Annex 2

REQUIREMENTS FOR HEPATITIS B VACCINES
MADE BY RECOMBINANT DNA TECHNIQUES

(Requirements for Biological Substances No. 45)

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GENERAL CONSIDERATIONS

Advances in molecular genetics and nucleic acid chemistry have made it possible to identify genes coding for biologically active substances, to analyse them in detail, to transfer them within and
between organisms, and to obtain gene expression under controlled conditions with efficient synthesis of the encoded product. A gene that codes for a specific product can be isolated and propagated by insertion into a suitable vector with the aid of highly specific restriction endonuclease enzymes (which cleave the vector DNA at predetermined sites) and ligases (which join the gene insert to the vector). The vector can then be introduced into host organisms, and individual clones that carry the desired gene can be selected and propagated in mass culture.

A gene is characterized by a specific nucleotide sequence in one strand of the double-stranded DNA molecule. When the strands are separated, each forms a template for the synthesis of a complementary copy, thus providing a mechanism for the faithful reproduction of genes with conservation of the linear sequence of the four types of mononucleotides. The process of decoding the genetic information and of synthesizing the gene product occurs in two phases, of which the first is transcription of the DNA coding strand in the form of messenger RNA, and the second translation of the information carried by the messenger RNA molecule into an amino acid sequence. The factors affecting the expression of foreign genes introduced into prokaryotic and eukaryotic cells are complex; the efficient and controlled expression of stable, cloned DNA sequences is an important field of current research.

Hepatitis B virus has several characteristics that distinguish it from the other families of DNA viruses. It has an outer coat (more substantial than a membrane or envelope) consisting of protein, lipid, and carbohydrate and bearing a unique antigen complex, hepatitis B surface antigen (HBsAg). Its nucleic acid consists of a circular DNA genome of relative molecular mass about 2 million, part of which is double-stranded and part single-stranded—an unusual feature among viruses. Virus recovered from the plasma of a hepatitis B carrier has been used to prepare viral DNA; that DNA has been cloned in Escherichia coli and the gene coding for HBsAg has been isolated.

This gene has been inserted into yeast and mammalian cells by means of appropriate expression vectors. Purified antigens obtained from transfected cultures containing it have been shown to induce antibodies in mice and guinea-pigs and have been formulated into vaccines. Electron microscopy has revealed that the purified HBsAg used for these vaccines exists as particles 15–30 nm in diameter, with the morphological characteristics of free surface antigen in plasma.
and of the purified antigen now used in plasma-derived hepatitis B vaccines. Some of the vaccine formulations containing these materials have already been shown to be immunogenic in mice, chimpanzees and other monkeys, and human beings, with antigenic potencies similar to those of vaccine made from plasma-derived antigen.¹

The requirements that follow apply to the control and testing of hepatitis B vaccine made by recombinant DNA methods, and have been formulated to take account of the scale-up required for commercial production. Particular emphasis is placed on introducing “in-process” control, which has been highly effective for other bacterial and viral vaccines, rather than relying entirely on tests on the end-products. General requirements, such as tests for potency, purity, toxicity, pyrogenicity, and sterility, will apply as much to hepatitis B vaccines made by recombinant DNA methods as to those derived from human plasma. Certain tests will be required on every production batch of vaccine, whereas others will be required only to establish the validity, acceptability, and consistency of a given manufacturing process.

A detailed description of how the product is made should be given. Evidence should be presented to show that any HBsAg made by recombinant DNA techniques possesses the characteristics of an immunogen that protects against hepatitis B virus.

Rigorous identification and characterization of recombinant DNA-derived vaccines will be required. The ways in which these products differ chemically, structurally, biologically, or immunologically from the naturally occurring antigen must be fully documented. Such differences could arise during processing at the genetic or post-translational level, or during purification. Differences between batches of the product may result from genetic instability during serial cultivation. Microbial contamination during fermentation may occur, and tests for contaminants must be thorough.

Special attention should be given to purity because:

— unwanted gene products may be co-expressed unexpectedly with the HBsAg, for example if transcription is initiated at several sites, or if changes occur during culture that affect transcription,

initiation, or termination processes or favour the expression of
other genes in the vector or the host cell;
—biologically active extraneous components, such as DNA,
proteins, and endogenous retroviruses derived from the host-cell
system may be found in the final product;
—agents used in the purification process (column matrices,
antibodies) may give rise to specific contaminants in the final
product.

