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Hepatitis B vaccines prepared from yeast by recombinant DNA techniques: Memorandum from a WHO Meeting*

*A meeting of experts was convened on 19–21 November 1984 in Geneva, Switzerland, to advise the World Health Organization on the production of hepatitis B vaccine prepared from yeast (*Saccharomyces cerevisiae*), using recombinant DNA technology. This vaccine development follows the advances in molecular genetics that have permitted genes coding for biologically active substances to be identified, analysed with fine precision, transferred within and between host organisms, and expressed under controlled conditions so as to obtain efficient synthesis of the product which they encode. The gene for hepatitis B surface antigen (HBsAg, the coat protein of the virus) has been cloned and inserted into several expression vectors. These recombinants have been introduced into and have been expressed in yeast cells. The meeting, which included representatives from seven manufacturers, presented preliminary guidelines for the control and testing of hepatitis B vaccine produced in yeast.*

HEPATITIS B — THE DISEASE

Hepatitis B virus is one of at least three hepatitis viruses causing a systemic infection with pathological changes in the liver (hepatitis A, hepatitis B, and non-A, non-B hepatitis). Hepatitis B is the most important of all the viral hepatitides. It accounts for half of all clinical hepatitis seen in some countries and is responsible for much of the mortality, with an acute case-fatality rate of about 1%. From 5% to 10% of patients infected with hepatitis B become chronic carriers. In addition to the disability associated with the acute clinical disease, chronic liver disease, cirrhosis and hepatocellular carcinoma are now recognized sequelae of unresolved hepatitis B infection. Indeed, in some areas of Asia and sub-Saharan Africa, hepatocellular carcinoma, ostensibly attributable to hepatitis B infection, ranks as a leading cause of cancer deaths among males.

The reservoir of hepatitis B virus resides mainly in a population of chronic carriers in the world, now estimated to number more than 200 million, with approximately the following geographical distribution: a prevalence of 0.1% or less in northern Europe, North America and parts of Australia; 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 Middle East; and a prevalence of up to 20% in some parts of Africa, Asia and the Pacific area.

Infection is transmitted to susceptible persons through close contact with the blood, or other body fluids of chronic infectious carriers or persons suffering acute infection. In low-incidence countries, the risk of hepatitis B infection is still high among certain groups of health-care personnel, patients receiving treatment by maintenance haemodialysis or blood products made from large pools of human plasma, residents of institutions for the mentally handicapped, prisoners, abusers of illicit injectable drugs, and the sexually promiscuous. In high-incidence areas, such as south-east Asia, transmission from mother to child in the perinatal period is the major mode of infection, supplemented by horizontal transmission between other family contacts; whereas in other high-incidence areas, such as Africa, the main mode of transmission is horizontal.

The incubation period for hepatitis B is long. Six weeks to six months may elapse between exposure to infection and onset of clinical symptoms. The illness usually begins with fatigue and anorexia and sometimes is accompanied by myalgia and abdominal discomfort. Later, jaundice, dark urine, light-coloured stools, and tender hepatomegaly may appear. In other cases, the onset may be rapid, with appearance of jaundice early, in association with fever, chills, and leukocytosis. In some cases, jaundice may never be recognized and the patient may be aware only of a "flu-like" illness. An important point is that persons with mild, anicteric illness are just as likely to become

* This Memorandum was drafted by the signatories listed on page 61. A French translation of this article will appear in a later issue of the *Bulletin*. Requests for reprints should be addressed to Division of Communicable Diseases, World Health Organization, 1211 Geneva 27, Switzerland.

chronic carriers as those who present with clinical disease.

Hepatitis B virus (HBV) has been identified as a 42-nm particle containing double-stranded DNA. Infection with HBV is manifested by at least three antigenic markers: hepatitis B surface antigen (HBsAg), the core antigen (HBcAg), and the *e* antigen (HBeAg), resulting from replication of the virus in the hepatocytes. The surface antigen is found as 18–22-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.

HBV 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.

