530)

4.1 Preparation

Human gamma-interferon (HuIFN-γ) was prepared at the Medical College of Wisconsin by the method of Johnson, et al. (1) by induction with staphylococcal enterotoxin A, 0.02 µg/ml, in human leukocytes (obtained by plateletpheresis) in culture medium RPMI 1640 supplemented with 100 g/l fetal bovine serum. The supernatant fluids were collected after incubation for four days at 36 °C, and clarified by low-speed centrifugation. These lots of crude HuIFN-γ were stored at −70 °C and subsequently thawed, pooled, partially purified to a specific activity of 7 × 10⁷ units/mg protein by chromatography on controlled pore glass. Then after the addition of 1.5 mg/ml human serum albumin they were dialysed exhaustively against 0.1 mol/litre potassium phosphate buffer at pH 7. The HuIFN-γ was stored at −70 °C while its antiviral activity was being determined. It was then thawed, filter-sterilized, aseptically diluted into sterile buffer containing human serum albumin (to provide a final concentration of 30 mg/ml), and dispensed by a high-precision Hamilton dispenser in 1.00-ml portions into glass ampoules for freeze-drying. The reproducibility of the filling, as measured by the weight of liquid dispensed into 13 preweighed vials (distributed throughout the operation), was 0.12% (coefficient of variation). The HuIFN-γ was kept chilled throughout the dilution and dispensing process and was placed immediately in the precooled freeze-drying chamber. After freezing, the ampoules were dried to a residual moisture of about 3%, filled with argon, and heat-sealed at atmospheric pressure. The last ampoule filled in each group of 24 was marked and tested for sterility and antiviral activity after freeze-drying. The ampoules are stored at −70 °C, but can be shipped at ambient temperatures.

4.2 Recommendations for reconstitution

Add 1.0 ml of sterile distilled water to the lyophilized powder, taking care that no loss of material takes place in the neck or stem of the ampoule. Portions of this reconstituted material can either be stored at −70 °C, or an appropriate dilution can be made in the
freeze-drying buffer (see above), supplemented with 30-mg/ml human serum albumin, or in the serum-containing culture medium used in the biological assay. All liquid samples should be stored at 

−70 °C or lower. In view of the relative lability of HuIFN-γ, it is recommended that aliquots of the reconstituted and diluted material be stored in a frozen state so that repeated freezing and thawing can be avoided.

4.3 Stability

In the linear non-isothermal accelerated degradation test (2) (in which the material is progressively heated from 50 °C to 90 °C over a 28-hour period) the freeze-dried reference preparation lost about 70% of its activity during heating to 80 °C and about 81% as the temperature attained 90 °C. From the results of the predictive multiple isothermal accelerated degradation test (2) involving storage at 52°, 60°, and 68° for periods up to 270 days, the product is estimated to have unlimited stability at −20 °C and −70 °C. The length of time during which the product is predicted to lose one log of activity at temperatures above freezing is estimated from these data to be: 0.24 years at 56°C, 2.9 years at 37°C, 30.3 years at 20°C, and 413 years at 4°C.

4.4 Test results

No mycoplasma, bacteria, or fungi could be cultured from the preparation before freeze-drying or in the many samples tested after freeze-drying. The preparation contained no detectable viruses, as indicated by absence of cytopathic effect during blind passage in cultures of human heteroploid A549 or diploid BUD-8, mouse L, or hamster BHK-21 cells. Also, there was no interference with EMC virus replication in the same cell cultures. No staphylococcal enterotoxin A was detected by a sensitive radioimmunoassay.¹ The product, diluted to contain 1 mg of human serum albumin per ml, was characterized before freeze-drying. It was found to be non-sedimentable at 100,000 g and more than 99% of the interferon was inactivated by trypsin in 1 hour, and 93% was inactivated by heating at 56°C for 30 minutes. At pH 2, 60% was inactivated in 6 hours

¹ M. Bergdoll. Personal communication.
and 87% in 24 hours. The product was not neutralized by antisera to either HuIFN-α (NIH GO26-502-568) or HuIFN-β (NIH GO28-501-568). However, it was neutralized by antisera to HuIFN-γ produced by Dr I. Braude, Meloy Labs, Springfield, VA, and by that prepared at the Medical College of Wisconsin against purified HuIFN-γ. When subjected to sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis in phosphate buffer (method of Weber & Osborne), the HuIFN-γ gave a single peak of activity corresponding to a relative molecular mass of 27 000. It should be noted that only 0.14% activity was recovered. Upon isoelectric focusing a single sharp peak was observed at pH 8.3.

The potency was determined from the data contributed by the eight laboratories that had performed two or more titrations of the preparation using a microtitration modification of the proposed reference bioassay technique (Table 4, page 63) (3, 4). The reference bioassay involved the reduction in yield of infectious EMC virus in the A549 line of human lung carcinoma cells; EMC virus yields were measured in L cells. The mean of the GMT values reported by each laboratory (total number of titrations = 46) was 3.61 log units/ampoule with a standard deviation (SD) of 0.22 log, corresponding to about 1.7-fold variation. The titration of the HuIFN-γ by routinely used bioassays of different types with various virus-cell combinations (mostly dye-uptake measurements of cytolysis) gave GMT values ranging from 2.6 to 4.2 log units/ampoule, with a mean GMT of 3.47 log units/ampoule and inter-laboratory standard deviation of 0.61 log. Greater detail is provided in Table 4. Negligible activity on cells of heterologous species was observed as indicated by the following unadjusted titres obtained by the EMC virus haemagglutinin yield-reduction method (5): human A549 cells (9.7 × 10⁴ laboratory units (LU)/ml); mouse L cells (< 10 LU/ml); bovine EBTr cells (< 10 LU/ml); and the FEA line of feline cells (< 150 LU/ml).

4.5 Titre assignment

On the basis of the data provided by the eight participating laboratories, the proposed HuIFN-γ preparation was assigned a potency of 4000 Reference Units per ampoule (see Table 5).
4.6 Use of the proposed international standard for gamma-interferon, human

This international standard is intended for comparison of the sensitivities of bioassays used in different laboratories for the measurement of the antiviral activity of HuIFN-γ. HuIFN-α or HuIFN-β standards cannot be substituted for HuIFN-γ because the dose–response curves for different types of interferon are not parallel (3, 4, 6–8). Note that this preparation should be used for the calibration of only those laboratory preparations that have dose–response curves parallel to the dose–response curve of this preparation (1–5, 7).

Each laboratory should measure the potency of the proposed standard simultaneously with an internal laboratory standard in five or more titrations done on separate occasions. It is recommended that the name and unitage of the standard (in Reference Units, or in International Units should this proposed standard be accepted by WHO) should be stated along with the logarithm of the observed geometric mean titre (GMT), its standard deviation, and the number of titrations done to obtain this result, thereby indicating the sensitivity of the assay (3, 4, 6–8).

The potency of the laboratory standard in Reference Units (RU) (or International Units) is calculated in relation to the proposed international standard as follows:

\[
\frac{\text{assigned potency (in RU)}}{\text{of the international standard}} \times \frac{\text{potency (in LU) of the laboratory standard}}{\text{observed from GMT}} = \frac{\text{potency (in RU) of the laboratory standard}}{\text{(1)}}.
\]

Similarly, the laboratory standard may be used to determine the potency of a test sample as follows:

\[
\frac{\text{assigned potency (in RU)}}{\text{of the laboratory standard}} \times \frac{\text{potency (in LU) of the test sample}}{\text{observed from GMT}} = \frac{\text{potency (in IU) of the test sample}}{\text{test sample}}.
\]
REFERENCES


<table>
<thead>
<tr>
<th>Assay method</th>
<th>Results obtained in different laboratories</th>
<th>Summary of results</th>
</tr>
</thead>
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<td>1</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EMC virus yield-reduction*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of titrations</td>
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<td>5</td>
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<tr>
<td>GMT (log)</td>
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<td>SD (log)</td>
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<td>Other assay methods</td>
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<td></td>
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<td>GMT (log)</td>
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<tr>
<td>SD (log)</td>
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*The reference biosassay method measured the reduction in the yield of encephalomyocarditis (EMC) virus in the human A549 cell line. The yield of infectious EMC virus was measured by titrations in L cells using a cytopathic-effect end-point. A standard protocol modified from that originally recommended (4, 9) detailing the steps in the microtitration method was provided to all participating laboratories. EMC virus and both cell lines were provided by Dr S.E. Grosberg’s laboratory at the Medical College of Wisconsin.

*In this column the geometric mean titre (GMT) and standard deviation (SD) are based on the GMT values obtained from titrations calculated from the raw data provided by each laboratory. An SD value calculated so as to provide a combined estimate of random variation between titrations within the individual laboratory is 0.38 log, corresponding to about 2.4-fold variation.

*In this column the GMT and SD are based on the total number of titrations obtained from all laboratories.

*The assigned potency of Ga23-901-532, in relation to the International Reference Preparation of interferon, Human, Leukocyte (69/19), is 25 000 or 4.40 log IU International Units/ampoule (see Table 5).

* A dash indicates that no titrations were done, and NA — not available.
<table>
<thead>
<tr>
<th>Assay method</th>
<th>Results obtained in different laboratories</th>
<th>Summary of results</th>
</tr>
</thead>
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<tr>
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<tr>
<td><strong>EMC virus yield-reduction</strong></td>
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</tr>
<tr>
<td>Number of titrations</td>
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<td>5</td>
</tr>
<tr>
<td>GMT (log)</td>
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<td>SD (log)</td>
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<td></td>
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<tr>
<td>Number of titrations</td>
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<tr>
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<td>SD (log)</td>
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</table>

*The reference bioassay method measured the reduction in the yield of encephalomyocarditis (EMC) virus in the human A549 cell line. The yield of infectious EMC virus was measured by titrations in L cells using a cytopathic-effect end-point. A standard protocol modified from that originally recommended (6, 7) detailing the steps in the microtitration method was provided to all participating laboratories. EMC virus and both cell lines were also provided by Dr. S.E. Grossberg's laboratory at the Medical College of Wisconsin.

*In this column the geometric mean titre (GMT) and standard deviation (SD) are based on the GMT values obtained from titrations calculated from the raw data provided by each laboratory. An SD value calculated so as to provide a combined estimate of random variation between titrations within the individual laboratory is 0.31 log corresponding to about 2-fold variation.

*In this column the GMT and SD are based on the total number of titres obtained from all laboratories.

*The assigned potency of Gxa01-901-535, in relation to the International Reference Preparation of Interferon, Human, Leukocyte (62/18), is 9000 or 3.95 log IU International Units/ampoule.

*A dash indicates that no titrations were done, and NA = not available.
Table 3. Summary of results of the international collaborative study of the human leukocyte interferon reference preparation
(NIH catalogue number Ga23-902-530)

<table>
<thead>
<tr>
<th>Assay method</th>
<th>Results obtained in different laboratories</th>
<th>Comparison of GMT results in different laboratories*</th>
<th>All tests in all laboratories*</th>
</tr>
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<td>2</td>
<td>3</td>
</tr>
<tr>
<td>GMT (log)</td>
<td>5.11</td>
<td>4.52</td>
<td>4.54</td>
</tr>
<tr>
<td>SD (log)</td>
<td>0.36</td>
<td>0.15</td>
<td>0.18</td>
</tr>
</tbody>
</table>

EMC virus yield-reduction

Number of titrations | 10 | 5 | 2 | 6 | 8 | 5 | 5 | 3 | 8 | 44 |
SD (log)             | 0.36 | 0.15 | 0.18 | 0.15 | 0.13 | 0.75 | 0.46 | 0.52 | 0.47  | 0.61  |

Other assay methods

Number of titrations | 24 | 5 | -- | -- | 7 | 6 | 5 | 3.64 | 3.93 |
SD (log)             | 0.13 | 0.09 | -- | -- | 0.29 | 0.18 | NA* | 0.12 | 0.56 |

*The reference bioassay method measured the reduction in the yield of encephalomyocarditis (EMC) virus in the human A549 cell line; yield of infectious EMC virus was measured by titrations in L cells using a cytopathic-effect end-point. A standard protocol modified from that originally recommended (4, 9) detailing the steps in the microtitration method was provided to all participating laboratories. EMC virus and both cell lines were also provided by Dr S.E. Grossberg's laboratory at the Medical College of Wisconsin.

*In this column the geometric mean titre (GMT) and standard deviation (SD) are based on the GMT values obtained from titres calculated from the raw data provided by each laboratory. An SD value calculated so as to provide a combined estimate of random variation between titrations within the individual laboratory is 0.47 log, corresponding to about 3-fold variation.

*In this column the GMT and SD are based on the total number of titres obtained from all laboratories.

*The assigned potency of Ga23-902-530, in relation to the International Reference Preparation of Interferon, Human, Leukocyte (69/19), is 12 000 or 4.68 log₁₀ International Units/ampoule (see text).

*A dash indicates that no titrations were done, and NA -- not available.
Table 4. Summary of results of the international collaborative study to calibrate the HuLFN-γ reference preparation (NIH catalogue number Gg23-901-530)

<table>
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<th>Assay method</th>
<th>Results obtained in different laboratories</th>
<th>Summary of results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>EMC virus yield-reduction*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of titrations</td>
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<td>2</td>
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<td>GMT (log)</td>
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<td>0.31</td>
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<tr>
<td>Other assay methods</td>
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<td></td>
</tr>
<tr>
<td>Number of titres</td>
<td>12</td>
<td>-</td>
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<td>GMT (log)</td>
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<td>-</td>
</tr>
<tr>
<td>SD (log)</td>
<td>0.15</td>
<td>-</td>
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</table>

*The reference bioassay method measured the reduction in the yield of encephalomyocarditis (EMC) virus in the human A549 cell line. The yield of infectious EMC virus was measured by titrations in 5 cells using a cytopathic-effect end-point. A standard protocol modified from that originally recommended (3, 7) detailing the steps in the microtitration method was provided to all participating laboratories. EMC virus and batch cell lines were also provided by Dr S.E. Grossberg’s laboratory at the Medical College of Wisconsin.

*In this column the geometric mean titre (GMT) and standard deviation (SD) are based on the GMT values obtained from titres provided by each laboratory. An SD value calculated so as to provide a combined estimate of random variation between titrations within the individual laboratory is 0.22 log, corresponding to about 1.7-fold variation.

*In this column, the GMT and SD are based on the total number of titres obtained from all laboratories.

*The assigned potency of Gg23-901-030 is 4000 or 3.60 log, Reference Units/ampoule.

*A dash indicates that no titrations were done.
<table>
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<tr>
<th>HuIFN sample</th>
<th>IFN type</th>
<th>Observed (LU/ampoule)</th>
<th>Log₁₀ ± SD</th>
<th>Antilog</th>
<th>Corrected* or assigned (LU/ampoule)</th>
<th>Log₁₀</th>
<th>Antilog</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRP 69/19 (Le)</td>
<td>α</td>
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<td>7691</td>
<td>3.70</td>
<td>5000</td>
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<tr>
<td>Ga23-901-530 (Le)</td>
<td>α</td>
<td>4.279 ± 0.669</td>
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<td>12000</td>
<td></td>
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<tr>
<td>Ga23-901-532 (Ly)</td>
<td>α</td>
<td>4.590 ± 0.381</td>
<td>38905</td>
<td>4.40*</td>
<td>25000*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gax01-901-535(u0A)</td>
<td>α</td>
<td>4.134 ± 0.313</td>
<td>13614</td>
<td>3.95*</td>
<td>9000*</td>
<td></td>
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</tr>
<tr>
<td>Gg23-901-530</td>
<td>γ</td>
<td>3.81 ± 0.25</td>
<td>4044</td>
<td>3.60</td>
<td>4000</td>
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</tbody>
</table>

*LU = International Units; LU = Laboratory Units as determined by the proposed reference bioassay (EMC virus infectivity yield reduction in human A549 cells).