The product arising from the recombinant system must be shown
to elicit specific antibody responses to HBsAg in laboratory animals,
including, where possible, a non-human primate species. HBsAg of
diverse subtypes should be used to characterize the specificity of the
response fully.

One approach to evaluating the protective potential of
recombinant DNA-derived vaccines in human beings is through
immunization and challenge studies in chimpanzees. The
requirement for a test of vaccine efficacy in chimpanzees should be
at the discretion of the national control authority but, if possible, on
at least one occasion, chimpanzees given the recommended human
immunization schedule should be shown to be protected against
challenge with hepatitis B virus. In some studies, more than 1000
chimpanzee infectious doses of hepatitis B virus of homotypic and
heterotypic subtypes, given intravenously, have been used as a
challenge.

The vaccine should reliably induce antibody responses to HBsAg
in human recipients. The frequency and titre of the antibody
responses should be at least equivalent to those induced by plasma-
derived vaccines that fulfil WHO requirements. The aims of
immunogenicity studies in human subjects should be to define the
quantity of antigen and number of doses required to elicit reliably
antibody responses to HBsAg. The titre, duration, and quality of the
responses should be clearly defined. In vitro tests of antigenicity may
be difficult to standardize, and it is therefore proposed that the
content of pure HBsAg in the product should be used as the basis for
comparing immunogenicity in mice and responses in human
subjects. The relationship between the antigenicity of the product in
in vitro tests, in mouse immunogenicity tests, and in human beings
should be established. Studies in human subjects should be designed
to provide information on the frequency and severity of any local
and systemic adverse reactions to vaccination. Vaccine recipients in
such studies should be representative of the intended target group for immunization in terms of age and risk.

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 should be included permitting modifications of the manufacturing requirements on the condition that it can be demonstrated, to the satisfaction of the national control authority, that such modifications ensure a degree of safety and potency of the vaccine 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 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 recombinatum*. 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 hepatitidis B recombinatum* is a preparation of purified hepatitis B surface antigen (HBSAg) that has been produced by recombinant DNA techniques. The preparation shall satisfy all the requirements formulated below.
1.3 International reference materials

International standards and reference reagents are needed for the control of HBsAg or to determine antigenic content and potency.

The reference materials mentioned below are available but are not specified for use in tests in these requirements.

For the assay of antigenic content by techniques such as radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), single radial immunodiffusion, and rocket electrophoresis, an international reference material consisting of an aqueous preparation without an adjuvant is required (see Part A, section 7.3). Such a preparation was established in 1985 as the first International Standard for Hepatitis B Surface Antigen ad Subtype (100 IU per ampoule).

The International Reference Reagent of Plasma-Derived Hepatitis B Vaccine for Immunogenicity Studies was established in 1986. It is a liquid preparation containing approximately 20 μg/ml of plasma-derived hepatitis B surface antigen and contains aluminium hydroxide as an adjuvant.

Both the above-mentioned reference materials are available on request from the National Institute for Biological Standards and Control, Potters Bar, England.

For the calibration of techniques used to measure antibody responses to hepatitis B vaccines, an international reference preparation is available. This International Reference Preparation of Hepatitis B Immunoglobulin (established in 1977) is dispensed in ampoules containing 50 IU of hepatitis B immunoglobulin (from fractionated human plasma, and 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

*Hepatitis B virus*: a 42-nm double-shelled virus particle, originally known as the Dane particle, which contains the DNA genome of the virus.

*HBsAg*: hepatitis B surface antigen, comprising a complex of antigens associated with the virus envelope and subviral forms (22-nm spherical and tubular particles). Native HBsAg is encoded by envelope gene sequences (S plus pre-S) in the viral DNA. Recombinant DNA-derived hepatitis B vaccines may contain the S gene product or products of the S/pre-S combination.

*Cell seed lot*: a quantity of cells stored frozen at −70 °C or below in aliquots of uniform composition, one or more of which would be used for the production of a manufacturer’s working cell bank.
Manufacturer's working cell bank (MWCB): a quantity of cells, derived from one or more ampoules of the cell seed, stored frozen at −70°C or below in aliquots of uniform composition.

In normal practice, a cell seed is expanded by serial subculture up to a passage number (or population doubling, as appropriate) selected by the manufacturer, at which point the cells are combined to give a single pool and preserved cryogenically to form the MWCB.

Production cell culture: a collection of cell cultures being used for biological production that have been derived from one or more ampoules of the MWCB.

Single harvest: the biological material prepared from a single production run.

