Title: NOVEL VACCINE

Abstract: The invention relates to the use of a hepatitis B antigen free or substantially free of thiomersal, in the manufacture of a prophylactic or therapeutic hepatitis B vaccine for intradermal delivery.
NOVEL VACCINE

This invention relates to novel vaccines and to novel methods of vaccination and vaccine delivery. In particular the invention relates to hepatitis B vaccine formulations for delivery into the dermis of an individual, for the treatment or prophylaxis of hepatitis B virus (HBV) infections.

Chronic hepatitis B virus (HBV) infection, for which there is currently limited treatment, constitutes a global public health problem of enormous dimensions. Chronic carriers of HBV, estimated to number more than 300 million world-wide, are at risk for development of chronic active hepatitis, cirrhosis and primary hepatocellular carcinoma.

It would be desirable to provide a vaccine that targets the cell mediated immune system for example by targeting the antigen to the dendritic cells and langerhans cells that reside in the skin, particularly in the dermis. Cell mediated immunity appears to assist viral clearance and recovery from illness and may provide better cross protection between strains than antibodies. Cell mediated immunity may be critical for viral clearance in chronic hepatitis B virus carriers. It has also been described in the literature that intradermal administration allows for the induction of mucosal immunity at the level of the mucosal surfaces. The intradermal route can offer benefits compared to the parenteral route in particular since one of the routes of transmission of hepatitis B virus is sexual transmission via the mucosa. It is also desirable to provide an alternative way of administering hepatitis B vaccines, in particular a way that is pain-free or less painful than i.m. injection, and does not involve the associated negative effect on patient compliance because of "needle fear".

Furthermore, it would be desirable to reduce the amount of antigen needed for a dose of hepatitis vaccine.

Many vaccines which are currently available require a preservative to prevent deterioration. A frequently used preservative is thiomersal which is a mercury-containing compound. Some concerns have been raised about the use of mercury in vaccines, although commentators have stressed that the potential hazards of thiomersal-containing vaccines should not be overstated (Offit; P.A. JAMA Vol.283;No:16). Nevertheless it would be advantageous to find new and potentially safer methods of preparation of vaccines to replace the use of thiomersal in the manufacturing process. There is thus a need to develop vaccines which are thiomersal-free, in particular hepatitis B vaccines.

In a first aspect the present invention provides the use of a hepatitis B antigen free or substantially free of thiomersal, in the manufacture of a prophylactic or therapeutic hepatitis B vaccine for intradermal delivery.
In another aspect the present invention provides the use of a hepatitis B antigen prepared by a method which comprises purification of the antigen in the presence of a reducing agent comprising a free –SH group, in the preparation of a vaccine for intradermal delivery. The hepatitis B antigen is preferably stable and substantially free of thiomersal.

The antigen is substantially free of thiomersal when thiomersal is not detectable in the purified antigen product using absorption spectrophotometry of mercury, as described herein.

The hepatitis antigen preparation preferably comprises less than 0.025 µg mercury per 20µg protein (hepatitis antigen), suitably as measured by absorption spectrophotometry.

Accordingly the present invention relates to a method for producing an intradermal hepatitis B vaccine, the vaccine comprising a purified hepatitis B surface antigen and having less than 0.025 µg mercury per 20µg hepatitis B antigen, wherein the antigen is purified in the presence of a reducing agent having a free –SH group.

Preferably the vaccine is free of a preservative, preferably free of a thiomersal preservative.

Preferably the antigen is produced by a purification method which is carried out in the absence of thiomersal, and the purified antigen is completely free of thiomersal. Alternatively the purification may be carried out in the presence of thiomersal, which is then removed or largely removed to leave substantially no thiomersal. For example the antigen may be treated with a reducing agent comprising a free –SH group to remove the thiomersal.

Accordingly the invention also relates to a method for producing an intradermal hepatitis B surface antigen suitable for use in a vaccine, the method comprising purification of the antigen in the presence of a reducing agent having a free -SH group, wherein the antigen is purified in the presence of thiomersal before treatment with the reducing agent.

Preferably the hepatitis B antigen employed in the invention is stable, suitably substantially as stable as a hepatitis antigen in the presence of thiomersal, for example as outlined in Example 1 herein.