*Corrected against the assigned titre (3.70 log₁₀ or 5000 International Units) of the WHO International Reference Human Leukocyte Interferon Preparation 69/19.
Annex 2

REQUIREMENTS FOR DIPHTHERIA TOXOID, PERTUSSIS VACCINE, TETANUS TOXOID, AND COMBINED VACCINES

(Requirements for Biological Substances No. 8 and 10)
Addendum 1984

An informal consultation, held in Geneva from 12 to 14 December 1983, discussed the potency assay of diphtheria and tetanus toxoids for both single toxoids as well as combined vaccines. The opportunity was taken to look at a number of other tests but no attempt was made to review the entire Requirements (WHO Technical Report Series, No. 638, 1979, Annex I; Addendum 1980 in WHO Technical Report Series, No. 658, 1981, Annex 13; and Addendum 1983 in WHO Technical Report Series, No. 700, Annex 1).

The following revisions were made:

REQUIREMENTS FOR DIPHTHERIA TOXOID

Section A.3.3.4  Detoxification and purification of toxin (page 46)

The non-mandatory paragraphs (small print) imply that the test of toxicity reversal applies to each single harvest. In fact, the test applies only to the bulk purified toxoid. The two paragraphs in small print should, therefore, be deleted, and a new section should be added at the end of section A.3.4.4.

Insert: A.3.4.5  Test of toxicity reversal

Each bulk purified toxoid shall be tested to ensure that reversal has not taken place on storage.

Methods for the detection of reversal are suggested in the Manual (4, Appendix D.9).

1 Page numbers refer to WHO Technical Report Series, No. 638.
In some countries the following test is applied to check the possibility of toxicity reversal. One sample is diluted to 200 Lf/ml (with 0.0167 mol/l phosphate-buffered saline), and another sample to a concentration equivalent to that of the final bulk. The latter sample is kept standing at 37°C for 20 days. Then 5 ml of each sample is injected subcutaneously into at least 5 guinea-pigs, which are then observed for 6 weeks (as described in Part A, Section A.3.4.3).

Section A.5.7 Test for pH (page 52)

Many of the diphtheria toxoids available today have a pH slightly higher than 6.7. Therefore, replace the non-mandatory paragraph (small print) by the following:

The pH should be between 6.7 and 7.0. In some countries this test is applied to the final bulk only.

SUMMARY PROTOCOL FOR DIPHTHERIA TOXOID, PRODUCTION AND TESTING

Replace the text under “3. Bulk purified toxoid” (page 56) by the following:

Results of test for antigenic purity
(Lf/mg protein N) ..............................................

Test of toxicity reversal (on toxoid diluted to reach the concentration of the final bulk)

Concentration (Lf/ml)
of the toxoid solution ..............................................
Incubation temperature ..............................................
Length of time of incubation ..............................................
Volume injected into each guinea-pig ..............................................
Route of injection ..............................................
No. of guinea-pigs injected ..............................................
Time of observation of guinea-pigs ..............................................
Result of the test ..............................................
REQUIREMENTS FOR TETANUS TOXOID

Section A.3.3.4  *Detoxification and purification of toxin* (page 87)

The non-mandatory paragraphs (small print) imply that the test of toxicity reversal applies to each single harvest. In fact, the test applies only to the bulk purified toxoid. The two paragraphs in small print should, therefore, be deleted, and a new section should be added at the end of section A.3.4.4.

*Insert: A.3.4.5 Test of toxicity reversal*

Each bulk purified toxoid shall be tested to ensure that reversal has not taken place on storage.

Methods for the detection of reversal are suggested in the Manual (II, Appendix T.11).

In some countries the following test is applied to check the possibility of toxicity reversal. One sample is diluted to 200 LF/ml (with 0.0167 mol/l phosphate-buffered saline) and another sample to a concentration equivalent to that of the final bulk. The latter sample is kept standing at 37°C for 20 days. Then 5 ml of each sample are injected subcutaneously into each of at least 5 guinea-pigs, which are then observed for 21 days (as described in Part A, Section A.3.4.3).

Section A.3.5.9  *Test for pH* (page 91)

Many of the tetanus toxoids available today have a pH slightly higher than 6.7. Therefore, replace the non-mandatory paragraph (small print) by the following:

The pH should be between 6.7 and 7.0.

SUMMARY PROTOCOL FOR TETANUS TOXOID, PRODUCTION AND TESTING

*Replace the text under “3. Bulk toxoid”* (page 97) *by the following:*

Purified/Unpurified

<table>
<thead>
<tr>
<th>Nature of bulk toxoid</th>
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<tr>
<td>Results of test for antigenic</td>
<td></td>
</tr>
<tr>
<td>purity, if applicable</td>
<td></td>
</tr>
<tr>
<td>(LF/mg protein N)</td>
<td></td>
</tr>
</tbody>
</table>

67
Test of toxicity reversal (on toxoid diluted to reach the concentration of the final bulk)

Concentration (Lf/ml) of the toxoid solution

Incubation temperature

Length of time of incubation

Volume injected into each guinea-pig

No. of guinea-pigs injected

Time of observation of guinea-pigs

Result of the test

SPECIAL TESTS FOR DPT VACCINE

Section A.14 Test for pH (page 105)

Many of the combined vaccines available today have a pH slightly above or below 6.7.

*Therefore, replace the section by the following:*

The pH of DPT vaccine should be between 6.0 and 7.0.

SUMMARY PROTOCOL FOR DIPHTHERIA–PERTUSSIS–TETANUS VACCINE

DIPHTHERIA TOXOID, Information on Manufacture

*Replace the text under “3. Bulk purified toxoid” (page 107) by the following:*

Results of test for antigenic purity (Lf/mg protein N)

Test of toxicity reversal (on toxoid diluted to reach the concentration of the final bulk)

Concentration (Lf/ml) of the toxoid solution
<table>
<thead>
<tr>
<th>Incubation temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length of time of incubation</td>
</tr>
<tr>
<td>Volume injected into each guinea-pig</td>
</tr>
<tr>
<td>Route of injection</td>
</tr>
<tr>
<td>No. of guinea-pigs injected</td>
</tr>
<tr>
<td>Time of observation of guinea-pigs</td>
</tr>
<tr>
<td>Result of the test</td>
</tr>
</tbody>
</table>

**TETANUS TOXOID, Information on Manufacture**

*Replace the text under “3. Bulk toxoid” (page 108) by the following:*

<table>
<thead>
<tr>
<th>Purified/Unpurified</th>
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<tbody>
<tr>
<td>Nature of bulk toxoid</td>
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<tr>
<td>Results of test for antigenic purity, if applicable (Lf/mg protein N)</td>
</tr>
<tr>
<td>Test of irreversibility (on toxoid diluted to reach the concentration of the final bulk)</td>
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<tr>
<td>Concentration (Lf/ml) of the toxoid solution</td>
</tr>
<tr>
<td>Incubation temperature</td>
</tr>
<tr>
<td>Length of time of incubation</td>
</tr>
<tr>
<td>Volume injected into each guinea-pig</td>
</tr>
<tr>
<td>No. of guinea-pigs injected</td>
</tr>
<tr>
<td>Time of observation of guinea-pigs</td>
</tr>
<tr>
<td>Result of the test</td>
</tr>
</tbody>
</table>

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Annex 3

REQUIREMENTS FOR HEPATITIS B VACCINE (REVISED) PREPARED FROM PLASMA

(Requirements for Biological Substances No. 31)
(Revised 1984)

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INTRODUCTION

Many viruses may infect the liver of animals and man and may produce severe diseases. The general term “viral hepatitis” refers to infection caused by hepatitis A virus or hepatitis B virus, which can now be differentiated by specific laboratory tests for antigens and antibodies associated with these infections, and by more recently identified and unrelated types of hepatitis referred to as “hepatitis non-A, non-B”. This form of hepatitis (non-A, non-B) is caused by more than one agent.

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Viral hepatitis is a major public health problem occurring endemically in all parts of the world. Acute viral hepatitis is a systemic or generalized infection with the liver as the target organ. The clinical picture therefore includes inapparent or subclinical infection, mild gastrointestinal symptoms of the anicteric form of the disease, acute illness with jaundice, severe prolonged jaundice, and acute fulminant hepatitis.

Hepatitis A is usually spread by person-to-person contact by the faecal–oral route, and outbreaks also result most frequently from faecal contamination of water and food. The virus, which has been identified as an RNA virus with the characteristics of members of the Enterovirus genus, has recently been cultivated in tissue culture and progress has been made towards the production of a vaccine.

There is substantial evidence that hepatitis B may progress to chronic liver disease, including chronic persistent hepatitis, chronic active (aggressive) hepatitis, and cirrhosis. About 80% of hepatocellular carcinomas are ascribed to chronic infection with hepatitis B virus.

Hepatitis B virus (HBV) has been identified as a 42-nm particle (known as the Dane particle) containing double-stranded DNA. Infection with hepatitis B virus is manifested by at least three antigenic components: hepatitis B surface antigen (HBsAg), the core antigen (HBeAg), and the e antigen (HBeAg), resulting from replication of the virus in the hepatocytes. Infection also produces high titres of anti-HBcIgM. The surface antigen is most frequently found as 20–22 nm spherical particles (sometimes slightly larger or smaller) and as tubular forms, and possesses a common determinant a and generally at least two mutually exclusive subdeterminants d or y and w or r. Other subspecificities have also been recognized.

Hepatitis B virus has not yet been cultivated in cell culture and small laboratory animals are not susceptible to infection. The infection can be transmitted to certain of the apes, of which the chimpanzee is the only available susceptible animal model for hepatitis B and for both short- and long-incubation forms of non-A, non-B hepatitis.

The importance of hepatitis B may also be considered under a variety of headings, which include its effects on every field of medical practice, the impact that it has on blood transfusion services, and its association with progression to chronic liver disease. In addition, infection with hepatitis B may be followed by the persistent carrier state. Such a carrier state may be associated with liver damage. It
is estimated that the number of carriers in the world amounts to more than 200 million, with approximately the following geographical distribution, particularly among blood donors: northern Europe, North America, and parts of Australia; a prevalence of 0.1% or less; a prevalence of up to 5% in central and eastern Europe, with a higher frequency in southern Europe and countries bordering the Mediterranean; a similar frequency of 5% or more in parts of Central and South America; a frequency of 5–10% in the Gulf States; and a prevalence of up to 20% in some parts of Africa, Asia, and the Pacific area.

The importance of the parenteral and inapparent parenteral routes of transmission of hepatitis B virus is now well established, and although various body fluids, such as saliva, menstrual and vaginal discharges, seminal fluid, and serous exudates, have been shown to be important in the spread of infection, infectivity appears to be especially related to blood and some blood products. Transmission of hepatitis B infection from carrier mothers to their babies can occur during the perinatal period and the first years of life, and appears to be an important factor determining the prevalence of the virus infection in some regions.

Because of the urgent need for a hepatitis B vaccine, particularly for groups that are at increased risk of acquiring infection (1), WHO Requirements were formulated in 1980 (2). Since it has been shown that the separated viral coat proteins, containing hepatitis B surface antigen, lead to the production of protective antibody, it is now possible to use purified and inactivated 22-nm spherical hepatitis B surface antigen particles, or subunits derived from the surface antigen, as vaccines. However, the preparation of such vaccines for use in man from human viral antigens not grown in cell culture, but obtained from the plasma of infected persons—namely, from persistent carriers of hepatitis B antigens—continues to be an entirely new approach in vaccine production and demands special consideration in the tests applied to the production and quality control of the vaccines. Still more important, it has now been shown that human blood and plasma may harbour a number of infectious agents, including hepatitis B. Particular attention, therefore, must be given to the selection of the donors of the plasma, the process of separation of the antigen, and the inactivation procedures to ensure that all potential infectious agents that may still be present after the purification of the antigen have been inactivated.
The development of vaccines

The virus of hepatitis B still eludes reliable propagation in the laboratory in vitro. For this reason the preparation of a vaccine from virus grown in cell cultures is not possible. The discovery of “Australia antigen” in the serum of an Australian aborigine and the subsequent demonstration that this antigen is in fact the surface antigen of hepatitis B virus in an infected carrier of hepatitis B opened the door to the means of detecting hepatitis B carriers, for laboratory diagnosis, and for vaccine development even in the absence of any means of propagating the virus in the laboratory.

In 1971, Krugman first used this knowledge of the relationship of the hepatitis B surface antigen to hepatitis B and prepared a “vaccine” for preventing the disease. This preparation consisted of boiled, diluted, infectious serum from a hepatitis B carrier. Though crude and nonstandardized and now known not to have been completely inactivated, the material did induce antibodies against hepatitis B surface antigen (anti-HBs) in human subjects and the inoculated subjects were partially protected against the disease when challenged.

These important developments provided a stimulus for studies in a number of laboratories to prepare inactivated HBsAg vaccines using HBsAg purified from plasma obtained from antigenaemic carriers of hepatitis B. Vaccines of varying degrees of purity and technological complexity have been prepared in a number of laboratories and some have been tested in humans. Current scientific information indicates that the only hepatitis B antigen eliciting protective immunity against infection with HVB is HBsAg. This antigen, therefore, is the single essential component of hepatitis B vaccine.

Source plasma could contain infectious agents that possess a wide range of physico-chemical and biological characteristics and various degrees of susceptibility or resistance to different modes of inactivation. Consequently, to ensure as far as possible the inactivation of a wide range of infectious agents, it is desirable that, in addition to separation and purification, at least two treatment procedures, based on different inactivation principles, be applied during the vaccine manufacturing process. However, it should be recognized that certain treatments or combinations of treatments can adversely affect the antigenicity and protective efficacy of such vaccines.
Whatever the procedure used, it is universally accepted that the vaccines must be safe (i.e., free from demonstrable virus and other microbial agents), potent (i.e., capable of eliciting antibody against the virus in animals and in man by the administration of a standardized dose of antigen) and efficacious (i.e., protective against the disease).

A matter of some concern with regard to the use of human blood and some products made from human blood or plasma is the occurrence in epidemic proportions of acquired immunodeficiency syndrome (AIDS) which leads to tumours, such as Kaposi sarcoma and opportunistic infections that would otherwise be suppressed. This syndrome has been recognized to occur in many parts of the world (3) with high mortality.

There have been a number of significant findings in the production of hepatitis B vaccine since the WHO Requirements were first formulated in 1980, and the steps now considered to be essential include both physical and chemical or biological methods. In choosing methods of purification and inactivation, it is important to appreciate that hepatitis B surface antigen is a relatively stable glycoprotein that can withstand fairly harsh treatment. This permits the production of an essentially pure vaccine that can be well defined and for which assurances as regards safety can be given.

Hepatitis B vaccines prepared to date have been incorporated in an adjuvant such as alum, and all are assayed for their ability to stimulate anti-HBs in animals, including mice, monkeys, chimpanzees, and guinea-pigs. An extinction assay performed in mice has been developed for quantification of the immunizing potency of batches of vaccine, and a proposed WHO standard is now being investigated in an international collaborative study for this purpose.

Controlled studies in chimpanzees have shown the efficacy of several vaccines in preventing hepatitis B following challenge with human hepatitis B virus. Cross-protection studies carried out in chimpanzees have shown that subtypes are not of major importance in vaccine composition. In one country, the use of the vaccine in the staff of a renal dialysis unit has shown considerable protection against ay infection by ad vaccine. The reverse is also true because of the common a component.

It is now recognized that safe and effective hepatitis B vaccines are being prepared using infected plasma as starting material. Alternative methods for the production of hepatitis B surface
antigen, however, are under investigation. Polypeptide vaccines have been produced from plasma and such vaccines are now being developed from other sources of antigens, such as those obtained by recombinant DNA technology and chemical synthesis. With the latter vaccines, it will be necessary to add a carrier in order that the short amino acid sequence being synthesized is immunogenic and gives a broad spectrum of protection against all subtypes as well as giving lasting immunity. When such vaccines from alternative sources become available, it will be necessary to either revise these requirements or formulate new ones. These revised requirements refer only to hepatitis B vaccine prepared from human plasma.

Each of the following sections constitutes a recommendation. The parts of each section that are printed in large type have been written in the form of requirements, so that, if a health administration so desires, these parts as they appear may be included in definitive national requirements. The parts of each section that are printed in small type are comments and recommendations for guidance.

Should individual countries wish to adopt these requirements as the basis of their national regulations concerning hepatitis B vaccine, it is recommended that a clause be included permitting modifications of manufacturing requirements on the condition that it can be demonstrated, to the satisfaction of the national control authority, that such modified requirements ensure that the degree of safety and the potency of the vaccine are at least equal to those provided by the requirements formulated below. The World Health Organization should then be informed of the action taken.