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 HBsAg, and 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 and during the preparation of the finished vaccine. A final lot must therefore consist of finished material distributed into containers 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) (1, p. 11) shall apply to establishments manufacturing hepatitis B vaccine, with the addition of the following directives:

(a) Production areas shall be decontaminated before they are used for the manufacture of hepatitis B vaccine.

(b) Hepatitis B vaccine shall be produced by staff who have not handled animals or infectious microorganisms in the same working day. The staff shall consist of persons who have been examined medically and have been found to be healthy and not to be carriers of hepatitis B.

(c) No cultures of microorganisms or eukaryotic cells other than those approved by the national control authority shall be
introduced into or handled in the production area at any time during manufacture of the vaccine.

(d) 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 is drawn 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) (I, p. 13) regarding the training and experience of persons in charge of production and testing and of those assigned to various positions of responsibility in the manufacturing establishment, and to the registration of such personnel with the national control authority.

The preparation of hepatitis B vaccine shall be based strictly on the cell seed lot system. A description of the system used should be provided, including the number of vials of seed available and details of their storage. Particular attention should be paid to the stability of the expression vector and to the plasmid copy number in the seed stock under conditions of storage and recovery.

Full details of the cell culture process used in manufacture should be provided to the national control authority with particular reference to tests to monitor microbial contamination in the cell culture vessels. Information on the sensitivity of methods of detecting such contamination and the frequency of testing should be provided, together with criteria for the rejection of contaminated materials.

The yield of HBsAg shall be monitored during the course of individual production runs. Criteria, based on yield, for the acceptance of culture harvests for further processing into vaccine shall be defined, and consistency of production shall be established by testing at least five consecutive lots prepared by the same procedures.

3. Validation and control of manufacturing procedures

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

A full description of the biological characteristics of the host cell and expression vectors used in production should be given. This should include details of: (a) potential retrovirus-like particles in and genetic markers of the host cell; (b) the construction, genetics, and structure of the expression vector; and (c) the origin and identification of the gene that is being cloned.

The physiological measures used to promote and control the expression of the cloned gene in the host cell should be described in detail.

Data that demonstrate the stability of the expression system during storage of the MWCB and beyond the passage level used for production should be provided. Any instability of the expression system occurring in the seed culture or after a production-scale run, for example involving rearrangements, deletions, or insertions of nucleotides, must be documented. Unstable preparations must not be used until approval to continue use has been obtained from the national control authority.

3.2 Biochemical characterization of recombinant vector

The nucleotide sequence of the gene insert and of adjacent segments of the vector and restriction-enzyme mapping of the vector containing the gene insert shall be provided as required by the national control authority.

3.3 Purification procedures

The methods used to purify the HBsAg from culture harvests should be fully described. The capacity of each step of the purification procedure to remove and/or inactivate substances other than HBsAg derived from the host cell or culture medium, including, in particular, virus particles, proteins, and nucleic acids, shall be evaluated. If individual contaminants are difficult to monitor, the results of pilot-scale studies to follow the removal of individual, deliberately added contaminants at appropriate stages of purification will provide valuable information.

If antibodies are used in the purification procedures, their origins and characteristics should be fully described. The degree of purity of monoclonal antibodies produced from hybridoma cell lines and the criteria for freedom from cell-derived DNA and murine viruses
should conform to the regulations laid down by the national control authority.

3.4 Characterization of gene products (HBsAg)

3.4.1 Particle characterization

The morphological characteristics of the HBsAg particles and degree of aggregation should be established by electron microscopy and by physicochemical methods, for example by gradient centrifugation. In addition, the protein, lipid, nucleic acid, and carbohydrate content should be measured.

3.4.2 Determination of protein content

The protein content (composition) in comparison with a reference reagent should be established by quantitative amino acid analysis or by another accurate method.

3.4.3 Protein characterization

An ultraviolet absorption spectrum should be recorded. The protein composition should be established by techniques such as sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions. The bands should be identified by sensitive staining techniques and, where possible, by specific antibodies to confirm the presence of the expected products of the hepatitis B virus envelope gene. The identity of the protein should be established by partial N-terminal and C-terminal sequence analysis.

3.4.4 Antibody responses

The recombinant DNA-derived vaccine should be shown to induce antibody responses in humans comparable to those elicited by plasma-derived vaccines that have proved effective in the field. The antibodies induced by the vaccine in human beings should be titrated and characterized with respect to their activities against relevant determinants of the hepatitis B virus envelope, for example group and subtype determinants.
3.4.5 Consistency of yield

Data on the consistency of yield between runs and during individual production runs shall be provided, and the national control authority shall approve the criteria for an acceptable production run.