Preferably the antigen is at least as immunogenic and antigenic as hepatitis B antigen manufactured in the presence of thiomersal as described in Example 1. Preferably the immunogenic hepatitis B antigen has a mean ELISA protein ratio greater than or equal to 1 and an RF1 content with at least a 2-fold lower IC50 value that that of the hepatitis B surface antigen manufactured in the presence of thiomersal. More preferably the hepatitis B antigen has a mean ELISA protein ratio greater than 1.5 and an RF1 content with at least a 3-fold lower IC50 value than that of the hepatitis B surface antigen manufactured in the presence of thiomersal.
Preferably the reducing agent is added during the antigen purification process, preferably after growth of cells expressing the antigen.

5 Preferably the reducing agent is selected from the group consisting of cysteine, dithiothreitol, β-mercaptoethanol or glutathione, with cysteine being most preferred.

Accordingly the present invention preferably provides the use of a stable immunogenic hepatitis B antigen with no or substantially no thiomersal prepared by a method which comprises purification of the antigen in the presence of cysteine, in the manufacture of a vaccine for the prophylaxis or therapy of hepatitis B infection. Such a thiomersal free vaccine has been shown, in human studies, to be more immunogenic than an equivalent thiomersal containing vaccine, in that antibody titres are increased in response to the thiomersal free vaccine.

10 Preferably, the cysteine, in solution or powder form, is added during the process to a final concentration of between 1 and 10mM, preferably 1 to 5 mM. More preferably, the cysteine is added to a final concentration of about 2mM.

15 Preferably the cysteine is L-cysteine.

The stable hepatitis B antigen may be prepared by subjecting the crude antigen to gel permeation chromatography, to ion-exchange chromatography and mixing it with a reducing agent having a free –SH group.

20 Preferably the ion-exchange chromatography is anion-exchange chromatography.

The invention further provides a hepatitis B vaccine composition containing substantially no thiomersal, in a dose volume suitable for intradermal delivery.

25 For administering the vaccines of the present invention to the skin, or more specifically into the dermis, the vaccine is in a low liquid volume, particularly a volume of between about 0.05 ml and 0.2 ml.

30 Preferably the volume of a dose of vaccine according to either aspect of the invention is between 0.025 ml and 0.25 ml, more preferably approximately 0.2 ml or less. Preferred dose volumes are a fraction of the conventional intramuscular dose, e.g. a half or less of the conventional dose, or one fifth or one tenth of the conventional dose, such as approximately 0.1 ml or approximately 0.2 ml. A 50 μl dose volume might also be considered. A 0.1 ml dose is approximately one fifth or approximately one tenth of the volume of a conventional intramuscular vaccine dose. The volume of liquid that can be administered intradermally depends in part upon the site of the injection. For example, for an injection in the deltoid region, 0.1 ml is the maximum preferred volume whereas in the lumbar region a large volume e.g. about 0.2 ml can be given.
Preferably the vaccines according to the invention are administered to a location between about 1.0 and 2.0 mm below the surface of the skin. More preferably the vaccine is delivered to a distance of about 1.5 mm below the surface of the skin.

The hepatitis B antigen of the invention may be used for either the treatment or prophylaxis of hepatitis B infections, especially treatment or prophylaxis, for example, of chronic hepatitis B infections.

The vaccines described herein may further comprise an adjuvant. Preferably the adjuvant is an aluminium salt or a preferential stimulator of TH1 cell response, or a combination of the two.

Preferably the antigen is a hepatitis B surface antigen.


As used herein the expression 'hepatitis B surface antigen' or 'HBsAg' includes any HBsAg antigen or fragment thereof displaying the antigenicity of HBV surface antigen. It will be understood that in addition to the 226 amino acid sequence of the HBsAg S antigen (see Tiollais et. al. Nature, 317, 489 (1985) and references therein) HBsAg as herein described may, if desired, contain all or part of a pre-S sequence as described in the above references and in EP-A- 0 278 940. HBsAg as herein described can also refer to variants, for example the 'escape mutant' described in WO 91/14703.

HBsAg may also refer to polypeptides described in EP 0 198 474 or EP 0 304 578.

Normally the HBsAg will be in particle form. In a particularly preferred embodiment the HbsAg will consist essentially of the HbsAg S-antigen mentioned hereinabove.

The vaccine may advantageously include a pharmaceutically acceptable excipient such as a suitable adjuvant. Suitable adjuvants are commercially available such as, for example, Merck Adjuvant 65 (Merck and Company, Inc., Rahway, NJ); aluminum salts such as aluminum hydroxide or aluminum phosphate; salts of calcium, iron or zinc; an insoluble suspension of acylated tyrosine; acylated sugars; cationically or anionically derivatized polysaccharides; polyphosphazenes; biodegradable microspheres; monophosphoryl lipid A and quill A. Cytokines, such as GM-CSF or interleukin-2, -7, or -12, may also be used as adjuvants.