The terms “national control authority” and “national control laboratory”, as used in these requirements, always refer to the country in which the final vaccine is manufactured and/or used.

PART A.
MANUFACTURING REQUIREMENTS

1. DEFINITIONS

1.1 International name and proper name

The international name shall be “Vaccinum hepatitidis B explasma humanum”. 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.

1.2 Descriptive definition

*Vaccinum hepatitis B explasma humanum* is a preparation of purified hepatitis B surface antigen (HBsAg) that has been inactivated to kill any virus coming through the separation process with the non-infectious HBsAg. The preparation shall satisfy all the requirements formulated below.

1.3 International reference preparations and international units

Two international reference preparations are required for determination of the purity and potency of hepatitis B surface antigen.

For the techniques used in the assessment of immunogenicity of vaccines, an international reference adjuvanted vaccine is required for the measurement of immune responses of animals and calibrated with its immune responses in human subjects (see Part A, section 5.5). Such a preparation is under study.

For the assay of purity of antigenic content by techniques such as radioimmunounassay, ELISA, or radial diffusion, an international reference aqueous preparation without an adjuvant is required (see Part A, section 3.6.2). Such a preparation is under study.

For the calibration of techniques used in the measurement of antibody responses to hepatitis B vaccines, an international reference preparation is available. This preparation is calibrated in terms of anti-HBs.

The International Reference Preparation of Hepatitis B Immunoglobulin (established in 1977) is dispensed in ampoules containing 50 IU of hepatitis B immunoglobulin (fractionated human plasma, freeze-dried). This preparation is in the custody of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, Netherlands.

1.4 Terminology

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

*Whole blood* (sometimes referred to as blood): the blood collected in an anticoagulant solution.

*Plasma*: the liquid part of blood collected in a receptacle containing an anticoagulant.
HBV: hepatitis B virus. A 42-nm double-shelled virus, originally known as the Dane particle.

HBsAg: hepatitis B surface antigen. The hepatitis B antigen found on the surface of the virus and on the accompanying unattached spherical (22-nm) and tubular particles.

HBeAg: hepatitis B core antigen. The hepatitis B antigen found within the core of the virus.

HBeAg: the e antigen has now been identified as a cryptic HBe antigen.

Anti-HBs: antibody to hepatitis B surface antigen.

Anti-HBe: antibody to hepatitis B core antigen.

Anti-HBe: antibodies to the e antigen(s).

Single donor plasma: plasma obtained from a single donation of whole blood or obtained by plasmapheresis.

Plasma pools: pools of single-donation plasmas that have been shown to be satisfactory before pooling.

Purified, inactivated HBsAg batch: purified HBsAg prepared from one or more plasma pools and inactivated by suitable procedures.

Final aqueous bulk: the final bulk before the addition of an adjuvant.

Final bulk: the finished biological material prepared from one or more batches of purified, inactivated HBsAg present in the container from which the final containers are filled.

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

2. GENERAL MANUFACTURING REQUIREMENTS

The general manufacturing requirements contained in the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (4, p. 11) shall apply to establishments manufacturing hepatitis B vaccine, with the addition of the following directives:

Production areas shall be decontaminated before they are used for the manufacture of hepatitis B vaccine. Completely separate areas shall be used for the separation and inactivation steps. All
separation and inactivation steps shall be carried out in closed systems and closely monitored.

The production of hepatitis B vaccine shall be conducted by staff who have not handled other infectious microorganisms or animals in the same working day. The staff shall consist of persons who shall be examined medically, and found to be healthy and not carriers of hepatitis B.

In some countries steps are taken to ensure that all such persons in the production areas are immune to hepatitis B as shown by the presence of anti-HBs.

Persons not directly concerned with the production processes, other than official representatives of the national control authority, shall not be permitted to enter the production area.

Particular attention shall be given to the recommendations contained in Part A, section 1 of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (4, p. 13) regarding the training and experience of persons in charge of production and testing and of those assigned to various areas of responsibility in the manufacturing establishment, as well as to the registration of such personnel with the national control authority.

3. PRODUCTION CONTROL

The general production precautions as formulated in the requirements of Part A, section 3 of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (4, p. 15) shall apply to the manufacture of hepatitis B vaccine.

3.1 The collection of blood and plasma

3.1.1 The selection of donors

Source materials for further processing are obtained from donations of blood or plasma. The medical criteria for accepting donors—criteria relating to the safety, purity, potency, and efficacy of the final products—must be the same for donors of whole blood (see Part A, section 3.1.2) components or blood components collected by plasmapheresis (5, Annex 1, p. 38), except that the
donors must be antigenaemic and need not meet the exclusions relating to hepatitis.

In some countries separate areas or special times are set aside for the collection of plasma known to be HBsAg positive.
In some countries donors with a high HBsAg content but negative for HBeAg are selected for the donation of plasma.
Records should be kept of the identity of the donors for the identification of each batch of vaccine.

3.1.2 Donors of whole blood

The physical fitness of a donor shall be determined by a licensed physician or a person under the direct supervision of a licensed physician. Donors shall be asymptomatic persons of either sex between the ages of 18 and 65 years, except that the findings in liver function tests may exceed normal limits.

In some countries the upper age limit is restricted to 50 for males and 45 for females.

3.1.3 Medical history

General. Before each donation questions shall be asked to determine that the donor is asymptomatic and has not suffered, or is not suffering, from any serious illness—e.g., malignant disease, diabetes, epilepsy, hypertension, renal disease, malaria.

Any donor who appears to be suffering from symptoms of acute or chronic disease, or who is receiving oral or parenteral medication, with the exception of vitamins or oral contraceptives, may not be accepted for donation unless approved by a physician. The values obtained in liver function tests may exceed normal limits.

Any donor who appears to be under the influence of alcohol or any drug or who does not appear to be providing reliable answers to medical history questions shall not be accepted.

National health authorities shall develop policies designed to prevent the transmission of other infectious diseases based on the prevalence of these diseases in the donor population and the susceptibility of recipients to the same diseases.

Minor surgery. Donors shall not have a history of tooth extraction or other minor surgery during a period of 72 hours prior to donation.
Pregnancy. Pregnant women shall be excluded from blood donation. In general, mothers shall also be excluded for the period of lactation and for at least six months after full-term delivery.

Immunization. Symptom-free donors who have recently been immunized may be accepted with the following exceptions:

— those receiving smallpox vaccine shall be excluded until the scab has fallen off or until two weeks after an immune reaction;
— those receiving attenuated vaccines for measles (rubeola), mumps, yellow fever, or poliomyelitis shall be excluded until two weeks after the last immunization or injection;
— those receiving attenuated rubella (German measles) vaccine shall be excluded until eight weeks after the last injection;
— those receiving rabies (therapeutic) vaccine or rabies immunoglobulin shall be excluded until one year after the last injection;
— those receiving passive immunization using animal serum products shall be excluded until four weeks after the last injection.

3.1.4 Physical examination

Donors shall have a weight, blood pressure, pulse rate, and temperature within normal limits. Donors with any measurements outside the established normal limits of weight, blood pressure, and pulse rate may be accepted only if approved by the responsible licensed physician.

The following recommendations may be useful for guidance:

1. Blood pressure. Systolic blood pressure between 12 and 14 kPa (90 and 180 mmHg); diastolic blood pressure between 6.7 and 13.3 kPa (50 and 100 mmHg).
2. Pulse. Between 50 and 100 beats per minute and regular.
3. Temperature. Oral temperature not exceeding 37.5°C.
4. Weight. Donors weighing less than 50 kg may be bled proportionately less than 450 ml per unit, provided all other donor requirements are met.

In some countries it is not required to take the body temperature but the decision to do so or not should be made by the national control authority.

Donors shall be free from any infectious skin disease at the venepuncture site and from skin punctures or scars indicative of addiction to narcotics.

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3.1.5 *Determination of haemoglobin or erythrocyte volume fraction*

The haemoglobin shall not be less than 125 g/l of blood for women and 135 g/l of blood for men. If erythrocyte volume fraction measurement is substituted for haemoglobin measurement the values shall be not less than 0.38 and 0.41 for men and women, respectively.

These limits are not universally accepted, and the national control authorities should raise or lower them when considered to be appropriate.

3.1.6 *Donors for plasmapheresis*

All phases of plasmapheresis, including explaining to donors what is involved in the process and obtaining their informed consent, shall be performed under the direct supervision of a licensed physician.

There are two groups of plasmapheresis donors: those who donate at a frequency comparable to that allowed for whole blood donations and those who donate more frequently. The former group shall be accepted on the basis of the above criteria for donors of whole blood.

In addition to these criteria, donors participating in the plasmapheresis programme shall be examined by a licensed physician on the day of the first donation, or no more than one week prior to the first donation. This examination shall include urine analysis and blood sampling for liver function tests, and determination of plasma proteins by electrophoresis or another suitable method.

On the day of each donation, in addition to meeting the requirements for whole blood donors, plasmapheresis donors shall be shown to have a total serum protein of no less than 60 g/l.

The medical evaluation of plasmapheresis donors shall be repeated at monthly intervals, or as specified by the national control authority.

Whenever a laboratory value other than a liver function test is found to be outside the normal limits or any important abnormalities are noted in a donor’s history or on physical examination, the donor shall be removed from the programme. The donor shall not return to the programme until the abnormal finding has returned to normal and the responsible physician has given approval.
If a plasmapheresis donor donates a unit of whole blood or does not have the red blood cells returned from a unit taken during the procedure, the donor shall be deferred for eight weeks unless special circumstances warrant approval by the responsible physician of earlier plasmapheresis. Plasmapheresis of donors of HBsAg-positive plasma will be permitted by the responsible physician, even if the liver function test values are above normal limits in individual donors.

The upper limits of these values should be specified by the national control authority.

Appropriate guidelines to define donor changes significant to justify discontinuation of plasmapheresis should be established by the responsible physician (5, p. 43).

The maximum volume of blood or plasma that may be taken in one year from chronic hepatitis B carriers shall be determined by the national control authority.

No guidelines have been established for the maximum volume of plasma that can be taken in any year from a chronic hepatitis B carrier.

In normal healthy subjects, some countries permit only 10 litres to be collected whereas in one country as much as 60 litres are allowed. Studies are needed to establish safe limits for the quantity of plasma to be collected from a chronic hepatitis B carrier. Such studies are under way.

Where plasmapheresis is regularly performed on HBsAg-positive donors, it is advisable to have equipment set aside specifically for this purpose.

3.2 Tests on single-donation plasma

Each single-donation plasma, whether obtained from whole blood or by plasmapheresis, shall be subjected to the following tests:

In some countries the tests are done on plasma pools, where this is approved by the national control authority.

In some countries it is not permitted to use source material containing detectable HBV or HBe antigen.

3.2.1 Sterility tests

Single-donation plasma shall be tested for sterility according to the requirements given in Part A, section 5 of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (6, p. 48).
3.2.2 Test for HBsAg

Each single donation shall be tested for the titre of HBsAg by a method approved by the national control authority.

Several tests are suitable for this purpose (1). Potency should be established with reference to an appropriate potency standard included in all assays.

3.2.3 Test for HBsAg subtype

In some countries, unless the subtype of a particular donor has been identified, each single donation is tested for HBsAg subtype by a method approved by the national control authority. The national control authority may require the data on the subtype composition.

Several tests (among them a gel diffusion test) have been shown to be suitable for this purpose (1).

3.3 Pooling of single-donation plasma

Only acceptable plasma shall be included in a plasma pool.

3.4 Tests on plasma pools

3.4.1 Sterility tests

A volume of at least 10 ml of each plasma pool shall be tested for sterility according to the requirements given in Part A, section 5 of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (6, p. 48).

3.4.2 Test for Mycobacterium tuberculosis

In some countries it is required that each plasma pool shall be tested for the presence of mycobacteria (human) by culture methods approved by the national control authority.

3.4.3 Tests for extraneous viruses

3.4.3.1 Tests in animals and fertile eggs

(a) Tests in adult mice

Each plasma pool shall be tested in adult mice for adventitious agents pathogenic to mice. Each of at least 10 adult mice, of 15–20 g
weight, shall be inoculated intracerebrally with 0.03 ml and at least 10 mice intraperitoneally with at least 0.5 ml of the plasma pool. The mice shall be observed for at least 21 days. All mice that die after the first 24 hours of the test or that show signs of illness shall be autopsied and examined for evidence of viral infection, both macroscopically by direct observation and by subinoculation of appropriate tissue suspensions by the intracerebral and intraperitoneal routes into at least five additional mice, which shall be observed for 21 days.

The plasma pool passes the test if at least 80% of the original inoculated mice survive the observation period and if no mice show evidence of infection with adventitious transmissible agents attributable to the plasma pool.

(b) Tests in suckling mice

Each plasma pool shall be tested in suckling mice for adventitious agents pathogenic to mice. Each of at least 20 mice less than 24 hours old shall be inoculated intracerebrally with 0.01 ml and intraperitoneally with at least 0.1 ml of the plasma pool. The mice shall be observed daily for at least 14 days. All mice that die after the first 24 hours of the test or that show signs of illness shall be autopsied and examined to determine the cause of death or illness.

In some countries a subinoculation is suggested and in others an additional blind passage is made of a suspension of the pooled emulsified tissue (minus skin and viscera) of all mice surviving the original 14-day test.

The plasma pool passes the test if at least 80% of the original inoculated mice survive the observation period and if no mice show evidence of infection with adventitious transmissible agents attributable to the plasma.

(c) Tests in embryonated eggs

A sample of at least 5 ml of each plasma pool shall be tested in a group of embryonated hen eggs by the allantoic route of inoculation and a similar sample in a separate group of eggs by the yolk-sac route of inoculation, using at least 0.25 ml of the pool per egg for each route of inoculation. The incubation of the eggs and the observation time shall be approved by the national control authority. The embryo shall remain normal throughout the observation period.
The plasma pool passes the test if there is no evidence of the presence of any adventitious agents attributable to the plasma pool.

3.4.3.2 Tests in cell cultures

A sample of at least 5 ml of each plasma pool shall be tested for adventitious agents by inoculation into simian cell cultures. Similar volumes of the plasma pool shall likewise be tested in human diploid cell cultures. The inoculated cell cultures and uninoculated control cultures shall be observed for at least 14 days.

In some countries larger volumes of the plasma pool are required to be tested.

Suitable simian cell cultures are prepared from the kidneys of Cercopithecus monkeys or from VERO cells. For the human cell cultures, either WI-38 or MRC-5 may be used.

The plasma pool passes the test if none of the cell cultures shows evidence of the presence of any adventitious agent attributable to the plasma pool.

3.4.3.3 Other tests

In some countries tests for the presence of HBV DNA or HBV DNA polymerase and reverse transcriptase are also carried out.

3.5 Concentration, purification, and inactivation

Each plasma pool shall be subjected to purification procedures before inactivation.

The method used for the concentration of the HBsAg should be approved by the national control authority. Such methods should have been shown to concentrate the HBsAg.

Precipitation by ammonium sulfate and polyethylene glycol have been found suitable.

The methods used for purification of the HBsAg shall be approved by the national control authority. The purification procedure shall be such that the bulk of extraneous substances shall be removed.

Purification has been effected by such methods as fractional precipitation, chromatographic techniques, and sequential isopyknic and rate zonal centrifugation (some of these only in combination with others). Digestion by pepsin followed by
dialysis has also been used for the removal of extraneous proteins.

After purification, the protein content shall be measured for both total and HBsAg-specific protein and the latter shall be compared with that of a reference preparation calibrated against the International Reference Preparation. The HBsAg protein in purified preparations shall be at least 95% of the total protein.

If the preparations do not meet this degree of purity then the national control authority should approve the degree of purity permitted and accept the responsibility for the use of this material.

It has been found suitable to measure the total concentration of protein by means of extinction coefficient $E_{280}^{1%=1}$, by micro-Kjeldahl, the Lowry technique, or other suitable method. The antigen content may be measured by an RIA assay.

The purity of the HBsAg may be measured by an electrophoretic technique.