Development of vaccines

Because of the urgent need for hepatitis B vaccine, particularly for persons who are at increased risk of acquiring infection, the WHO requirements for hepatitis B vaccine prepared from plasma were formulated in 1981 and revised in 1983.[a] 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 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—represents an entirely new approach in vaccine production and demands special consideration in the tests applied to the production and quality control of the vaccines. Even more important, it has now been shown that human blood and plasma may harbour a number of infectious agents including LAV (or HTLV III). Particular attention, therefore, must be given to the selection of the donors of the plasma, to the process of separation of the antigen, and to the inactivation procedures to ensure that all potential infectious agents which may still be present after the purification of the antigen have been inactivated.

In most countries, the supply of vaccine is potentially limited by the available sources of suitable plasma. In addition, extensive processing and safety testing have been necessary to ensure production of a vaccine that is pure and free of any extraneous living agent which might have been present in the starting plasma. Even though multiple inactivation treatments used in the antigen purification process have been shown to inactivate representatives of all major groups of animal viruses, concern over the theoretical possibility of a living organism (such as the etiological agent of acquired immune deficiency syndrome (AIDS)) being present in the plasma and surviving the purification and inactivation procedures has impeded general acceptance of hepatitis B vaccine. Nevertheless, the hepatitis B plasma-derived vaccines, which meet the WHO requirements revised in 1983, have been shown to be completely safe.[b]

HBV has a number of structural components that differentiate it from the other families of DNA viruses. This virus 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 double-stranded circular DNA genome of a relative molecular mass of approximately 2×10^6, part of which is single-stranded, which is an unusual feature among viruses. Virus has been recovered from the plasma of a hepatitis B carrier and DNA has been isolated from the viral particles. The viral DNA has been cloned in *Escherichia coli* and the surface antigen gene has been isolated.

PRINCIPLES OF VACCINE PRODUCTION BY RECOMBINANT DNA TECHNIQUES AND STANDARDIZATION AND CONTROL OF PRODUCTS

Advances in molecular genetics and nucleic acid chemistry have enabled genes coding for biologically active substances to be identified, analysed in fine detail, transferred within and between organisms, and expressed under controlled conditions so as to obtain efficient synthesis of the product for which they code. A gene which codes for a specific product can be isolated and propagated by inserting naturally occurring or synthetic DNA molecules into a suitable vector which is introduced into a host organism and selecting individual clones that carry the required gene; this is the process of gene cloning. A key step in the process involves the insertion of the gene into the vector with the aid of highly specific restriction endonuclease enzymes (which cleave the vector DNA at predetermined sites) and ligases (which join the gene to the vector).

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] Unpublished WHO document, WHO/BS/83/1391/revision 2, 1983.

[b] See *British medical journal*, **289**: 1243–1244 (1984).

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 mononucleotides. The process of decoding this information and the synthesis of the gene product occurs in two phases: first, transcription of the DNA coding strand in the form of a messenger RNA (mRNA) and, second, translation of the information carried by the mRNA molecule into an amino acid sequence. The factors affecting the expression of foreign genes introduced into a microorganism by a plasmid are complex; indeed, the efficient and controlled expression of stable, cloned DNA sequences is an important field of current research.

The guidelines given below should be considered in the control and testing of hepatitis B vaccine made by recombinant DNA methods and may help applicants for clinical trial certificates and product licences to assemble the information needed to support their applications. They have been formulated bearing in mind the scale-up required for commercial production. Particular emphasis is placed on "in-process" control, a concept that has been highly effective in the control of, for example, bacterial and viral vaccines, rather than relying entirely on tests on the final end-product. It is to be expected that certain general requirements, such as tests for potency, purity, abnormal 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 of these requirements are given in WHO requirements for plasma-derived hepatitis B vaccine[c] or in other WHO documents. However, the novelty of the recombinant-DNA-derived vaccines and the procedures used in their manufacture dictate the use of reasoned caution in their control. Certain tests will be required on every production batch of vaccine but others will be required only to establish validity, acceptability, and consistency of a given manufacturing process.