4. Manufacturer’s working cell bank (MWCB)

4.1 Origin of cell banks

Only cells approved by and registered with the national control authority shall be used to produce HBsAg. The national control authority shall have responsibility for approving the cell seed. A short history of the cell banks shall be provided.

4.2 Characteristics of cell seed lot

The characteristics of the cell seed (host cell in combination with the expression vector system) shall be fully described, and information given on the absence of extraneous agents and on genetic homogeneity. The nucleotide sequence of the HBsAg gene insert and its flanking regions shall be specified where relevant. A peptide map and/or terminal amino acid sequence of the gene products shall be obtained.

4.3 Phenotypic indicators of purity and genetic consistency of recombinant cultures

Cells must be maintained in a frozen state that allows recovery of viable cells without alteration of genotype. The cells should be recovered from the frozen state, if necessary, in selective media such that the genotype and phenotype consistent with the unmodified host and unmodified recombinant DNA vector are maintained and are clearly identifiable. The cell seed must be identified by means of appropriate tests.

4.4 Sterility tests

Tests for bacteria, fungi, and mycoplasmas shall be performed in accordance with the requirements of Part A, section 5 of the revised
Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (2, p. 49).

4.5 Additional tests for MWCB of mammalian cells

The cells shall have been characterized as specified under the Requirements for Biological Substances No. 37 (Requirements for Continuous Cell Lines Used for Biologicals Production) (3).

The national control authority shall have the responsibility for approving the cell seed.

5. Production precautions

5.1 Production cell cultures

Only cell cultures derived from the MWCB shall be used for production. All processing of cells shall be done in an area in which no cells or organisms are handled other than those directly required for the process.

5.2 Culture conditions for production cell cultures

Production cell cultures shall be grown under conditions agreed with the national control authority. These conditions shall include details of the culture system used, the cell doubling time, the number of subcultures or the duration of the period of subcultivation permitted, and the incubation temperature.

Cell cultures shall be monitored for freedom from microbial contamination as required by the national control authority.

5.3 Cell culture medium

If serum is used for the propagation of cells for vaccine production, it shall be tested by methods approved by the national control authority, to demonstrate freedom from bacteria, fungi, viruses, and mycoplasmas, according to the requirements given in Part A, sections 5.2 and 5.3 of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (2, p. 49) as well as freedom from pathogens of the species of origin of the serum.
In some countries, sera are examined for freedom from phages.

Antibiotics of the β-lactam type shall not be used at any stage in the production.

Minimal concentrations of other suitable antibiotics may be used if approved by the national control authority. Nontoxic pH indicators may be added, e.g., phenol red in a concentration of 20 mg/l.

6. Single harvests

6.1 Sterility

Microbial contamination in the cell culture vessels shall be monitored during and at the end of the production runs by methods approved by the national control authority.

6.2 Consistency of yield

The yield of HBsAg from each single harvest shall be shown to be within the limits approved by the national control authority (see Part A, section 3.4.5).

6.3 Plasmid retention

A sample of cells that are representative of each harvest must be tested to confirm that the recombinant phenotype has been retained. The method used shall be approved by the national control authority.

7. Purification

The purification procedure can be applied to a single harvest, a part of a single harvest, or a pool of single harvests. The maximum number of single harvests that may be pooled shall be fixed by the national control authority. The antigen must be purified before adsorption on to an adjuvant. Adequate purification may require several purification steps based on different principles. This will minimize the possibility of co-purification of extraneous cellular materials. The methods used for the purification of the HBsAg
should be appropriately validated (see Part A, section 3.3) and approved by the national control authority.

The experience of several manufacturers has shown that it is possible to produce batches in which HBsAg accounts for at least 95% of the total protein. One suitable method of analysing the proportion of potential contaminant proteins in the total protein of the product is separation of the proteins by polyacrylamide gel electrophoresis under both non-reducing and reducing denaturing conditions. Individual gels should be stained by Coomassie Blue and by silver stain, or a single gel may be successively stained by both methods. The silver stain is considerably more sensitive for the detection of very small quantities of proteins and is also useful for identifying any non-protein materials, such as nucleic acids, carbohydrates, and lipids, which may be present. The protein in each band can be quantified by densitometric analysis. The staining procedure should be sensitive enough to reveal a potential contaminant protein present at a level of 1% of total protein.