The purified and concentrated HBsAg shall then be subjected to procedures that have been shown to give rise consistently to inactivated batches of vaccine. The purification and inactivation procedure used shall be demonstrated to be capable of yielding defined preparations and inactivating all detectable infectious agents that may be found in human blood.

Much experience has now been gained in the consistent production of safe batches of vaccine. Reliance is placed predominantly on different methods (chemical treatment, physical separation, and heat treatment) in different countries.

In one country purification by zonal centrifugation is followed by three chemical treatment procedures:

(i) pepsin, 1 mg/ml at pH 2.0 held at 37°C for 18 hours;
(ii) urea, 8 mol/litre held at 37°C for four hours; and
(iii) formalin 1 : 4000 (1 : 10 000 formaldehyde) at 37°C for three days.

One country places the greatest reliance upon the separation of the HBsAg, including isopyknic zonal centrifugations through cesium chloride, followed by treatment with 1 : 4000 formalin (1 : 10 000 formaldehyde) at 30°C for 48 hours.

The approach in another country involves three isopyknic zonal centrifugation steps with KBr followed by rate zonal centrifugation through sucrose. The HBsAg is then heated at 60°C for 10 hours followed by treatment with 1 : 2000 formalin (1 : 5000 formaldehyde) at 37°C for four days.

Yet another country uses two heat treatment procedures (90 seconds at 101–104.5°C and 10 hours at 63°C).
The national control authority shall accept the responsibility for ensuring that the separation and inactivation procedures have been demonstrated to be capable of removing or killing all infectious agents that may be found in human blood. The national control authority shall also approve the number of steps that shall be used for inactivation and assume the responsibility for the procedure.

In the initial investigations of satisfactory inactivation procedures the inoculation of susceptible chimpanzees is considered to be necessary (see Appendix 1).

3.6 Tests on purified, inactivated HBsAg batches

3.6.1 Sterility tests

A volume of at least 10 ml of each batch shall be tested for sterility according to the requirements given in Part A, section 5 of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (6, p. 48).

3.6.2 Tests for HBsAg

The content of HBsAg shall be determined by a serological test in comparison with a reference preparation. It is important that the method of production gives a reproducible content of HBsAg. The lower limit of concentration permitted shall be determined by the national control authority.

Both radioimmunoassay and ELISA methods have been shown to be suitable for this purpose. Other tests such as single radial diffusion may be used.

The concentration of HBsAg shall be related to the total protein.

The total protein may be measured by the extinction coefficient $E_{1%}^{1%}$ by micro-Kjeldahl, or by the Lowry test.

3.6.3 Tests for extraneous substances

Tests shall be made for the presence of blood group substances and other blood proteins, including liver-specific membrane proteins, by methods approved by the national control authority.

Agglutinins and agglutinogens are tested for by haemagglutination. Immunelectrophoresis, agar gel diffusion, more sensitive immunoassays, and polyacrylamide gel
electrophoresis have been used to test for other extraneous proteins.

The preparation shall be free from detectable blood group substances.

The permitted concentration of serum proteins present in the vaccine shall be determined by the national control authority.

Tests shall be made for the presence of HBV DNA polymerase or HBV DNA by a method approved by the national control authority.

The preparation must be free from HBV DNA polymerase or HBV DNA.

3.6.4 Test for antigen purity

A test shall be made for purity of HBsAg by polyacrylamide gel electrophoresis (PAGE).

In reduced preparations there are usually two bands shown by polyacrylamide gel electrophoresis. One at 22 000–23 000 and another at 28 000–30 000 relative molecular mass.

The national control authority shall determine the electrophoretic pattern permitted as a demonstration of purity.

3.6.5 Tests for agent used for purification

A test shall be made for the presence of any potentially hazardous agent that may have been used in the course of purification of the HBsAg.

The method used and the permitted concentration shall be approved by the national control authority.

3.6.6 Test for inactivating agents

If the antigen has been treated with formaldehyde and/or other inactivating agents, then the material shall be tested for the presence of free formaldehyde and/or other agents. The method used and the permitted concentration shall be approved by the national control authority.

3.7 Final aqueous bulk

The final aqueous bulk consists of one or more purified, concentrated, and inactivated HBsAg batches. Only those batches
that have satisfied the requirements of Part A, sections 3.5 and 3.6 shall be included in the final bulk.

3.7.1 Test in chimpanzees

The national control authority shall determine how many of the initial lots of vaccine shall be tested for the presence of infectious hepatitis viruses in chimpanzees. The test shall be approved by the national control authority.

The national control authority of any country may take the responsibility for omitting this test. A test shown to be suitable is described in Appendix 1.

3.7.2 Sterility tests

A volume of at least 10 ml of the final bulk shall be tested for bacterial and mycotic sterility according to the requirements given in Part A, section 5.2 of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (6, p. 49).

3.7.3 Test for HBsAg

The quantity of HBsAg protein compared with the total protein in the final bulk shall be determined by a quantitative serological procedure. The lower limit of HBsAg protein and the limit of total protein per human dose shall be approved by the national control authority.

The tests referred to in Part A, section 3.6.2 have been found to be suitable. The samples for this test should be taken before the addition of preservative.

3.7.4 Pyrogenicity test

Each final bulk shall be tested for pyrogenicity by the intravenous injection of rabbits. Three or more healthy rabbits that have not been injected previously shall be used. Each rabbit shall receive, by injection into the ear vein, one human dose.

The criteria for passing the test shall be those specified in the International Pharmacopoeia (7, Appendix 43, p. 747).
3.8 Final bulk

3.8.1 Addition of adjuvant

Where the final bulk contains an adjuvant, the adjuvant and the concentration used shall be approved by the national control authority. Where aluminium salts are used, the concentration of aluminium shall not exceed 1.25 mg per single human dose.

At this stage more preservative may need to be added.

In some countries the alum used as an adjuvant is formed in the presence of the HBsAg, whereas in others preformed alum salts are added to the aqueous bulk. Where preformed aluminium adjuvants are used, it may not be possible to resolublize the aluminium compound, and the testing for purity and concentration of the HBsAg in the final bulk may not be possible.

It must be demonstrated, however, that all the HBsAg is present in the final bulk.

3.8.2 Tests for sterility

A volume of at least 10 ml of the final bulk shall be tested for bacterial and mycotic sterility according to the requirements given in Part A, section 5.2 of the revised Requirements for Biological Substances No. 6 (General Requirements for Sterility of Biological Substances) (6, p. 49).

3.8.3 Tests for preservative

The final bulk shall be tested for the presence of preservative. The method used and the permitted concentration shall be approved by the national control authority.

4. FILLING AND CONTAINERS

The requirements concerning filling and containers given in Part A, section 4 of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (4, p. 16) shall apply.

Care should be taken to ensure that the material of which the
container is made does not adversely affect the HBsAg under the recommended conditions of storage.

Adjuvanted HBsAg vaccine can only be stored at 5°C ± 3°C.

5. CONTROL TESTS ON FINAL PRODUCT

Samples shall be taken from each final lot for the tests in the following sections.

5.1 Sterility tests

The final lot shall be tested for sterility according to the requirements given in Part A, section 5 of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (6, p. 48).

5.2 Innocuity tests

Each final lot shall be tested for innocuity by appropriate tests in mice and guinea-pigs using parenteral injections. The tests shall be those approved by the national control authority.

5.3 Test for preservative

Each final lot shall be tested for the presence of preservative. The test used and the permitted concentration shall be approved by the national control authority.

5.4 Assay of adjuvant

Each final lot shall be assayed for the content of adjuvant. The method used and permitted concentration shall be approved by the national control authority. Where aluminium compounds are used the concentration of aluminium shall not be greater than 1.25 mg per single human dose.

5.5 Potency and identity test

The vaccine shall be identified as HBsAg by appropriate methods. An appropriate quantitative potency assay shall be performed on each final lot irrespective of how many filling lots are made. The vaccine potency shall be compared with that of a reference
preparation calibrated in international units.

A suitable quantitative extinction test in mice is as follows:

Each of a group of at least 20 suitable mice, five weeks of age, is vaccinated intraperitoneally with graded doses of adjuvanted hepatitis B vaccine diluted in the adjuvant used in the vaccine. Similar groups of mice are inoculated with the adjuvanted reference preparation. The mice are bled 42 days later and the sera are kept separate. Antibody determinations are performed by a sensitive quantitative test such as radioimmunoassay. The lower limit should be less than 25% response. The data are analysed according to seroconversion as well as according to the geometric mean titre of anti-HBs for each antigen dose. The strain of mice used for this test must give a steep dose response curve to the reference antigen.

Inbred or outbred mice with the H-2 or H-2q haplotype have been shown to be suitable.

In some countries a quantitative extinction test in guinea-pigs has been shown to be suitable. In any test a reference preparation calibrated in international units should be included.

The potency shall be measured in terms of quantity of vaccine (units) giving an antibody response in 50% of the animals. The national control authority shall determine the lower limit of potency.

6. RECORDS

The requirements given in Part A, section 6 of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (4, p. 17) shall apply.

7. SAMPLES

The requirements given in Part A, section 7 of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (4, p. 18) shall apply.

8. LABELLING

The requirements given in Part A, section 8 of the revised Requirements for Biological Substances No. 1 (General Requirements
The leaflet accompanying the package shall include the following information:

- the method used in the inactivation of the HBV;
- the nature and amount of any preservative, adjuvant, or stabilizer present in the vaccine;
- the volume of one recommended human dose, immunization schedules, and the recommended routes of administration; these shall be given for newborns, children, adults, and immunosuppressed individuals and shall be the same for a given vaccine for all regions of the world;
- the amount of protein contained in one recommended human dose;
- the amount of HBsAg contained in one recommended human dose; and
- a statement declaring whether the production process has been checked for safety by the inoculation of chimpanzees.

9. DISTRIBUTION AND SHIPPING

The requirements given in Part A, section 9 of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (4, p. 18) shall apply.

In addition, the condition of shipping shall be such that the vaccine does not freeze.

Temperature indicators should be packaged with each vaccine shipment to show that freezing did not occur.

If freezing has occurred then the vaccine should not be used.

10. STORAGE AND EXPIRY DATE

The requirements given in Part A, section 10 of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (4, p. 19) shall apply.

In addition, the conditions of storage shall be such that the vaccine does not freeze.
10.1 Storage conditions and stability

Before being distributed by the manufacturing establishment, or before being issued from a depot for the maintenance of reserves of vaccines, all vaccines in bulk form or in final containers shall be kept at $5 \pm 3^\circ C$. After distribution or issue, the vaccine shall be stored at a temperature not exceeding $8^\circ C$. The vaccine shall have been shown to maintain the HBsAg content of the human dose for a period equal to that between the date of issue and the expiry date. During storage the vaccine shall not be frozen.

10.2 Expiry date

The expiry date shall be fixed with the approval of the national control authority and shall relate to the date of the last satisfactory potency test, the date of this test being that on which the test system was inoculated.

PART B.

NATIONAL CONTROL REQUIREMENTS

1. GENERAL

The general requirements for control laboratories contained in Part B of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (4, p. 19) shall apply.

The national control authority shall:

— approve the medical evaluation of donors;
— approve the methods of sterility control;
— approve the tests for HBsAg concentration and define its minimum value;
— approve the methods for concentration, purification, and inactivation;
— approve the purity of the final product;
— approve the tests for extraneous substances and total protein;
— approve the tests for the agents used for concentration and purification, free formaldehyde, and other inactivating agents and preservatives;
— approve the test for the presence of infectious hepatitis viruses;
— approve the tests used for freedom from abnormal toxicity in the
final product;
— approve the adjuvant assay and define the permitted
concentration of adjuvant in the final product; and
— approve the animals used in the assay of potency.

The national control authority shall also provide national
reference preparations for the expression of activity of HBsAg
contained in a given quantity of protein.

Where chimpanzees are not used in the proof of safety of the
production process the national control authority must accept
responsibility for the use of the vaccine in man.

2. RELEASE AND CERTIFICATION

A hepatitis B vaccine shall be released only if it fulfils Part A of
the present Requirements.

A statement signed by the appropriate official of the national
control laboratory shall be provided at the request of the
manufacturing establishment and shall certify whether or not the
final lot of vaccine in question meets all national requirements as
well as Part A of the present requirements. The certificate shall state
the date of the last satisfactory HBsAg potency test, the lot number,
the number under which the lot was released, and the number
appearing on the labels of the containers. In addition, a copy of the
official national release document shall be attached.

The purpose of the certificate is to facilitate the exchange of
hepatitis B vaccine between countries.

AUTHORS

The Requirements for Hepatitis B Vaccine formulated in 1980 (2) were prepared
by the following WHO consultants and staff members:

Dr P. Brès, Virus Diseases, World Health Organization, Geneva, Switzerland
Dr R.J. Gerety, Hepatitis Branch, Bureau of Biologics, Food and Drug
Administration, United States Public Health Service, Bethesda, MD, USA
(Consultant)
Dr M. Hilleman, Director, Virus and Cell Biology Research, Merck Institute for
Therapeutic Research, Merck Sharp & Dohme Research Laboratories, West
Point, PA, USA (Consultant)
Dr R. Mauler, Behringerwerke AG, Marburg, Federal Republic of Germany (Consultant)
Dr J. Maynard, Hepatitis Laboratories Division, Centers for Disease Control, Phoenix, AZ, USA (Consultant)
Dr F.T. Perkins, Chief, Biologicals, World Health Organization, Geneva, Switzerland
Dr A.M. Prince, New York Blood Center, New York, NY, USA (Consultant)
Dr R.H. Purcell, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA (Consultant)
Dr J.D. van Ramshorst, Biologicals, World Health Organization, Geneva, Switzerland
Dr G. Sobeslavsky, Virus Diseases, World Health Organization, Geneva, Switzerland
Professor A. Zuckerman, Director, Department of Medical Microbiology, London School of Hygiene and Tropical Medicine, London, England (Consultant)

The revised Requirements for Hepatitis B Vaccine (1984) were formulated by a WHO informal consultation, with the following members and secretariat:

Members

Dr P. Adamovicz, Pasteur Institute Production, Marnes-la-Coquette, France
Dr P. Beasley, Department of Epidemiology, School of Public Health, University of Washington, Seattle, WA, USA
Professor C. de Bac, Institute of Tropical and Infectious Diseases, Rome, Italy
Professor F. Deinhardt, Max von Pettenkofer Institute, Munich, Federal Republic of Germany
Dr J. Desmyter, Professor of Virology, Catholic University of Louvain, Rega Institute, Louvain, Belgium
Dr S. Drew, Director, Biochemical Engineering, Merck & Co. Inc., Rahway, NJ, USA
Dr S. Funakoshi, Research Director, Green Cross Corporation, Osaka, Japan
Dr M.R. Hilleman, Director, Virus and Cell Biology Research, Merck Institute for Therapeutic Research, Merck Sharp & Dohme Research Laboratories, West Point, PA, USA
Dr F. Hyall, Pasteur Institute Production, Marnes-la-Coquette, France
Dr Kyong-Ho Kim, Director, Research and Development Laboratory, Korea Green Cross Corporation, Seoul, Republic of Korea
Dr P.E. Lemoine, Institute of Hygiene and Epidemiology, Ministry of Family and Public Health, Brussels, Belgium
Dr Li Ho-min, Director, National Institute for the Control of Pharmaceutical and Biological Products, Ministry of Health, Beijing, China
Dr J. Maynard, Assistant Director for Medical Science and Chief, Viral Disease Division, Centers for Disease Control, Atlanta, GA, USA
Dr R. Netter, Director General, National Health Laboratory, Paris, France
Dr K. Nishiohka, Director, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan
Professor Oon Chong Jin, National Centre for Reference and Research on Hepatitis and Related Diseases, National University of Singapore, University Department of Medicine, Singapore General Hospital, Singapore