A preferred adjuvant for use in the invention is non-toxic bacterial lipopolysaccharide derivative (LPS). A non-toxic derivative of LPS, monophosphoryl lipid A (MPL), produced
by removal of the core carbohydrate group and the phosphate from the reducing-end glucosamine, has been described by Ribi et al (1986, Immunology and Immunopharmacology of bacterial endotoxins, Plenum Publ. Corp., NY, p407-419).

5 A further detoxified version of MPL results from the removal of the acyl chain from the 3-position of the disaccharide backbone, and is called 3-O-Deacylated monophosphoryl lipid A (3D-MPL). It can be purified and prepared by the methods taught in GB 2122204B, which reference also discloses the preparation of diphosphoryl lipid A, and 3-O-deacylated variants thereof. Other purified and synthetic lipopolysaccharides have been described (US 6,005,099 and EP 0 729 473 B1; Hilgers et al., 1986, Int.Arch.Allergy.Immunol., 79(4):392-6; Hilgers et al., 1987, Immunology, 60(1):141-6; and EP 0 549 074 B1).

10 A preferred form of 3D-MPL is in the form of an emulsion having a small particle size less than 0.2μm in diameter, and its method of manufacture is disclosed in WO 94/21292. Aqueous formulations comprising monophosphoryl lipid A and a surfactant have been described in WO98/43670.

The bacterial lipopolysaccharide derived adjuvants to be used in the present invention may be purified and processed from bacterial sources, or alternatively they may be synthetic. For example, purified monophosphoryl lipid A is described in Ribi et al 1986 (supra), and 3-O-Deacylated monophosphoryl or diphosphoryl lipid A derived from Salmonella sp. is described in GB 2220211 and US 4912094. Other purified and synthetic lipopolysaccharides have been described (US 6,005,099 and EP 0 729 473 B1; Hilgers et al., 1986, Int.Arch.Allergy.Immunol., 79(4):392-6; Hilgers et al., 1987, Immunology, 60(1):141-6; and EP 0 549 074 B1). Particularly preferred bacterial lipopolysaccharide adjuvants are 3D-MPL and the β(1-6) glucosamine disaccharides described in US 6,005,099 and EP 0 729 473 B1.

20 Accordingly, the LPS derivatives that may be used in the present invention are those immunostimulants that are similar in structure to that of LPS or MPL or 3D-MPL. In another aspect of the present invention the LPS derivatives may be an acylated monosaccharide, which is a sub-portion to the above structure of MPL.

30 Saponins are known as adjuvants in vaccines for systemic administration. The adjuvant and haemolytic activity of individual saponins has been extensively studied in the art (Lacaille-Dubois, M and Wagner H. (1996) A review of the biological and pharmacological activities of saponins. Phytomedicine vol 2 pp 363-386). For example, Quil A (derived from the bark of the South American tree Quillaja Saponaria Molina), and fractions thereof, are described in US 5,057,540 and "Saponins as vaccine adjuvants", Kensil, C. R., Crit Rev Ther Drug Carrier Syst, 1996, 12 (1-2):1-55; and EP 0 362 279 B1. Particulate structures, termed Immune Stimulating Complexes (ISCOMS), comprising fractions of Quil A are haemolytic and have been used in the manufacture of vaccines (Morein, B., EP 0 109 942 B1; WO 96/11711; WO 96/33739). The haemolytic saponins
QS21 and QS17 (HPLC purified fractions of Quil A) have been described as potent systemic adjuvants, and the method of their production is disclosed in US Patent No.5,057,540 and EP 0 362 279 B1. Other saponins which have been used in systemic vaccination studies include those derived from other plant species such as Gypsophila and Saponaria (Bomford et al., Vaccine, 10(9):572-577, 1992).

In the formulations of the invention it is preferred that the adjuvant composition induces an immune response predominantly of the TH1 type. High levels of Th1-type cytokines (e.g., IFN-γ, TNFα, IL-2 and IL-12) tend to favour the induction of cell mediated immune responses to an administered antigen. Within a preferred embodiment, in which a response is predominantly Th1-type, the level of Th1-type cytokines will increase to a greater extent than the level of Th2-type cytokines. The levels of these cytokines may be readily assessed using standard assays. For a review of the families of cytokines, see Mosmann and Coffman, Ann. Rev. Immunol. 7:145-173, 1989.