High-performance liquid chromatography is another very useful method for determining the purity of a protein or peptide and for investigating its molecular configuration. A widely used method is reverse-phase high-performance liquid chromatography. A protein or peptide that elutes as a single symmetrical peak in two markedly different systems, including an ion-pair system, is generally of high purity.

7.1 Protein and other components of the vaccine

The total protein content of the vaccine should be determined (see also Part A, section 3.4.2).

The total concentration of protein may be determined by measuring absorbance at 280 nm by the micro-Kjeldahl method, the Lowry technique, or another suitable method.

Lipid and carbohydrate contents may also provide useful information.

If serum is used in the medium for the production cell cultures or at any stage in the purification process, for example as a reagent in immunoabsorption chromatography, tests shall be made for residual serum in the purified vaccine.

The concentration of animal serum in the vaccine shall be not more than 1 μl per litre of vaccine.

A concentration of 1 μl of animal serum per litre of vaccine is approximately equivalent to 50 ng of albumin per ml. Methods such as enzyme-linked immunosorbent assay (ELISA) and
7.2 Tests for agents used during purification or other phases of manufacture

A test shall be made for the presence of any potentially hazardous agent used in manufacture. The method used and the permitted concentration shall be approved by the national control authority.

7.2.1 Monoclonal antibodies

Where a monoclonal antibody is used in vaccine preparation, for example for immunological affinity chromatography to purify HBsAg, the product should be tested for residual antibody. The methods used and the permitted concentrations of antibody shall be approved by the national control authority.

Several national control authorities have drafted guidelines for the control of monoclonal antibody preparations used for the manufacture of biological products for human use.

7.3 Determination of HBsAg content

The HBsAg content of the purified preparation shall be determined by an appropriate method.

Tests that have been found suitable include radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), single radial immunodiffusion, and rocket electrophoresis in which the purified preparation is compared with a known standard. Analysis of the results by the parallel-line method has been found suitable for most of these techniques.

7.4 Test for antigenic identity (molecular and immunochemical identity)

A test shall be made for the molecular characteristics of the HBsAg gene product by polyacrylamide gel electrophoresis under reducing denaturing conditions.

In one country, the detection of pre-S antigen by SDS-PAGE is required.

The gene products shall be shown to possess antigenic determinants characteristic of HBsAg by means of tests with
monoclonal antibodies or other antibodies of defined specificity directed against epitopes of HBsAg known to be relevant to the protective efficacy of the vaccine. Such tests shall be approved by the national control authority.

7.5 Test for sterility of purified surface antigen

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) (2, p. 48).

7.6 Test for inactivating agents

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

8. Final bulk before addition of adjuvant (aqueous bulk)

The final aqueous bulk consists of one or more purified HBsAg batches. Only batches that have satisfied the requirements of sections 2–7 above shall be included in the final bulk. The national control authority shall determine the maximum number of batches that may be pooled.

8.1 Test for sterility

The final aqueous 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) (2, p. 49).

8.2 Test for HBsAg

The quantity of HBsAg as a proportion of the total protein in the final bulk shall be determined by an appropriate quantitative procedure. The protein load should be specified so as to ensure that
the assay is of reasonable sensitivity. The lower limit of the ratio of HBsAg to total protein shall be approved by the national control authority. The final aqueous bulk should contain at least 90% of the total protein as HBsAg.

In one country, the amount of pre-S antigen is determined by means of a suitable quantitative assay.

8.3 Test for DNA

The amount of residual cell or plasmid DNA in each batch of vaccine should be determined by sensitive methods which must be validated and approved by the national control authority. Suitable tests for the homologous vector used in the production of the HBsAg should be included. Maximum acceptable levels of DNA per vaccine dose shall be approved by the national control authority.

The WHO Study Group on Biologicals concluded that the probability of risk associated with heterogeneous contaminating DNA in a product derived from a continuous cell line is negligible when the amount of such DNA is 100 pg or less in a single dose given parenterally (4).

9. Final bulk

9.1 Addition of adjuvant

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

A preservative may need to be added at this stage.

9.2 Tests on final bulk

9.2.1 Tests for sterility

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) (2, p. 49).

9.2.2 Tests for preservative

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

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9.2.3 Assay for adjuvant

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

10. 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) (I, 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 storage conditions. Adjuvanted HBsAg vaccine must be stored at 5–8°C.

11. Control of final lot

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

11.1 Sterility test

Each 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).

11.2 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 approved by the national control authority.

11.3 Test for pyrogenic substances

Each final lot shall be tested for pyrogenic substances. The test shall be approved by the national control authority.
11.4 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.