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Dr P. Paroz, Federal Office of Public Health, Control of Immunobiological Products, Bern, Switzerland
Dr J.C. Petricciani, Director, Office of Biologics, Blood and Blood Products, National Center for Drugs and Biologics, Food and Drug Administration, Bethesda, MD, USA
Dr P. Prunet, Pasteur Institute Production, Marnes-la-Coquette, France
Dr R. Purcell, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA
Dr H. Recsink, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, Netherlands
Dr G.C. Schild, National Institute for Biological Standards and Control, London, England
Dr H. Shimoo, Director, Department of Enteroviruses, National Institute of Health, Tokyo, Japan
Dr T. Takahashi, Head, Division of Hepatitis Research, Kitasato Institute, Tokyo, Japan
Professor W. van Aken, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, Netherlands
Dr H.J.M. van de Donk, National Institute for Public Health, Bilthoven, Netherlands
Dr Zhao Kai, Director, Department of Viral Hepatitis, National Vaccine and Serum Institute, Beijing, China
Professor A.J. Zuckerman, Department of Microbiology, London School of Hygiene and Tropical Medicine, London, England

Secretariat
Dr T.A. Bektimirov, Chief, Virus Diseases, WHO, Geneva, Switzerland
Dr F. Assaad, Director, Division of Communicable Diseases, WHO, Geneva, Switzerland
Dr J. Stjernswärd, Chief, Cancer, WHO, Geneva, Switzerland
Dr F.T. Perkins, Chief, Biologicals, WHO, Geneva, Switzerland
Dr P. Sizaret, Scientist, Biologicals, WHO, Geneva, Switzerland

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Dr P.E. Lemoine, Institute of Hygiene and Epidemiology, Brussels, Belgium
Dr J. Desmyter, Professor of Virology, Catholic University of Louvain, Rega Institute, Louvain, Belgium
Dr Xiang Jianzhi, Head, Division of Science and Education, Shanghai Institute of Biological Products, Shanghai, China
Dr W. Aeg. Timmerman, De Bilt, Netherlands
Dr M.R. Hilleman, Director, Virus and Cell Biology Research, Merck Institute for Therapeutic Research, Merck Sharp & Dohme Research Laboratories, West Point, PA, USA
Professor W.G. van Aken, Medical Director, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, Netherlands
Professor A.J. Zuckerman, Director, Department of Medical Microbiology, London School of Hygiene and Tropical Medicine, London, England
Professor F. Deinhardt, Max von Pettenkofer Institute, Munich, Federal Republic of Germany
Dr A. Gray, Director, Biologics, Merck Sharp & Dohme Research Laboratories, West Point, PA, USA
Dr C. Guthrie, Operations Director, Commonwealth Serum Laboratories, Parkville, Victoria, Australia
Dr K. Nishioka, Vice President, The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan
Dr J. Furesz, Director, Bureau of Biologies, Drugs Directorate, Virus Laboratory, Tunney’s Pasture, Ottawa, Ontario, Canada
Dr K. Chalifour, Director, Virus Research Institute, Department of Medical Sciences, Ministry of Public Health, Yod-se, Bangkok, Thailand
Dr V.F. Davey, Group Director, Science, Commonwealth Serum Laboratories, Parkville, Victoria, Australia
Dr J.C. Petricciani, Director, Blood and Blood Products, Office of Biologics, National Center for Drugs and Biologics, Food and Drug Administration, Bethesda, MD, USA
Dr S.O. Vyasov, D.I. Ivanovskij Institute of Virology, Moscow, USSR
Dr F. Hyall, Scientific Directorate, Pasteur Institute, Marnes-la-Coquette, France
Dr P. Prunet, Scientific Directorate, Pasteur Institute, Marnes-la-Coquette, France
Dr R. Nettet, Director General, National Health Laboratory, Paris, France
Dr A. Chippaux, Director, Department for the Control of Viral Vaccines and Blood Products, National Health Laboratory, Paris, France
Dr Körner, Behringwerke AG, Marburg, Federal Republic of Germany
Dr Praner, Behringwerke AG, Marburg, Federal Republic of Germany
Professor Oon Chong Jin, University Department of Medicine, Singapore General Hospital, Singapore

REFERENCES

Appendix 1

SAFETY AND EFFICACY TESTING OF HEPATITIS B VACCINE

Safety and consistency

In view of the concern about the possibility of infectious agents being present in the plasma used for the production of hepatitis B vaccine it is recommended that the national control authority ensure that the procedures of the collection of plasma and the production process, including purification and inactivation, are reproducible and will give rise to consecutive lots that will not differ with respect to safety.

When this has been achieved, the national control authority shall determine the number of lots tested for consistency of production and safety; this should never be less than five consecutive lots prepared by the same production procedures. These first five lots shall be tested individually without pooling. Ideally, the chimpanzees used for testing would have been under observation for at least six months before inoculation and shown to satisfy the conditions listed below. The chimpanzees shall:

(a) be free from hepatitis B virus infection, past or present, as shown by sensitive techniques (negative tests for HBsAg, anti-HBs, and anti-HBc);

(b) have normal levels of aminotransferases in at least eight specimens taken during the eight consecutive weeks before starting the study;

(c) have had at least two normal liver biopsies taken during the eight weeks preceding the start of the study;

(d) be housed in adequate isolation quarters and attended by persons free from hepatitis B infection;

(e) have never received blood or blood products of human origin.

In some countries more than 15 consecutive lots have been tested in chimpanzees by the manufacturers and evaluated by the control authorities before the vaccine has been licensed. The national control authority shall assume responsibility for this.

The number of chimpanzees and number of human doses given as a single intravenous injection shall be determined by the national control authority. The number of chimpanzees inoculated intravenously shall not be less than two.
After the initial five lots some countries permit the combination of several bulks of vaccines for this test.

The number of final aqueous bulks represented in the sample should be determined by the national control authority who should assume responsibility for this. In no case should this be more than five final bulks. The aliquots taken from the lots should be the same for each lot.

In one country each of a total of four acceptable chimpanzees is injected as follows:

(i) each of two receives the equivalent of one human vaccine dose by the intravenous route;
(ii) each of two receives the equivalent of 10 human vaccine doses by the intravenous route.

During the observation period of six months after inoculation the tests shall include:

(a) weekly determination of alanine aminotransferase (ALT) (this shall remain normal for each individual chimpanzee, and any abnormal finding shall be demonstrated to be unrelated to viral hepatitis);

(b) weekly determinations of the markers of HBV and HAV infection, using sensitive serological tests;

(c) weekly weight determinations and daily checks of general health;

(d) biopsies for light microscopic examination to search for evidence of hepatitis taken monthly and at any time that the chimpanzees show any abnormality.

If after six months’ observation the chimpanzees have shown normal alanine aminotransferase values throughout with no histological or serological evidence of viral hepatitis, the vaccine passes the chimpanzee safety test.

Vaccines that fail the test shall not be used in man. In such a case it shall be considered that the consistency has been broken and must be re-established by the testing of a further five consecutive lots. The reasons for failing the test shall be investigated and reported to the national control authority.

The first five consecutive lots that have been shown to comply with these requirements and have been shown to be safe in chimpanzees shall be subject to clinical trials in man.

The national control authority shall determine how the safety of the vaccine shall be established in man.
Whether or not chimpanzees are available, the national control authority must take the responsibility for granting permission for clinical trials in man that may be required to establish the suitability of each vaccine lot.

In one country the clinical tests in man were carried out according to the following protocol:
Each of the lots were administered to at least 20 seronegative volunteers at low risk to hepatitis B. Each volunteer was given a single human dose of vaccine and observed for six months. Thereafter, a full course of immunization was given. During the observation period serological measurements and clinical observations were made and biomedical tests were done at least once a month to determine whether the volunteers had developed viral hepatitis or a seroconversion indicating an infection with HBV.

If throughout the six months of observation the 20 volunteers on each of the consistency batches showed no abnormalities, the procedures of purification and inactivation as used in the production of the consistency batches were regarded as being capable of removing infectious agents as well as of killing hepatitis B virus. The national control authority determined how many additional lots should be tested in chimpanzees and evaluated in clinical trials in man before accepting the particular production procedures as safe for general use.

Careful surveillance of the vaccine in general use shall be carried out to detect the possible presence of extraneous agents in the vaccine with longer incubation periods, such as the agent causing the acquired immunodeficiency syndrome.

A test for efficacy

The national control authority shall be responsible for the approval of the protocol for the test for efficacy of the hepatitis B vaccine. The protocol shall include:

(a) members required in the target group;
(b) age and medical status of the target group;
(c) dose and route of administration; and
(d) period of observation.
INTRODUCTION

Varicella, also known as chickenpox, is an acute and highly contagious disease of childhood. It is of ubiquitous distribution, occurs in epidemic waves, and infects nearly all persons by 20 years of age. The disease is characterized by vesicular and papular rash
that may be accompanied by fever, malaise, aches, and arthralgia. The disease is usually more severe in adults than in children. Recovery from primary infection is commonly followed by the establishment of latent infection. Reactivation of latent virus may occur resulting in herpes zoster (shingles), particularly in patients with cancer, those receiving immunosuppressive chemotherapy, and the elderly. Zoster is characterized by vesicular rash along the cutaneous distribution of the involved nerves, and by neuralgia with localized inflammation of the nerves and ganglia.

Because of associated complications, varicella virus infections are of greater importance than is generally recognized. Varicella may be accompanied by severe complications particularly in immunosuppressed patients but also occasionally in normal children or adults. These complications include pneumonia, encephalitis, acute cerebellar ataxia, Reye's syndrome, and hepatitis. There is some evidence that in utero infections may cause congenital malformations. Zoster may also be associated with severe complications, including persistent neuralgia, paralysis, pneumonia, meningoencephalitis, and ocular involvement.

The antiviral drugs at present available are partially effective for the treatment of varicella infection and its complications. Hyperimmune globulin administered passively after known exposure may be of value in aborting or attenuating the infection but this is of limited applicability. Vaccines therefore have an important potential role in the control of varicella virus infections.

Several vaccine strains of attenuated varicella virus have been developed and compared, and the OKA strain has been shown to have the most desirable attributes of low virulence while inducing adequate antibody response and protection against the disease. These studies have included both normal and immunosuppressed persons. The OKA strain was developed by Dr M. Takahashi and colleagues at the Research Institute for Microbial Diseases, Osaka University, Osaka, Japan.

The target groups which might be considered for immunization may include: (a) immunosuppressed persons, especially persons with cancer and those who are on immunosuppressive therapy; (b) non-immune adults; (c) susceptible patients in hospital at risk of exposure; (d) susceptible health care professionals; (e) non-pregnant teenage females; and (f) susceptible children.

Further studies of the vaccine are in progress.
GENERAL CONSIDERATIONS

The OKA strain is the only strain of varicella virus currently considered suitable for vaccine production. It was isolated from a vesicle of a boy (OKA) who had chickenpox but who was otherwise healthy. The virus was isolated in human embryonic lung cells, and attenuated by serial passage in both human and guinea-pig embryo cells before further passages in human diploid cells, after which clinical investigations in at least seven countries were initiated. So far some 7000 subjects have been given the virus, including healthy children and adults as well as subjects with underlying disease including some with leukaemia. The virus has been shown to be safe and immunogenic and the doses used have ranged from 500 to 11,000 plaque-forming units given by the subcutaneous route. Accordingly, these requirements have been formulated for the OKA strain only. Varicella virus is very heat labile. Therefore, special precautions should be taken to maintain viability throughout the manufacture and distribution procedures for the vaccine. It is important to ensure that the vaccine is available only in the freeze-dried form.

It is important to establish clearly defined, well-standardized techniques for the assay of the infectious virus content of vaccines. There is a need for an international reference preparation against which infectious virus content can be compared and the World Health Organization will provide such a reference preparation as soon as possible. Similarly, a reference preparation for the measurement of antibody responses to the vaccine is needed (see Appendix 1).

Studies of marker tests, by which the vaccine virus may be reliably differentiated from wild strains, are being carried out. Temperature sensitivity of growth of wild and attenuated strains is too similar for this to be a useful marker. Differential growth capacity of the attenuated and wild strains in guinea-pig embryo cells and human diploid cells has shown more promise but requires further evaluation. Examination of the DNA restriction endonuclease maps has also shown promise.

Each of the following sections constitutes a recommendation. The parts of each section that are printed in large type have been written in the form of requirements so that, if a health administration so desires, these parts as they appear may be included in definitive national requirements. The parts of each section that are printed in
small type are comments and recommendations for guidance. In order to facilitate the international distribution of vaccine made in accordance with these requirements a summary protocol for the recording of the results of the tests is included in Appendix 2.

Should individual countries wish to adopt these requirements as the basis of their national regulations concerning varicella vaccine, it is recommended that a clause be included permitting modifications of manufacturing requirements on the condition that it can be demonstrated, to the satisfaction of the national control authority, that such modified requirements ensure a degree of safety and potency of the vaccine at least equal to those provided by the requirements formulated below. It is desirable that the World Health Organization should then be informed of the action taken.

The terms “national control authority” and “national control laboratory”, as used in these requirements, always refer to the country in which the final vaccine is manufactured and/or used.

PART A.
MANUFACTURING REQUIREMENTS

1. DEFINITIONS

1.1 International name and proper name

The international name shall be “Vaccinum varicella vivum”. 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.

1.2 Descriptive definition

Vaccinum varicella vivum is a preparation of live attenuated varicella virus grown in human diploid cell cultures. The preparation shall satisfy all the requirements formulated below.

The strain shown to be most useful so far is the OKA strain. At present, live varicella vaccine is stabilized by lyophilization and should be available for distribution only in that form.
1.3 International reference preparation and international unit

Since no international standards or reference preparations of live varicella vaccine have yet been established, no requirements for potency based on such standards or preparations can be formulated. National control authorities should provide a reference preparation of live varicella virus for use in tests of virus concentration (see Part A, sections 3.4.1 and 5.2).

1.4 Terminology

_Virus seed lot_. A quantity of virus processed together and of uniform composition. Seed lots are derived from the virus used in the preparation of an original vaccine shown to be immunogenic and safe in man.

_Cell culture lot_. A number of cell cultures derived from the same pool of cells, processed, and prepared together.

_Merchant's working cell bank (MWCB)_A. A quantity of human diploid cells derived from a single ampoule of the cell seed and of uniform composition stored frozen at $-70^\circ C$ or below in aliquots, one of which would be used for the production of each single harvest.

_Production cell culture_. A collection of cell cultures at the population doubling level used for virus growth that have been derived from a single ampoule of the MWCB.

_Single harvest_. A virus suspension harvested in one continuous operation from one cell culture lot, all the cultures having been inoculated at the same time.

_Virus pool_. A preparation made from a single harvest or from a number of single harvests mixed and processed together.

_Final bulk suspension_. A quantity of vaccine after completion of preparations for filling, present in the container from which the final containers are filled.

_Vaccine lot_. All finished material in final containers which has been derived from the same final bulk suspension and which has, therefore, a uniform composition.

_Filling lot (final lot)._ A vaccine lot, or a part of it, which is homogeneous with respect to the risk of contamination during filling or drying. A filling lot, therefore, consists of finished material filled in one working session and dried together.
2. GENERAL MANUFACTURING REQUIREMENTS

The general requirements for manufacturing establishments contained in the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (1) shall apply to establishments manufacturing live varicella vaccine with the addition of the following:

Visitors not directly concerned with the production processes shall not be permitted to enter areas used for processing live varicella vaccine; others having business in such areas shall be admitted only under supervision.

Continuous cell lines shall not be introduced into areas used for the production of live varicella vaccine.

Production and control shall be organized as two separate units of the manufacturing establishment with independent responsibilities.

Personnel involved in the production of the vaccine shall be shown to be immune to varicella.

3. PRODUCTION CONTROL

3.1 Control of source materials

3.1.1 Virus strains

The OKA strain of varicella virus used in the production of live varicella vaccine shall be identified by historical records which shall include information on the origin of the strain and its subsequent manipulation. The virus shall at no time have been passed in continuous cell lines and shall be shown in monkeys to be free from neurovirulence. The seed lot shall have been shown to yield live varicella vaccines of adequate immunogenicity and safety in man and shall be approved by the national control authority.

The OKA strain was isolated from a vesicle from a case of varicella occurring in an otherwise healthy boy (named OKA). The virus isolated in primary human embryo lung cells was passaged 11 times at 34°C in cells from the same stored cell suspension. After a further 12 passages at 37°C in guinea-pig embryo cells, the virus harvested was tested in children for its suitability. After three more passages at 37°C in human diploid cells the virus strain was considered to be suitable for vaccine
production. Thus, from first isolation the virus was passaged through 26 sequential passages. A further two passages were made in human diploid cells (passage 28) which has been used for distribution from which the vaccine is being made. The clinical data upon which the safety and efficacy of this strain has been based are from vaccine made within 10 passages of the 28th passage virus.