General considerations

A detailed description of the strategy by which the product is made should be given. Evidence should be presented to show that the intended gene product, that is the HBsAg, in the form made by recombinant DNA technology, possesses the characteristics required of it.

Rigorous identification and characterization of the yeast-derived product by biological and chemical methods will be of major concern. The way in which the product differs structurally, biologically or immunologically from the naturally occurring antigen must be fully documented. Such alterations could arise either at the genetic or post-translational level or during purification. Variations between batches of the product may result from the genetic instability of the plasmid or its loss from yeast cells during fermentation. Microbial contamination during fermentation needs to be thoroughly tested.

Special attention should be given to purity because:

(*a*) unwanted gene products may be co-expressed unexpectedly with the HBsAg; such products might arise because transcription initiates at several sites in a plasmid, or because changes in the plasmid might occur during fermentation which affect transcription, initiation, or termination processes favouring the expression of other genes in the plasmid;

(*b*) the presence, in the product, of extraneous components derived from the host-cell system, not encountered in plasma-derived vaccine, may have undesired consequences when administered to man.

(*c*) agents used in the purification process (column matrices, antibodies) may give rise to specific contaminants in the final product.

Strategy for cloning and expressing the gene

(1) A full description of the host cell and expression vectors used in production should be given. This should include details of the construction, genetics, and structure of the expression vector and the origin and identification of the gene that is being cloned.

(2) The nucleotide sequence of the gene insert and also of adjacent segments of the plasmid (or other vector used in production) should be provided. Restriction enzyme mapping of the plasmid containing the gene insert may also provide useful information.

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

(4) For each lot, an assessment should be made of the proportion of cells at the end of fermentation which possess the plasmid.

Manufacturing procedures

(1) A description of the seed-lot 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 in the seed stock under conditions of storage and recovery. Instability of the plasmid in the seed culture, which may involve rearrangements, deletions, or insertions of nucleotides, must be documented.

(2) Full details of the fermentation process used to manufacture the product should be provided to the national control authority with particular reference to the frequency, degree, and nature of any microbial

[c] See footnote *a*.

contamination in the fermentation vessels at the end of fermentation runs, including information on the sensitivity of methods used to detect contamination.

(3) Data on consistency of the yield of the HBsAg in all fermentation runs should be presented together with a description of the assays used to obtain the data.

(4) The structure of the expression vector and the characteristics of the host cell at the end of full-scale fermentation cycles should be established on a number of occasions and any changes documented.

(5) Methods used to purify the HBsAg should be described. The capacity of the purification procedure to remove host-cell proteins, nucleic acid, and other impurities should be demonstrated, as should the reproducibility of the purification process. Pilot-scale studies may be valuable in this context. Chemical and biological substances added during manufacture and processing should be fully documented and their presence or absence in the final product justified and documented. The sensitivity of the tests to detect yeast proteins, DNA, and other contaminants should be well documented.

(6) Precise characterization of the bulk aqueous product should be undertaken by appropriate biological, immunological, and physicochemical methods. Sufficient sequence information to characterize the product should be obtained on at least one occasion. If the gene insert specifies *N*-terminal methionine, then account should be taken of its possible presence in the product. Particular attention should also be paid to the amino acid sequences of the *N*- and *C*-terminal regions. A comparison of the sequence of the product with the sequence of natural antigen should be made wherever possible. Physicochemical characterization of the product should include techniques to establish its conformational structure, e.g., the antigen particle size.

VACCINES UNDER DEVELOPMENT

Following the original demonstration by Valenzuela et al.[d] of the expression of HBsAg in yeast (*Saccharomyces cerevisiae*) by recombinant DNA techniques, several manufacturing organizations have exploited this technique for vaccine development. Seven manufacturers reported to the meeting the progress in hepatitis B vaccine development. HBV DNA sequences coding for HBsAg obtained from cloned HBV DNA, or, in one case, a partially synthetic sequence, were incorporated into various recombinant plasmids designed for expression in yeast. Promoter, terminator, and selectable marker sequences were incorporated in appropriate juxtaposition in the expression vectors. Among promoters used by the various manufacturers were yeast glyceraldehyde-3 phosphate dehydrogenase and phosphatase (PHO.5). Genes used for selection of yeast carrying the recombinant plasmids include TrpI or Leu 2. The cloned DNA molecules from HBV of subtypes *adw*, *ayw* or *adr* were used as sources of surface antigen genes. The HBV gene inserted coded for the 226 amino acids of the HBsAg product.