11.5 Assay for adjuvant

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

11.6 Potency and identity tests

The vaccine shall be identified as envelope antigens of hepatitis B virus by appropriate methods. An appropriate quantitative test for antigen content and an immunogenicity assay shall be performed on samples representative of the final filling lots. The vaccine potency shall be compared with that of a reference preparation, and the national control authority shall determine the lower limit of potency.

A suitable quantitative extinction test in mice is as follows. Several groups of at least 20 suitable mice, five weeks of age, are tested. Each mouse is vaccinated intraperitoneally with the adjuvanted hepatitis B vaccine diluted in the adjuvant used in the vaccine; a different graded dose is used for each group of mice. Similar groups of mice are inoculated with the adjuvanted reference preparation. The reactivity of positive sera towards important epitopes on HBsAg should be verified by means of appropriate tests. Blood samples are taken from the mice after sufficient time has elapsed to permit the development of an adequate antibody response, and individual sera are assayed for antibodies to products of the envelope gene of hepatitis B virus by means of sensitive quantitative tests such as radioimmunoassay. The concentrations of vaccine tested should be selected to permit the calculation of 50% seroconversion to antibodies against HBsAg. The strain of mice used for this test must give a suitable dose–response curve with the reference and test antigens. In any test, a calibrated reference preparation should be included and the potency of the vaccine expressed in terms of it.
12. 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) (I, p. 17) shall apply.

13. 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) (I, p. 18) shall apply.

14. 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) (I, p. 18) shall apply, with the addition of the following directive.

The leaflet accompanying the package shall include the following information:

— the nature of the cells used to produce the antigen;
— the nature and amount of any preservative, adjuvant, or stabilizer present in the vaccine;
— the volume of one recommended human dose, the immunization schedules, and the recommended routes of administration (this information shall be given for newborn babies, children, adults, and immunosuppressed individuals, and shall be the same for a given vaccine for all regions of the world);
— the amount of total protein contained in one recommended human dose; and
— the amount of HBsAg protein contained in one recommended human dose.

15. Distribution and shipping

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

In addition, the conditions of shipping shall be such as to ensure that the adjuvanted vaccine does not freeze.

Temperature indicators should be packaged with each vaccine shipment to show whether freezing occurs. If freezing has occurred, the vaccine should not be used.

16. 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) (I, p. 19) shall apply.

16.1 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 vaccine in bulk form or in final containers shall be kept at 5-8°C. The vaccine shall also be stored at this temperature after distribution or issue. The vaccine shall have been shown to maintain its potency for a period equal to that between the date of issue and the expiry date. During storage the vaccine shall not be frozen.

16.2 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, i.e., the date on which the animals were inoculated with the vaccine.

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, p. 19) shall apply.
The national control authority shall:

— approve the methods for producing HBsAg by recombinant DNA techniques;
— approve the tests for HBsAg concentration and define its minimum value;
— approve the methods used for purification;
— approve the purity of the final product;
— approve the tests for extraneous substances and total protein;
— approve the tests for preservative and the agents used for purification and in other aspects of manufacture;
— approve the tests used to determine whether the final product is free from abnormal toxicity;
— approve the adjuvant assay and define the permitted concentration of adjuvant in the final product;
— approve the types of animals used in the assay of potency; and
— approve the data showing that the vaccine produces an adequate antibody response (titre, duration, and quality) in human beings.

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

Before releasing a vaccine, the national control authority shall be satisfied that the results of all tests, including those on individual batches during the process of manufacture, are satisfactory and that consistency has been established.

2. Release and certification

A hepatitis B vaccine shall be released only if it satisfies Part A of these 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 these 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 AND ACKNOWLEDGEMENTS

These Requirements for Hepatitis B Vaccines Made by Recombinant DNA Techniques are based on four previous drafts; for the authors of the first three of these drafts and corresponding acknowledgements, please see WHO Technical Report Series, No. 760, 1987, Annex 6. For the fourth draft, acknowledgements are also due to Dr G.R.E. Swaniker, University of Papua New Guinea, Boroko, Papua New Guinea. The fourth draft was prepared in January 1988 by Dr V. Grachev, Scientist, and Dr D. Magrath, Chief, Biologicals, WHO, Geneva, Switzerland.