3.1.2 Original cells and cell cultures of MWCB for virus propagation

The human diploid cells used for the propagation of varicella virus shall be those approved by and registered with the national control authority. The cells shall have been characterized with respect to their geneology, growth characteristics, genetic markers (HLA), viability during storage, and karyology, and they shall have been shown, by tests in animals and eggs, to be free from detectable adventitious agents. The supernatant fluids shall also have been shown by tests in cell cultures to be free from adventitious agents.

The cells of the MWCB shall also have been shown to be free from extraneous agents, and shown to be diploid and stable with respect to karyology and morphology by the tests outlined in this section.

In some countries the cells are examined also by ultra-thin sections and by negative staining under the electron microscope.

3.1.2.1 Tests in animals and eggs for extraneous agents

The tests of the MWCB in animals and eggs for adventitious agents shall include the inoculating of each of the following groups of animals with the cells by the intramuscular route, using at least $10^7$ cells divided equally between the animals in each group:

- 2 litters of suckling mice, totalling at least 10 animals, less than 24 hours old;
- 10 adult mice of 15–20 g weight;
- 5 guinea-pigs of 350–450 g weight; and
- 5 rabbits.

At least $10^6$ viable cells shall be injected into the allantoic cavity of 10 embryonated chicken eggs 9–11 days old. In one country it has been found that the inoculation of the yolk sac of 6–7-day-old embryos with incubation for 9 days and a subculture with an incubation also for 9 days is a sensitive method of the isolation of fastidious bacteria.
The animals shall be observed for at least four weeks and the embryonated chicken eggs shall be examined after not less than three days. Any animals that are sick or show any abnormality shall be investigated to establish the cause of illness. The allantoic fluids of the fertile eggs shall be tested with erythrocytes from guinea-pig and chick or other avian species for the presence of haemagglutinins.

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

The cells are suitable if at least 80% of the animals or eggs remain healthy and survive the observation period and none of the animals or eggs shows evidence of the presence in the cell cultures of any extraneous agents.

3.1.2.2 Freedom from tumorigenicity

The cells shall also be shown to be free from potential tumorigenicity by appropriate tests in animals approved by the national control authority.

Suitable tests using immunosuppressed animals may be made as follows. Approximately $10^6$ cells obtained from cultures at the same passage levels as those used for vaccine production are injected into: (a) newborn mice or hamsters treated with antilymphocyte serum; (b) athymic mice (nude nu/nu genotype); or (c) thymectomized mice irradiated and reconstituted (T-B). Some of the same group of animals should be inoculated with a similar dose of HeLa or KB cells and it should be shown that tumour formation is caused by the inoculation of the neoplastic tissue, thus demonstrating the ability of the strain of animals to give rise to tumours. The animals should be observed for not less than three weeks. Any other test using animals treated with immunosuppressive agents and with equal sensitivity to neoplastic cells may be used.

The cells are suitable for vaccine production if at least 80% of the animals inoculated with cells remain healthy and survive the observation period, and none of the animals shows evidence of tumour formation from the cells.

3.1.2.3 Chromosomal characterization

At least four samples shall be examined, prepared as described in Part A, section 3.1.2.4 from the cell seed at approximately equal
intervals over the life span of the cell line during serial cultivation. Each sample shall consist of 1000 metaphase cells.

It is also recommended that photographic reconstruction should be employed in the preparation of chromosome-banded karyotypes of 50 metaphase cells per 1000-cell sample using either G-banding or Q-banding techniques. The incidence of karyotypic abnormalities (pseudo-diploidy, inversions, translocations, etc.) that are detectable with the greater resolution provided by banding should be approved by the national control laboratory.

3.1.2.4 Chromosomal monitoring—preparation and testing

For the determination of the general character of each pool in the manufacturer’s working cell bank, a minimum of 500 cells in metaphase shall be examined at the production level or at any passage thereafter for frequency of polyploidy and for exact counts of chromosomes, frequency of breaks, structural abnormalities, and other abnormalities such as despiralization or marked attenuations of the primary or secondary constrictions.

For vaccine production, examination of the cells is usually made between the twenty-seventh and thirty-third population doubling. The national control authority should determine the level of cell population doubling allowable.

For cells examined in metaphase the upper limits of acceptability (upper fiducial limits at 95% (Poisson)) for abnormalities for a 1000- and 500-cell sample are as follows:¹

<table>
<thead>
<tr>
<th>Abnormality</th>
<th>1000 cells</th>
<th>500 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromatid and chromosome breaks</td>
<td>47/1000</td>
<td>26/500</td>
</tr>
<tr>
<td>Structural abnormalities</td>
<td>17/1000</td>
<td>10/500</td>
</tr>
<tr>
<td>Hyperploidy</td>
<td>8/1000</td>
<td>5/500</td>
</tr>
<tr>
<td>Hypoploidy</td>
<td>180/1000</td>
<td>90/500</td>
</tr>
<tr>
<td>Polyploidy</td>
<td>30/1000</td>
<td>17/500</td>
</tr>
</tbody>
</table>

All cells showing abnormalities shall be subjected to detailed examination, and records shall be maintained of the detailed criteria

¹ These upper limits are based on extensive experience with the examination of WI-38 and MRC5 cells reported to and examined by the Ad Hoc Committee on Karyological Controls of Human Cell Substrates, which met in 1978 at Lake Placid, NY, USA. These values will not necessarily be applicable if another human cell strain is used.

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applied to particular abnormalities evaluated in the karyotype analysis.

Stained slide preparations of the chromosomal monitoring of the working cell bank pool, or photographs of these, shall be maintained permanently as part of the record of the batch of vaccine and for monitoring successive batches made from that cell pool.

It is desirable that a portion of the sample of pooled cell substrate removed from the culture vessels should be stored frozen so as to retain viability. This would be available for future reference for karyology or for any other purpose relating to the batch of vaccine.

Only those cell pools of the MWCB that have normal karyology shall be used for vaccine production.

3.1.3 Virus seed lot system

The production of vaccine shall be based on the virus seed lot system. Seed lots shall be prepared in the same kind of cells as that used for the production of the final vaccine.

Each seed lot shall be identified as varicella virus by appropriate methods (see Part A, section 5.1).

Each seed lot shall be shown by appropriate tests to be free from all demonstrable extraneous viable microbial agents and shall be tested according to, and shall satisfy the requirements of, Part A, section 3.3. In addition, each seed lot shall pass the tests described below.

A seed lot if freeze dried shall be stored at a temperature lower than $-20^\circ C$. If it is not lyophilized, the seed lot shall be stored at a temperature of $-60^\circ C$ or lower.

All vaccine lots shall contain varicella virus derived only from cultures inoculated with seed virus. The virus in the final vaccine shall not have been passaged in cell cultures beyond the 38th passage level from the original isolated virus. (The virus being distributed for vaccine production is of the 28th passage level (see section 3.1.1 on page 107).)

Virus beyond the 38th passage level from the original isolated virus has not been clinically tested.
3.1.3.1 Tests for neurovirulence of the virus seed lot

Each seed lot shall be shown to be free from neurovirulence by tests in monkeys susceptible to varicella.

Immediately prior to the test, each monkey should be shown to be serologically negative for varicella. At least 10 monkeys should be employed in each test. The material under test should be given to each monkey by the inoculation of 0.5 ml into the thalamic region of each hemisphere. The total amount of varicella virus inoculated into each monkey should not be less than the amount contained in the recommended single human dose of vaccine. Monkeys should be observed for 17–21 days for symptoms of paralysis and other evidence of neurological involvement. Animals that die within 48 hours of injection may be replaced. The test is invalid and should be repeated if more than 20% of the monkeys die from nonspecific causes. At the end of the observation period each monkey should be autopsied and histopathological examinations should be made of appropriate areas of the brain for evidence of central nervous system involvement.

The material passes the test if there is no clinical or histopathological evidence of involvement of the central nervous system attributable to the inoculated virus.

In some countries the national control authorities advise the inoculation of some monkeys with sterile control fluids to serve as controls.

3.2 Production precautions

The general production precautions as formulated in Part A, section 3, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (1, p. 15) shall apply to the manufacture of live varicella vaccine with the addition of the following.

3.2.1 Cell cultures used for vaccine production

Only human diploid cell cultures derived from a cell seed (MWCIN) approved by the national control authority shall be used for vaccine production. The production of each single harvest shall be initiated from one or more new ampoules of the cell seed. All processing of the cell seed and subsequent cell cultures shall be done in an area in which no other cells are handled. The cell cultures shall be used only if no changes have occurred in their growth
characteristics and if no changes from the normal karyology have been shown to occur within the total number of population doublings that correspond to the average finite life of the cells as determined under the particular conditions of the production establishment (see Part A, section 3.1.2.3).

   It is advisable to ensure that both the trypsin and calf serum used in the preparation of the cell suspensions and growth are free from extraneous agents.

   It is important that the karyological pattern should have been shown not to differ from that established for the cell seed for the total number of population doublings of the cell cultures and that no changes from the normal karyology should have been shown to occur within the total number of population doublings that would be used for vaccine production.

The cells shall, however, not be used beyond two-thirds of the total number of population doublings corresponding to the average finite life of the cells.

   At least 5% of the cell suspension (not less than 500 ml) of the concentration employed for seeding the vaccine production cultures shall be used to prepare control cultures.

   In countries were the technology of large-scale production has been developed, the national control authorities should determine the size of the sample of cells to be examined.

   The treatment of cells set aside as control material shall be similar to that of the production cell cultures, but they shall remain uninoculated as control cultures for the detection of extraneous viruses.

   These control cell cultures shall be incubated under the same conditions as those for the inoculated cultures for at least two weeks and shall be examined during this period for evidence of cytopathic changes. For the test to be valid, not more than 20% of the control cell cultures shall have had to be discarded for nonspecific, accidental reasons.

   At the end of the observation period, the control cell cultures shall be examined for degeneration caused by an extraneous agent.

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

   In some countries fluids are collected from the control cells at the time of virus harvest and at the end of the observation
period; such fluids may then be pooled before testing for extraneous viruses.

Cell cultures for vaccine production shall be grown under aseptic conditions.

If animal serum is used in the maintenance medium for growth of the cell cultures, the cells shall be washed free from serum so that the final vaccine shall not contain more animal serum than 1 mg/kg. Human serum shall not be used. Penicillin shall not be used in the tissue culture medium.

Minimum concentrations of other suitable antibiotics may be used.

The control cultures grown from the 500 ml of control cell suspension shall be observed microscopically for changes attributable to the presence of adventitious agents for a period of not less than 14 days beyond the time of inoculation of the production vessels with varicella virus. At 14 days, fluids collected from the control cultures as well as cell sheets from a proportion of the control vessels shall be tested for the presence of adventitious agents by the tests described below. The results of the tests on control cultures are considered satisfactory only if there is no evidence of adventitious agents and if at least 80% of the control vessels are available for observation and testing at the end of the observation period.

3.2.1.1 Test for haemadsorbing viruses

The cell sheets of one-quarter to one-third of the control vessels shall be examined at the end of the observation period for the presence of haemadsorbing viruses by the addition of guinea-pig erythrocytes.

3.2.1.2 Test for adventitious agents in cell cultures

Samples of at least 5 ml each of the fluids pooled from the control cultures shall be inoculated into human and simian cell culture systems and into the cell culture system used for vaccine production and shall be tested by the method described in Part A, section 3.3.3.

3.2.1.3 Identity test

An identity test is performed on the control cell cultures by tests approved by the national control authority.

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Suitable tests are isozymes, HLA, or other immunological tests or karyotype of at least one metaphase spread of chromosomes.

The cells shall be shown to be of human origin.

3.3 Harvesting and disruption of cells

The infected cells constituting a single harvest shall be washed, released from the glass or other support surface, and pooled. The cell suspension shall be disrupted by sonication and tested in animals and cell cultures for the presence of extraneous agents by the following tests.

3.3.1 Sterility tests

A volume of at least 10 ml of each single harvest of disrupted cells shall be tested for bacterial and mycotic sterility according to the procedures given in Part A, sections 5.2 of Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (2), as well as for mycoplasma by a method approved by the national control authority.

Tests for mycoplasma should be done using both solid and liquid media that have been shown to be capable of growing mycoplasma.

3.3.2 Test for Mycobacterium tuberculosis

Each virus pool shall be tested for the presence of Mycobacterium tuberculosis (human, bovine, and avian) by culture methods appropriate for the detection of the organisms most likely to be found in the cell culture system used.

3.3.3 Tests in tissue cultures

In the tests that require prior neutralization of the varicella virus, the antiserum used shall not be of human, simian, or bovine origin. The immunizing antigen used for the preparation of the antiserum shall be produced in tissue culture cells free from extraneous microbial agents that might elicit antibodies inhibitory to the growth of any extraneous agents that may be present in the varicella virus pool and shall not be made using the OKA strain.

A volume of each virus pool equivalent to at least 500 human doses of vaccine, or 50 ml, whichever represents the greater volume,
shall be tested for adventitious agents by inoculation into cells of human and simian origin. The human cells shall include both continuous and diploid cells and the simian cells shall be derived from monkey kidneys. The tissue cultures shall be observed for at least 14 days.

Suitable cells for this are VERO, LLCMK, HeLa, and MRC5.

The virus pool passes the tests if at least 80% of the cell cultures remain viable and none of the tissue cultures shows evidence of the presence of any adventitious agents attributable to the virus pool.

3.3.4 Test in adult mice

Each virus pool shall be tested in adult mice for adventitious agents pathogenic to mice. Each of at least 10 adult mice, of 15–20 g weight, shall be inoculated intracerebrally with 0.03 ml and intraperitoneally with at least 0.5 ml of the virus pool. The mice shall be observed for at least 21 days. All mice that die after the first 24 hours of the test or that show signs of illness shall be autopsied and examined for evidence of viral infection, both macroscopically by direct observation and by subinoculation of appropriate tissue suspensions by the intracerebral and intraperitoneal routes into at least five additional mice, which shall be observed for 21 days.

The virus pool passes the test if at least 80% of the original inoculated mice survive the observation period and if no mice show evidence of infection with adventitious transmissible agents attributable to the virus pool.

In some countries the national control authority may permit the manufacturer to omit this test and others may permit a 60% survival of the original cultures.

3.3.5 Test in suckling mice

Each virus pool shall be tested in suckling mice for adventitious agents pathogenic to mice. Each of at least 20 mice less than 24 hours old shall be inoculated intracerebrally with 0.03 ml and intraperitoneally with at least 0.1 ml of the virus pool. The mice shall be observed daily for at least 14 days. All mice that die after the first 24 hours of the test or that show signs of illness shall be autopsied and examined for evidence of viral infection, both macroscopically by direct observation and by subinoculation of appropriate tissue
suspensions by the intracerebral and intraperitoneal routes into at least five additional suckling mice, which shall be observed daily for 14 days.

In some countries, in addition to the above test, a blind passage is made of a suspension of the pooled emulsified tissue (minus skin and viscera) of all mice surviving the original 14-day test.

The virus pool passes the test if at least 80% of the original inoculated mice survive the observation period and if no mice show evidence of infection with adventitious transmissible agents attributable to the virus pool.

In some countries the national control authority may permit the manufacturer to omit this test.

3.3.6 Test in guinea-pigs

Each virus pool shall be tested for adventitious agents by the intraperitoneal inoculation of 5.0 ml of the virus pool into each of at least five guinea-pigs of 350–450 g weight. The animals shall be observed for at least 42 days for signs of disease. All guinea-pigs that die after the first 24 hours of the test or that show signs of illness shall be examined by autopsy macroscopically, and the tissues shall be examined both microscopically and by culture for evidence of infection. Animals that survive the observation period shall be examined in a similar manner.

The virus pool passes the test if at least 80% of the guinea-pigs survive the observation period and if none of the animals shows evidence of infection with any adventitious transmissible agents attributable to the virus pool.

In some countries the national control authority may permit the manufacturer to omit this test.