The HBsAg produced by large-scale yeast fermentation remained cell-associated. The HBsAg was released by mechanical disruption of yeast cells and was purified by a variety of methods applied sequentially including (*a*) immunological affinity, (*b*) hydrophobic interaction, (*c*) ion exchange, and (*d*) gel exclusion chromatography. Isopycnic and rate sedimentation centrifugation were also employed. These procedures resulted in products with purity of greater than 90% with respect to protein content.

HBsAg particles obtained from plasma are relatively uniform in size (about 22 nm) and contain lipids and carbohydrates of host-cell origin. The HBsAg preparations from yeast were in the form of complex particles of about 15–30 nm in diameter (average, 17–20 nm) containing exclusively a non-glycosylated monomeric HBsAg polypeptide of relative molecular mass (M_r) 23–25 000 associated with host-cell lipids.

After adsorption to $Al(OH)_3$, the yeast product was shown to be capable of eliciting an anti-HBs response in laboratory animals. Chimpanzees immunized with at least two of the experimental vaccines were shown to resist a live HBV challenge of a thousand infectious doses.

The immunogenic potency of the yeast product in animals was similar to that of vaccines made from plasma. Clinical studies of the new vaccine are in progress in several countries.

RECOMMENDATIONS

1. It is recommended that monoclonal antibodies should be used to identify epitopes on HBsAg involved in protection and to standardize the content of these epitopes in the recombinant vaccines. Facilities to examine other candidate monoclonal antibodies for their ability to protect chimpanzees are available at the Food and Drug Administration of the USA.

2. A standing group of experts should be appointed at the international level to advise national health authorities, on request, on the use of the new hepatitis vaccines for clinical and field trials for the control of viral hepatitis. The group should also be available to

[d] *Nature* (*London*), **298**: 347 (1982).

consult and collaborate closely with manufacturers in the planning and execution of clinical and field studies, subject to approval by the WHO Secretariat Committee on Research Involving Human Subjects (SCRIHS).

3. The provisional WHO requirements for hepatitis B vaccines produced in yeast by recombinant DNA technology should be considered by the WHO Expert Committee on Biological Standardization as soon as possible.

4. A group of experts to establish standardized methods that are specific and sensitive to detect nucleic acids in biological products, and to establish reference standards for this purpose, should be convened at an international level.

5. Comparative studies on hepatitis B vaccines from yeast should be carried out. Manufacturers are invited (and those present at the meeting have agreed) to submit to WHO samples of up to three production lots of purified aqueous HBsAg product (bulk aqueous vaccine) and up to three lots of final vaccine. Any information provided on these materials will be treated in strict confidence by WHO. WHO will code these samples and submit them to the Office of Biologics Research and Review, the Food and Drug Administration, USA, and the National Institute of Biological Standards and Control, England, for comparative laboratory studies which will include:

(*a*) *in vitro* assays of antigen content;

(*b*) tests for epitope specification, e.g., with a panel of monoclonal antibodies to HBsAg;

(*c*) immunogenicity studies in mice in comparison with the proposed WHO reference hepatitis B vaccine to include tests of the mouse antisera for antibodies against specific +a+ epitopes (synthetic);

(*d*) physicochemical and molecular characteristics of the products.

The results of the studies should be submitted by the testing laboratory to WHO under code. WHO will inform each of the manufacturers of the results of the tests on their own samples, together with the coded results of other samples.

The minimal quantity of product required for the tests are:

(*a*) 2×20 ml of each bulk vaccine at a concentration of not less than 50–100 g HBsAg protein/ml;

(*b*) 2×20 ml of each final vaccine.

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