REFERENCES

Appendix

SUMMARY PROTOCOL FOR PRODUCTION AND TESTING OF HEPATITIS B VACCINES MADE BY RECOMBINANT DNA TECHNIQUES

Based on Requirements for Biological Substances No. 45
(Requirements for Hepatitis B Vaccines Made by Recombinant DNA Techniques)

Identification of final lot

Name and address of manufacturer

International name and proprietary name of vaccine

Lot number of final product

Date of manufacture of final lot

Date of filling containers

Number and nature of containers (ampoules or vials)

Date of last potency test

Number of doses in each container

Volume of single dose

Expiry date

3.1 Validation and control of manufacturing procedures

3.1 Strategy for cloning and expressing the gene

Details of:

(a) potential retrovirus-like particles in and genetic markers of the host cell

(b) construction, genetics, and structure of the expression vector

(c) origin and identity of the gene that was cloned

1 Numbers refer to the corresponding numbered sections in the text of the Requirements.

2 Section 3.1 does not necessarily need to accompany each batch of vaccine. If necessary, it should be typed on a separate sheet.
3.2 Biochemical characterization of recombinant vector

Nucleotide sequence of the surface-antigen gene insert

Restriction-endonuclease mapping of the recombinant vector, where relevant

3.3 Purification procedures

Methods used to purify the HBsAg

Results

Origins and characteristics of antibodies, if used

3.4 Characterization of gene products (HBsAg)

3.4.1 Particle characterization

Morphological characteristics of the particles

Results of examination by electron microscopy

Degree of aggregation

Method

Results

Quantity of protein

Method

Results

Quantity of lipid

Method

Results

Quantity of nucleic acid

Method

Results

Quantity of carbohydrate

Method

Results
3.4.2 Determination of protein content
   Method
   Results

3.4.3 Protein characterization
   Ultraviolet absorption spectrum
   Protein composition
      Method
      Results
   Identity of the protein by partial
   N-terminal and C-terminal analysis
      Method
      Results

3.4.4 Antibodies induced by the vaccine in human beings
   Titres
   Characteristics

3.4.5 Consistency of yield
   Between runs
   During individual runs

4. Manufacturer’s working cell bank (MWCB)

4.1 Origin of cell banks
   Date of establishment of cell banks
   Quantity of cells stored
   Passage level of the MWCB
   Storage conditions

4.2 Characteristics of cell seed lot
   Purity and homogeneity of the cell seed lot
      Method
      Results
   Genetic characteristics of the cell seed lot
      Method
      Results
   Purity of recombinant DNA vector
      Method
      Results

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Genetic characteristics of recombinant DNA vector
Methods
Results

Nucleotide sequence of HBsAg gene insert

Peptide map of gene product (HBsAg)

Terminal amino acid sequence of gene product

### 4.3 Phenotypic indicators of purity and genetic consistency of recombinant cultures

Tests on cells after recovery from preserved state
Methods
Results

Identity of cell seed
Method
Results

### 4.4 Sterility tests

Results

### 4.5 Additional tests for MWCB of mammalian cells

*Tests for adventitious agents*

Tests in suckling mice
- No. and weight of animals
- Quantity injected (intramuscular)
- Observation period
- Results of injection (pathological changes, no. survived, etc.)

Tests in adult mice
- No. and weight of animals
- Quantity injected (intramuscular)
- Observation period
- Results of injection (pathological changes, no. survived, etc.)

Tests in guinea-pigs
- No. and weight of animals
- Quantity injected (intramuscular)
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<td><strong>Observation period</strong></td>
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<td><strong>Results of injection</strong></td>
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<td>(pathological changes, no. survived, etc.)</td>
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<td><strong>Tests in rabbits</strong></td>
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<tr>
<td>No. and weight of animals</td>
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<tr>
<td>Quantity injected (intramuscular)</td>
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<td>Observation period</td>
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<td>Results of injection (pathological changes, no. survived, etc.)</td>
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<td><strong>Tests in embryonated chicken eggs</strong></td>
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<td>No. and age of eggs</td>
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<td>Quantity of cells injected</td>
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<td>Observation period</td>
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<td>Results of injection (pathological changes, no. survived, etc.)</td>
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<tr>
<td><strong>Tests in adult mice</strong></td>
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<td>No. and weight of animals</td>
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<td>Quantity injected (intracerebral)</td>
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<td>Observation period</td>
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<td>Results of injection (pathological changes, no. survived, etc.)</td>
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<td><strong>Tests for bacteria, fungi, and mycoplasmas</strong></td>
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<td>Media used</td>
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<td>Results</td>
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<tr>
<td><strong>Morphological tests</strong></td>
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<td>Examination by light microscopy</td>
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<td>Examination by electron microscopy (if this test has not been done on the cell seed)</td>
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<td><strong>Tests on cell cultures</strong></td>
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<td>Percentage of MWCB tested</td>
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<tr>
<td>Tests performed on cell system</td>
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<tr>
<td>Period of observation of cell cultures</td>
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<tr>
<td>Results</td>
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<tr>
<td><strong>Tests for haemagglutinating viruses</strong></td>
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<tr>
<td>Percentage of MWCB tested</td>
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<tr>
<td>Method</td>
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<tr>
<td>Results</td>
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</tbody>
</table>
Tests for retroviruses
  Percentage of MWC8 tested
  Method
  Results