3.3.7 Test in embryonated hens’ eggs

In some countries a test is made in embryonated hens’ eggs. A volume of each virus pool, equivalent to at least 100 human doses of vaccine, or 10 ml, whichever represents the greater volume, is tested in a group of embryonated eggs by the allantoic route of inoculation, using 0.5 ml of inoculum per egg. In one country it has been found that the inoculation of the yolk sac of 6–7-day-old embryos with incubation for 9 days and
a subculture with incubation also for 9 days is a sensitive method for the isolation of fastidious bacteria.

The virus pool passes the test if there is no evidence of the presence of any adventitious agent attributable to the virus pool.

3.4 Clarification and control of clarified virus pool

The disrupted cell suspension after appropriate testing shall be clarified by a method that will ensure the removal of all intact tissue cells.

A suitable test for the presence of residual intact cells is as follows: centrifuge 50 ml of the sample at 2000 rpm (750 g) for 30 minutes and resuspend the sediment in 0.9 ml (approximately 100-fold concentration). The concentrated suspension is examined microscopically for the presence of intact cells.

3.4.1 Virus titration

The live virus content of each single harvest shall be determined by titration in cell cultures using a reference preparation of live varicella virus for comparison (see Part A, section 1.3).

3.5 Control of final bulk suspension

The final bulk suspension shall be prepared from one or more clarified virus pools which satisfy the tests of Part A, sections 3.3 and 3.4.

Only stabilizers, diluents, or other substances approved by the national control authority shall be added to or combined with the vaccine and shall have been shown by appropriate tests to have no deleterious effects on the product in the amounts used. Antibiotics shall not have been added at any stage of manufacture after harvesting, and none shall be added to the final bulk suspension.

All manipulations shall be carried out under aseptic conditions.

It is desirable that the final bulk suspension after preparation should be filled and lyophilized without delay. If this is not possible, the final bulk suspension should be stored at $-70^\circ C$ or lower until it is filled and lyophilized.

3.5.1 Sterility tests

A volume of at least 20 ml of each final bulk suspension shall be tested for bacterial and mycotic sterility according to the
requirements given in Part A, sections 5.2 of Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Products) (2).

4. FILLING AND CONTAINERS

The requirements for filling and containers given in Part A, section 4 of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories (1, p. 16) shall apply.

In general, only single-dose containers should be used.

5. CONTROL TESTS ON FINAL PRODUCT

Samples shall be taken from each filling lot for the following tests.

5.1 Identity test

An identity test shall be performed on a pool of three labelled containers from each filling lot by appropriate methods.

Methods such as test in tissue culture of neutralized virus using specific antiserum are suitable (see Part A, section 3.3).

5.2 Test for virus concentration

A test for live virus concentration shall be done on each freeze-dried filling lot by titration in a suitable tissue culture system using a reference preparation of live varicella virus for comparison (see Part A, section 1.3).

It is desirable to titrate individually the contents of five or more separate containers.

5.3 Innocuity tests

Each filling lot shall be tested for abnormal toxicity by appropriate tests that shall be approved by the national control authority.
5.4 Inspection of final containers

Each container in each filling lot shall be inspected visually and those showing abnormalities shall be discarded.

5.5 Sterility tests

At least 10 ampoules shall be tested for bacterial and mycotic sterility according to the requirements given in Part A, sections 5.2 of Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Products) (2).

5.6 Residual moisture

The residual moisture in a representative sample of each freeze-dried lot shall be determined by a method approved by the national control authority. The moisture content shall be not greater than 2%.

6. RECORDS

The requirements given in Part A, section 6, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (1, p. 17) shall apply.

7. SAMPLES

The requirements given in Part A, section 7, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (1, p. 18) shall apply.

8. LABELLING

The requirements given in Part A, section 8, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (1, p. 18) shall apply with the addition of the following.
The label on the carton enclosing one or more final containers or the leaflet accompanying the container, shall contain the following additional information:

— a statement that the vaccine fulfils the requirements of this document;
— the nature of the preparation, i.e., the designation of the strain of varicella virus contained in the vaccine and the origin of the tissue used in the preparation of the vaccine;
— the nature and quantity of any residual antibiotic present in the vaccine;
— a statement concerning the photosensitivity of the vaccine, cautioning that both lyophilized and reconstituted vaccine should be protected from light;
— the volume and nature of the diluent\(^1\) to be added to reconstitute the vaccine; and
— a statement that after the vaccine is reconstituted it should be used without delay.

9. DISTRIBUTION AND SHIPPING

The requirements given in Part A, section 9, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (I, pp. 18–19) shall apply. Shipment should be at temperatures of 8 \(^\circ\)C or below.

10. STORAGE AND EXPIRY DATE

The statements concerning storage temperature and expiry date appearing on the label and the leaflet as required in Part A, section 10 of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (I, p. 19) shall be based on experimental evidence and shall be submitted for approval to the national control authority.

\(^1\) The diluent used should be that supplied by the manufacturer and must not contain any preservative.
10.1 Storage conditions

Before distribution the manufacturer shall store lyophilized vaccine below \(-20°C\). After distribution live varicella vaccine shall be stored at all times at a temperature below \(8°C\).

10.2 Expiry date

The expiry date shall be fixed with the approval of the national control authority and shall relate to the date of the last satisfactory test for virus concentration, the date of this test being that on which the test system was inoculated.

PART B

NATIONAL CONTROL REQUIREMENTS

1. GENERAL

The general requirements for control laboratories contained in Part B of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) (I, pp. 19–22) shall apply.

The national control authority shall give directions to manufacturers concerning the varicella vaccine seed virus to be used in vaccine production and concerning the recommended human dose.

In addition, the national control authority shall provide a reference preparation of live varicella virus (see Part A, section 1.3) for tests for virus concentration (see Part A, sections 3.4.1 and 5.2) and shall specify the requirement for virus content that shall be fulfilled in order to achieve adequate immunization in man using the recommended human dose.

2. RELEASE AND CERTIFICATION

A vaccine shall be released only if it fulfils Part A of the present requirements. A statement signed by the appropriate official of the national control laboratory shall be provided at the request of the
manufacturing establishment and shall certify whether the lot of vaccine in question meets all national requirements as well as Part A of the present requirements. The certificate shall state the date of the last satisfactory test for virus concentration, the lot number, the number under which the lot was released, and the number appearing on the labels of the containers. In addition, a copy of the official national release document shall be attached. The purpose of the latter certificate is to facilitate the exchange of live varicella vaccine between countries.

AUTHORS

The Requirements for Varicella Vaccine (Live) were formulated by the following WHO consultants and staff members:

Dr T.A. Bektimirov, Chief, Virus Diseases, World Health Organization, Geneva, Switzerland
Dr M.R. Hilleman, Director, Virus and Cell Biology Research, Merck Institute for Therapeutic Research, Merck Sharp & Dohme Research Laboratories, West Point, PA, USA (Consultant)
Dr C. Huygelen, Smith Kline-RIT, Rixensart, Belgium (Consultant)
Dr F.T. Perkins, Chief, Biologicals, World Health Organization, Geneva, Switzerland
Dr G. Quinnan, Office of Biologics, National Center for Drugs and Biologics, Food and Drug Administration, Bethesda, MD, USA (Consultant)
Dr G.C. Schild, National Institute for Biological Standards and Control, London, England (Consultant)
Dr P. Sizaret, Biologicals, World Health Organization, Geneva, Switzerland
Dr M. Takahashi, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan (Consultant)

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Dr S.C. Arya, King Fahad Central Hospital, Jizan, Saudi Arabia
Dr V.F. Davey, Group Director, Science, Commonwealth Serum Laboratories, Parkville, Victoria, Australia
Dr I. Di Tommaso, Responsible Head of Establishment, “Sclavo” Tuscan Institute for Serotherapy and Vaccine Production, Siena, Italy
Professor S.G. Dzagurov, Director, Tarasevich State Research Institute for the Standardization and Control of Medical Biological Preparations, Ministry of Health, Moscow, USSR
Dr J. Furesz, Director, Bureau of Biologics, Drug Directorate, Virus Laboratory, Tunney’s Pasture, Ottawa, Canada

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REFERENCES

Tests used to demonstrate immune responses to vaccination with varicella vaccine should be specific, sensitive and, when positive, should correlate with resistance to disease. The most widely available test for antibodies to varicella virus is the complement-fixation (CF) test. It is an insensitive test and has not been useful for evaluating vaccine effects. More sensitive tests that have been used include: (a) neutralization test (NT); (b) fluorescent antibody to membrane antigen (FAMA); (c) immune adherence haemagglutination (IAHA); and (d) enzyme-linked immunosorbent assay (ELISA). Among these, the one with which there is the greatest amount of experience is the FAMA test. This test yields excellent results with respect to sensitivity and specificity and a positive result correlates well with resistance to disease. However, the FAMA test is more difficult to perform than some of the other tests. The NT, IAHA, and ELISA tests are useful for evaluating responses to varicella virus vaccine if their sensitivity and specificity is first established by the laboratories performing the tests.

Skin testing for reactivity to varicella virus antigens has been performed by some investigators. Although useful information has been obtained in some cases, well-standardized preparations are not generally available and the testing is done only on a research basis.

The most commonly used in vitro test of cell-mediated immunity to varicella virus is the lymphocyte proliferation assay. Most healthy volunteers given varicella virus vaccine develop cell-mediated immunity, as measured by this procedure. However, the test is neither highly sensitive nor completely specific and should not be used as the primary method of evaluating vaccine responses.
Appendix 2

SUMMARY PROTOCOL FOR VARICELLA VACCINE (LIVE)

Based on Requirements for Biological Substances No. 36
Requirements for Varicella Vaccine (Live)

Name and address of manufacturer

Proprietary name of vaccine
Lot number of vaccine

Virus Strain

Name
Neurovirulence in monkeys:
  Date of inoculation
  Result
Has the virus strain been approved by the national control authority?

Manufacturer's Working Cell Bank

Identity of the human diploid cell line
Characteristic genetic markers

Tests for extraneous agents

<table>
<thead>
<tr>
<th></th>
<th>After four weeks, no. of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of animals alive after one day</td>
</tr>
<tr>
<td>Intramuscular</td>
<td></td>
</tr>
<tr>
<td>inoculation of</td>
<td></td>
</tr>
<tr>
<td>at least 10^7</td>
<td></td>
</tr>
<tr>
<td>cells divided</td>
<td></td>
</tr>
<tr>
<td>equally between</td>
<td></td>
</tr>
<tr>
<td>the animals in</td>
<td></td>
</tr>
<tr>
<td>each group of:</td>
<td></td>
</tr>
<tr>
<td>Suckling mice</td>
<td></td>
</tr>
<tr>
<td>Adult mice</td>
<td></td>
</tr>
<tr>
<td>Guinea-pigs</td>
<td></td>
</tr>
<tr>
<td>Rabbits</td>
<td></td>
</tr>
</tbody>
</table>
Inoculation of $10^6$ cells into allantoic cavity of embryonated eggs:

- No. of chicken embryos alive/dead three after injection
- Species used for providing red blood cells for haemagglutination
- No. of amniotic fluids containing/not containing haemagglutinins

**Test for tumorigenicity**

Species of animals used for the test

Characteristics of animals (newborn, treated with anti-lymphocyte serum, athymic, or thymectomized)

<table>
<thead>
<tr>
<th></th>
<th>Malignant cell line</th>
<th>Human diploid cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cells injected per animal</td>
<td>. . .</td>
<td>. . .</td>
</tr>
<tr>
<td>No. of animals inoculated</td>
<td>. . .</td>
<td>. . .</td>
</tr>
<tr>
<td>No. of animals alive after three weeks</td>
<td>. . .</td>
<td>. . .</td>
</tr>
<tr>
<td>No. of animals with tumour after three weeks</td>
<td>. . .</td>
<td>. . .</td>
</tr>
</tbody>
</table>

**Chromosomal characterization**

Period of the life span (number of population doubling) at which the test was made

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cells examined</td>
<td>. . .</td>
</tr>
<tr>
<td>No. of chromatid and chromosome breaks</td>
<td>. . .</td>
</tr>
<tr>
<td>No. of structural abnormalities</td>
<td>. . .</td>
</tr>
<tr>
<td>No. of hyperploid cells</td>
<td>. . .</td>
</tr>
<tr>
<td>No. of hypoploid cells</td>
<td>. . .</td>
</tr>
</tbody>
</table>

**Virus Seed Lot**

No. of passages since the first isolation of virus

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Methods used for identification</td>
<td></td>
</tr>
<tr>
<td>Results of tests for the absence of:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>bacterial contamination</td>
<td></td>
</tr>
<tr>
<td>mycotic contamination</td>
<td></td>
</tr>
<tr>
<td>mycoplasma contamination</td>
<td></td>
</tr>
</tbody>
</table>
— *Mycobacterium tuberculosis*
— adventitious agents

**Antiserum used for virus neutralization**

| Code number |  
| Species of origin |  

**Tests in cell cultures**

<table>
<thead>
<tr>
<th>Identity</th>
<th>% cells viable after 14 days</th>
<th>Presence of adventitious agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Continuous line</td>
<td>. . .</td>
<td>. . .</td>
</tr>
<tr>
<td>Diploid line</td>
<td>. . .</td>
<td>. . .</td>
</tr>
<tr>
<td>Simian kidney cells</td>
<td>. . .</td>
<td>. . .</td>
</tr>
</tbody>
</table>

**Tests in mice**

<table>
<thead>
<tr>
<th>Suckling</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of mice alive at day 1</td>
<td>. . .</td>
</tr>
<tr>
<td>No. of mice alive at day 21</td>
<td>. . .</td>
</tr>
<tr>
<td>No. of dead or sick mice between days 1 and 21</td>
<td>. . .</td>
</tr>
<tr>
<td>No. with adventitious agents</td>
<td>. . .</td>
</tr>
<tr>
<td>No. without adventitious agents</td>
<td>. . .</td>
</tr>
<tr>
<td>No. of mice alive and well at day 21</td>
<td>. . .</td>
</tr>
<tr>
<td>No. with adventitious agents</td>
<td>. . .</td>
</tr>
<tr>
<td>No. without adventitious agents</td>
<td>. . .</td>
</tr>
</tbody>
</table>

**Tests in guinea-pigs**

|  
|  
|  

|  
|  
|  

|  
|  
|  

**Tests in embryonated eggs**

| Total volume of virus inoculated |  
| Corresponding number of human doses |  
| No. of embryonated eggs inoculated |  

128
Incubation age at inoculation  
Route of injection  
Result of injection after 9 days  
Was a subculture in embryonated eggs necessary?  
If yes, what was the result?  

**Neurovirulence tests**  
Monkey species  
Date on which monkeys were inoculated  
No. of human doses injected into each monkey  
Route of injection  
Time of observation of animals  
No. of animals alive 48 hours after injection  

<table>
<thead>
<tr>
<th>No.</th>
<th>No. with CNS infection</th>
<th>No. with CNS infection in which varicella virus presence could be excluded</th>
</tr>
</thead>
</table>

Monkeys dead between 2 days after injection and end of the observation period  
Monkeys alive at the end of the observation period  

**Single Harvests**

No. of population doubling at which virus was inoculated  

**Control cell cultures**  
Antibiotics used in tissue culture medium (if used) 
Concentration  
Volume of control cell culture at the time of virus inoculation  
This volume represents a % of cells used for vaccine production of  
Species from which the serum used for cell growth derived  
% of cells discarded for accidental reasons within 2 weeks  
Result of examination after 2 weeks for degeneration caused by extraneous agents  

---

1 Provide information for each single harvest.
Tests for haemadsorbing viruses
Result of test on fluids

Test on cell sheets
— proportion of control vessels examined
— result of the test

Tests for adventitious agents

<table>
<thead>
<tr>
<th></th>
<th>Human cell cultures</th>
<th>Simian cell cultures</th>
<th>Cell cultures similar to those used for vaccine production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume of pooled fluid examined in</td>
<td>. . .</td>
<td>. . .</td>
<td>. . .</td>
</tr>
<tr>
<td>% of cells viable after two weeks</td>
<td>. . .</td>
<td>. . .</td>
<td>. . .</td>
</tr>
<tr>
<td>Presence of adventitious agents</td>
<td>. . .</td>
<td>. . .</td>
<td>. . .</td>
</tr>
</tbody>
</table>

Identity of control cells
Tests used to establish identity
Results of tests

Infected cells after disruption
Reference of single harvest
Results of tests for the absence of:
— bacterial contamination
— mycotic contamination
— mycoplasma contamination
— Mycobacterium tuberculosis
— adventitious agents.

Antiserum used for virus neutralization
Code No.
Species in which it was produced

Information on preparation examined
Volume
Corresponding total number of human doses

Tests in cell cultures

<table>
<thead>
<tr>
<th></th>
<th>Identity</th>
<th>% cells viable at the end of the test</th>
<th>Presence of adventitious agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Continuous line</td>
<td>. . .</td>
<td>. . .</td>
<td>. . .</td>
</tr>
<tr>
<td>Diploid line</td>
<td>. . .</td>
<td>. . .</td>
<td>. . .</td>
</tr>
<tr>
<td>Simian kidney cells</td>
<td>. . .</td>
<td>. . .</td>
<td>. . .</td>
</tr>
</tbody>
</table>

130
### Tests in mice

<table>
<thead>
<tr>
<th></th>
<th>Suckling</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of mice alive at day 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of mice alive at day 21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of mice dead or sick between days 1 and 21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. with adventitious agents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. without adventitious agents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of mice alive and well at day 21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. with adventitious agents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. without adventitious agents</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Tests in guinea-pigs

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of guinea-pigs alive at day 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of guinea-pigs alive at day 42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of guinea-pigs dead or sick between days 1 and 42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. with adventitious agents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. without adventitious agents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of guinea-pigs alive and well at day 42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. with adventitious agents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. without adventitious agents</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Tests in embryonated eggs

- Total volume of virus inoculated
- Corresponding number of human doses
- No. of embryonated eggs inoculated
- Incubation age at inoculation
- Route of injection
- Results of injection after 9 days
- Was a subculture necessary?
- If yes, what was the result?

### Presence of cells in the single harvest after filtration

- Which test was used?
- Result of the test

### Virus titration

- Virus preparation used as reference
- Virus titre (per ml)
Final Bulk

Code no. of final bulk

Information on composition of final bulk (blending)
No. of single harvests used for preparing the final bulk
For each single harvest, indicate:

<table>
<thead>
<tr>
<th>Single harvest no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
</tr>
<tr>
<td>Code no.</td>
</tr>
<tr>
<td>Volume</td>
</tr>
<tr>
<td>Virus titre (per ml)</td>
</tr>
<tr>
<td>Volume of diluent added</td>
</tr>
<tr>
<td>If stabilizer or other substances were added, indicate nature and concentration</td>
</tr>
</tbody>
</table>
Total volume of final bulk
No. of viral particles per ml in final bulk (if determined)

Tests for absence of contamination
Volume examined
Results of tests for absence of bacterial contamination
Results of tests for absence of mycotic contamination

Final Product

Total no. of containers
No. of human doses in each container

Identity test
No. of containers used to prepare the pool tested
Date of test
Result of test

Virus concentration
Date on which the test system was inoculated
No. of containers examined

<table>
<thead>
<tr>
<th>Container no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
</tr>
<tr>
<td>Total no. of viral particles in each container examined</td>
</tr>
<tr>
<td>Geometric mean</td>
</tr>
<tr>
<td>Which reference preparation was used for comparison?</td>
</tr>
</tbody>
</table>
Innocuity tests
Date of the tests ____________________
Describe briefly the tests and indicate the results

__________________________________

Result of inspection of final containers
Absence of bacterial contamination
No. of containers examined ____________
Date of the test ______________________
Media for bacterial sterility and result of test __________________________
Media for mycotic sterility and result of test __________________________

Residual moisture
Method used for determination __________________________
% of moisture content ________________

Labelling
Provide a container label
Provide either a label identical to those on the carton enclosing final containers or a leaflet accompanying containers

Expiry date _________________________
Provide a certificate from the national control laboratory indicating:
— that the vaccine lot meets all national requirements
— that the vaccine lot meets Part A of the WHO Requirements for Variella Vaccine (Live)
— number of final containers
— number under which the lot was released by the national control laboratory
— number appearing on the labels of the container.

Additional Tests
If additional tests were performed, give details (using separate sheets).
Annex 5

BIOLOGICAL SUBSTANCES: INTERNATIONAL STANDARDS, REFERENCE PREPARATIONS, AND REFERENCE REAGENTS

The list of international biological standards, international biological reference preparations, and international reference reagents previously included as annexes to the reports of the WHO Expert Committee on Biological Standardization are issued as a separate publication. The most recent list was published in 1984 and copies may be obtained direct (or through booksellers) from the agents shown on the back cover of this report or they may be ordered from: World Health Organization, Distribution and Sales Service, 1211 Geneva 27, Switzerland.

The Expert Committee, at its thirty-fifth meeting, made the following changes to the 1984 list:

ADDITIONS

Antibiotics

Sisomicin

35 200 IU/ampoule

First International Standard 1984

(This substance is held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, London NW3 6RB, England).

Antibodies

Cholera antitoxin, goat

2200 IU/ampoule

First International Standard 1984

(This substance is held and distributed by the International Laboratory for Biological Standards, State Serum Institute, 80 Amager Boulevard, DK-2300 Copenhagen S, Denmark).

### Antigens

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>House dust mite (Dermatophagoides pteronyssinus) extract</td>
<td>100,000 IU/ampoule</td>
<td>First International Standard 1984</td>
</tr>
</tbody>
</table>

(This substance is held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, London NW3 6RB, England).

### Blood products and related substances

<table>
<thead>
<tr>
<th>Product</th>
<th>Concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-C complete blood typing serum</td>
<td>100 IU/ampoule</td>
<td>First International Standard 1984</td>
</tr>
<tr>
<td>Anti-E complete blood typing serum</td>
<td>100 IU/ampoule</td>
<td>First International Standard 1984</td>
</tr>
</tbody>
</table>

(These substances are held and distributed by the International Laboratory for Biological Standards, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Pleinmanlaan 125, Amsterdam, Netherlands)

<table>
<thead>
<tr>
<th>Product</th>
<th>Concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabies immunoglobulin</td>
<td>59 IU/ampoule</td>
<td>First International Standard 1984</td>
</tr>
</tbody>
</table>

(This substance is held and distributed by the International Laboratory for Biological Standards, State Serum Institute, 80 Amager Boulevard, DK-2300 Copenhagen S, Denmark).

<table>
<thead>
<tr>
<th>Product</th>
<th>Concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prekallikrein activator (PKA)</td>
<td>85 IU/ampoule</td>
<td>First International Standard 1984</td>
</tr>
<tr>
<td>Beta-thromboglobulin (β-TG)</td>
<td>500 IU/ampoule</td>
<td>First International Standard 1984</td>
</tr>
<tr>
<td>Platelet factor 4 (PF₄)</td>
<td>400 IU/ampoule</td>
<td>First International Standard 1984</td>
</tr>
<tr>
<td>Human tissue plasminogen activator (t-PA)</td>
<td>1000 IU/ampoule</td>
<td>First International Standard 1984</td>
</tr>
</tbody>
</table>

(These substances are held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, London NW3 6RB, England).
## Endocrinological and related substances

<table>
<thead>
<tr>
<th>Substance Description</th>
<th>IU/ampoule</th>
<th>Standard Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha subunit of human pituitary luteinizing hormone</td>
<td>10</td>
<td>1984</td>
</tr>
<tr>
<td>Beta subunit of human pituitary luteinizing hormone</td>
<td>10</td>
<td>1984</td>
</tr>
</tbody>
</table>

(These substances are held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, London NW3 6RB, England.)

## Miscellaneous substances

<table>
<thead>
<tr>
<th>Substance Description</th>
<th>IU/ampoule</th>
<th>Standard Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interferon, human, lymphoblastoid (Namalwa)</td>
<td>25,000</td>
<td>1984</td>
</tr>
<tr>
<td>Interferon, human, rDNA (HuIFN-α2(αA))</td>
<td>9,000</td>
<td>1984</td>
</tr>
<tr>
<td>Interferon, human, (HuIFN-γ)</td>
<td>4,000</td>
<td>1984</td>
</tr>
<tr>
<td>Interferon, human, leukocyte (HuIFN-α(Le))</td>
<td>12,000</td>
<td>1984</td>
</tr>
<tr>
<td>Human liver ferritin protein</td>
<td>9.7 μg/ampoule</td>
<td>1984</td>
</tr>
</tbody>
</table>

(These substances are held and distributed by Development and Applications Branch, Microbiology and Infectious Diseases Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20205, United States of America.)

(These substances are held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, London NW3 6RB, England.)
Annex 6

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 formulated by international groups of experts and published in the Technical Report Series of the World Health Organization are listed hereunder:

<table>
<thead>
<tr>
<th>No.</th>
<th>Year of publication</th>
<th>Requirements</th>
</tr>
</thead>
<tbody>
<tr>
<td>178</td>
<td>1959</td>
<td>Requirements for Biological Substances:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>* 1. General Requirements for Manufacturing Establishments and Control Laboratories</td>
</tr>
<tr>
<td></td>
<td></td>
<td>* 2. Requirements for Poliomyelitis Vaccine (Inactivated)</td>
</tr>
<tr>
<td>179</td>
<td>1959</td>
<td>Requirements for Biological Substances:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>* 3. Requirements for Yellow Fever Vaccine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>* 4. Requirements for Cholera Vaccine</td>
</tr>
<tr>
<td>180</td>
<td>1959</td>
<td>Requirements for Biological Substances:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>* 5. Requirements for Smallpox Vaccine</td>
</tr>
<tr>
<td>200</td>
<td>1960</td>
<td>Requirements for Biological Substances:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>* 6. General Requirements for the Sterility of Biological Substances</td>
</tr>
<tr>
<td>237</td>
<td>1962</td>
<td>Requirements for Biological Substances:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>* 7. Requirements for Poliomyelitis Vaccine (Oral)</td>
</tr>
<tr>
<td>274</td>
<td>1964</td>
<td>WHO Expert Committee on Biological Standardization:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>* 8. Requirements for Pertussis Vaccine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>* 9. Requirements for Procaine Benzoylepenicillin in Oil with Aluminium Monostearate</td>
</tr>
<tr>
<td>293</td>
<td>1964</td>
<td>WHO Expert Committee on Biological Standardization:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>* 10. Requirements for Diphtheria Toxoid and Tetanus Toxoid</td>
</tr>
<tr>
<td>323</td>
<td>1966</td>
<td>WHO Expert Group: Requirements for Biological Substances (Revised 1965):</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1. General Requirements for Manufacturing Establishments and Control Laboratories</td>
</tr>
</tbody>
</table>

* Replaced by revised Requirements.
2. Requirements for Poliomyelitis Vaccine (Inactivated)
3. Requirements for Smallpox Vaccine
4. Requirements for Poliomyelitis Vaccine (Oral)

329 1966 WHO Expert Committee on Biological Standardization:
* 11. Requirements for Dried BCG Vaccine
12. Requirements for Measles Vaccine (Live) and Measles Vaccine (Inactivated)

361 1967 WHO Expert Committee on Biological Standardization:
9. Requirements for Procaine Benzylpenicillin in Oil with Aluminium Monostearate (Revisions adopted 1966)
13. Requirements for Anthrax Spore Vaccine (Live—for Veterinary Use)
14. Requirements for Human Immunoglobulin
15. Requirements for Typhoid Vaccine

384 1968 WHO Expert Committee on Biological Standardization:
16. Requirements for Tuberculin
* 17. Requirements for Inactivated Influenza Vaccine

413 1969 WHO Expert Committee on Biological Standardization:
† 4. Requirements for Cholera Vaccine (Revised 1968)
18. Requirements for Immune Sera of Animal Origin

444 1970 WHO Expert Committee on Biological Standardization:
19. Requirements for Rinderpest Cell Culture Vaccine (Live) and Rinderpest Vaccine (Live)
† 20. Requirements for Brucella abortus Strain 19 Vaccine (Live—for Veterinary Use)

444 1970 WHO Expert Committee on Biological Standardization:
* Development of a National Control Laboratory for Biological Substances
(A guide to the provision of technical facilities)

463 1971 WHO Expert Committee on Biological Standardization:
21. Requirements for Snake Antivenins

486 1972 WHO Expert Committee on Biological Standardization:
* 7. Requirements for Poliomyelitis Vaccine (Oral) (Revised 1971)

530 1973 WHO Expert Committee on Biological Standardization:
4. Requirements for Cholera Vaccine (Revised 1968) (Addendum 1973)
6. General Requirements for the Sterility of Biological Substances (Revised 1973)
* 17. Requirements for Inactivated Influenza Vaccine (Addendum 1973)
* 22. Requirements for Rabies Vaccine for Human Use

* Replaced by revised Requirements.
† Refer also to subsequent Addendum.
565 1975  WHO Expert Committee on Biological Standardization:  Recommendations for the Assessment of Binding-Assay Systems (Including Immunoassay and Receptor Assay Systems) for Human Hormones and their Binding Proteins  (A guide to the formulation of requirements for reagents and assay kits for the above assays and notes on cytochemical bioassay systems)  Development of national assay services for hormones and other substances in community health care

594 1976  WHO Expert Committee on Biological Standardization:  3. Requirements for Yellow Fever Vaccine (Revised 1975)  20. Requirements for Brucella abortus Strain 19 Vaccine (Live—for Veterinary Use) (Specification of tests used in the Requirements) (Addendum 1975)  † 23. Requirements for Meningococcal Polysaccharide Vaccine


626 1978  WHO Expert Committee on Biological Standardization:  Guidelines for the Preparation and Establishment of Reference Materials for Biological Substances


* Replaced by revised Requirements.  † Refer also to subsequent Addendum.
22. Requirements for Rabies Vaccine for Human Use (Revised 1980)
23. Requirements for Meningococcal Polysaccharide Vaccine (Addendum 1980)
29. Requirements for Rabies Vaccine for Veterinary Use
* 31. Requirements for Hepatitis B Vaccine
* 26. Requirements for Antibiotic Susceptibility Tests (Suggested changes 1980)
24. Requirements for Rubella Vaccine (Live) (Addendum 1980)
* 30. Requirements for Thromboplastins and Plasma used to Control Oral Anticoagulant Therapy
Guidelines for Quality Assessment of Antitumour Antibiotics
The National Control of Vaccines and Sera
Requirements for Immunoassay Kits

673 1982 WHO Expert Committee on Biological Standardization:
2. Requirements for Poliomyelitis Vaccines (Inactivated) (Revised 1981)
† 8 and 10. Requirements for Diphtheria Toxoid, Pertussis Vaccine, Tetanus Toxoid, and Combined Vaccines (Addendum 1981)
12. Requirements for Measles Vaccine (Live) (Addendum 1981)
† 26. Requirements for Antimicrobial Susceptibility Tests (Revised 1981)
32. Requirements for Rift Valley Fever Vaccine
A Review of Tests on Virus Vaccines

687 1983 WHO Expert Committee on Biological Standardization:
7. Requirements for Poliomyelitis Vaccine (Oral) (Revised 1982)
30. Requirements for Thromboplastins and Plasma used to Control Oral Anticoagulant Therapy (Revised 1982)
33. Requirements for Louse-Borne Human Typhus Vaccine (Live) Standardization of Interferons
26. Requirements for Antimicrobial Susceptibility Tests (Addendum 1982)

700 1984 WHO Expert Committee on Biological Standardization:
† 8 and 10. Requirements for Diphtheria Toxoid, Pertussis Vaccine, Tetanus Toxoid, and Combined Vaccines (Addendum 1983)
35. Requirements for Rift Valley Fever Vaccine (Live, Attenuated) for Veterinary Use
34. Requirements for Typhoid Vaccine (Live Attenuated, Ty 21a, Oral)

1985 WHO Expert Committee on Biological Standardization:
8 and 10. Requirements for Diphtheria Toxoid, Pertussis Vaccine, Tetanus Toxoid, and Combined Vaccines (Addendum 1984)
31. Requirements for Hepatitis B Vaccine prepared from Human Plasma (Revised 1984)
36. Requirements for Varicella Vaccine (Live) Standardization of Interferons

* Replaced by revised Requirements.
† Refer also to subsequent Addendum.

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