Tests for tumorigenicity
  Method
  Results

Identity tests
  Method
  Results

5. Production precautions

5.1 Production cell cultures

5.2 Culture conditions for production cell cultures
  Cell doubling time
  Number of subcultures
  Duration of permitted subculture
  Incubation temperature
  Sterility test
    Media
    Results

5.3 Cell culture medium

6. Single harvests

6.1 Sterility test performed at the end of the culture
  Media
  Results

6.2 Consistency of yield of HBsAg
  Method
  Results
6.3 Plasmid retention

Method

Results (proportion of cells still possessing the plasmid at the end of the culture)

7. Purification

Methods

Results

7.1 Protein and other components of the vaccine

Protein content
  Methods
  Results

Lipid content
  Methods
  Results

Carbohydrate content (as compared with total proteins)
  Methods
  Results (%)

Residual amounts of animal serum
  Methods
  Results

7.2 Tests for agents used during purification or other phases of manufacture

Methods

Results

7.2.1 Monoclonal antibodies

Methods

Results

7.3 Determination of HBsAg content

Methods

Results

7.4 Test for antigenic identity

Methods

Results

1 Only if immunoabsorption was used in the purification process.
7.5 Test for sterility of purified surface antigen

Methods
Results

7.6 Test for inactivating agents

Methods
Results

8. Final bulk before addition of adjuvant (aqueous bulk)

Volume of purified bulk
Number of batches pooled
Nature and volume of diluent added

8.1 Test for sterility

Method
Results

8.2 Test for HBsAg

Quantity of HBsAg
Method
Results
Ratio of HBsAg to total protein
Method
Results

8.3 Test for DNA

Method
Results

9. Final bulk

9.1 Addition of adjuvant

Volume of bulk
Nature and volume of adjuvant added
and final concentration
Nature and volume of preservative added
and final concentration
Composition of final bulk (mixing of all
ingredients) and identification number
9.2 Tests on final bulk

9.2.1 Tests for sterility
Method
Results

9.2.2 Tests for preservatives
Method
Results

9.2.3 Assay for adjuvant
Method
Results

10. Filling and containers
Date of filling
Quantity of containers
Volume of vaccine per container
Control for defective containers

11. Control of final lot

11.1 Sterility test
Method
Results

11.2 Inocuity tests

*In mice*
No. and weight of animals
Quantity injected
Observation period
Results

*In guinea-pigs*
No. and weight of animals
Quantity injected
Observation period
Results

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11.3 Test for pyrogenic substances
Method
Results

11.4 Test for preservative
Method
Results

11.5 Assay for adjuvant
Method
Results

11.6 Potency and identity tests
Identification of vaccine as HBsAg
Method
Results
Antigen content
Method
Results
Immunogenicity assay
Method
Results
Vaccine potency
Method
Results

Internal certification

Certification by person taking overall responsibility for production of the vaccine

I certify that lot no. ................. of the vaccine satisfies Part A of the Requirements for Hepatitis B Vaccines Made by Recombinant DNA Techniques published by WHO.

Signature
Name (typed)
Date

The protocol must be accompanied by a sample of the label and a copy of the leaflet.

Release certification by the national control authority

Whenever hepatitis B vaccines produced by recombinant DNA techniques are to be exported, they should be accompanied by a release certificate from the national control authority.
Sample release certificate

I hereby certify that batch no. .................. of hepatitis B vaccine produced by (name of producer) by recombinant DNA techniques meets all national requirements as well as Part A of the Requirements for Hepatitis B Vaccines Made by Recombinant DNA Techniques published by WHO.

The date of the last satisfactory potency test carried out by the national control laboratory is ..................

The final lot has been released by us under no. ..................

The number appearing on the label of the containers is ..................

Signature

Name (typed)  

Date

..........................................................