Accordingly, suitable adjuvants for use in eliciting a predominantly Th1-type response include, for example a combination of non-toxic LPS derivative such as monosophoryl lipid A, preferably 3-de-O-acetylated monosphophoryl lipid A (3D-MPL) together with an aluminium salt. Other known adjuvants which preferentially induce a TH1 type immune response include CpG containing oligonucleotides. The oligonucleotides are characterised in that the CpG dinucleotide is unmethylated. Such oligonucleotides are well known and are described in, for example WO 96/02555. Immunostimulatory DNA sequences are also described, for example, by Sato et al., Science 273:352, 1996. Another preferred adjuvant is a saponin, preferably QS21 (Aquilia Biopharmaceuticals Inc., Framingham, MA), which may be used alone or in combination with other adjuvants. For example, an enhanced system involves the combination of a monosophoryl lipid A and a saponin derivative, such as the combination of QS21 and 3D-MPL as described in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol, as described in WO 96/33739. Other formulations comprise an oil-in-water emulsion and tocopherol. A particularly potent adjuvant formulation involving QS21, 3D-MPL and tocopherol in an oil-in-water emulsion is described in WO 95/17210.

Another suitable adjuvant formulation including CpG and a saponin is described in WO 00/09159. Preferably the saponin in that particular formulation is QS21. Preferably the formulation additionally comprises an oil in water emulsion and tocopherol.

The vaccines described herein may comprise in addition to the hepatitis B surface antigen of the present invention, an adjuvant, and one or more antigens selected from the group consisting of: diphtheria toxoid (D), tetanus toxoid (T), acellular pertussis antigens (Pa or Pw - acellular or whole cell), inactivated polio virus (IPV), haemophilus influenzae antigen (Hib), hepatitis A antigen, herpes simplex virus (HSV), chlamydia, group B streptococcus (GBS), human papilloma virus (HPV), respiratory syncytial virus (RSV), hepatitis C virus (HCV), cytomegalovirus (CMV), influenza virus, streptococcus pneumoniae and neisseria antigens. Antigens conferring protection for other diseases may also be combined in the
vaccine formulation of the present invention. Particularly preferred combinations include hepatitis B surface antigen with D, T, P, with or without one or more of Hib, IPV and optionally comprising other antigens.

In such combinations, the hepatitis B surface antigen is preferably adsorbed onto aluminium phosphate.

In one particular embodiment, the vaccine formulation described herein comprises a hepatitis B surface antigen in conjunction with an adjuvant and an inactivated polio virus.

In a preferred embodiment the vaccine described herein comprises a hepatitis B surface antigen in combination with a hepatitis A antigen. Preferably the vaccine is suitable for administration in a two dose regimen. A suitable hepatitis A antigen is the commercially available HAV 175 strain (GlaxoSmithKline Biologicals). Preferably the hepatitis B plus A combination is adjuvanted with one or more aluminium salts, in particular with a combination of aluminium phosphate and aluminium hydroxide. Preferably the hepatitis B antigen is adsorbed onto aluminium phosphate. Preferably the amount of aluminium phosphate is between 0.015 to 0.1 mg, more preferably 0.02 to 0.08 mg, per μg hepatitis B surface antigen.

Preferred formulations for a hepatitis A plus B vaccine which are suitable for a two dose regimen are described in WO99/56772, incorporated herein by reference.

The present invention also provides a method of treatment and/or prophylaxis of hepatitis B virus infections, which comprises administering to a human or animal subject, suffering from or susceptible to hepatitis B virus infection, a safe and effective amount of an intradermal vaccine of the present invention for the prophylaxis and/or treatment of hepatitis B infection.

The invention further provides the use of an intradermal vaccine described herein in the manufacture of a medicament for the treatment of patients suffering from a hepatitis B virus infection, such as chronic hepatitis B virus infection.

The vaccines described herein will contain an immunoprotective or an immunotherapeutic quantity of the antigen and may be prepared by conventional techniques.

The invention provides in a further aspect a pharmaceutical kit comprising an intradermal administration device and a hepatitis B vaccine formulation as described herein. The device is preferably supplied already filled with the vaccine. Preferably the vaccine is in a liquid volume smaller than for conventional intramuscular vaccines as described herein, particularly a volume of between about 0.05 ml and 0.25 ml. Preferably the device is a short needle delivery device for administering the vaccine to the dermis.
Human skin comprises an outer "horny" cuticle, called the stratum corneum, which overlays the epidermis. Underneath this epidermis is a layer called the dermis, which in turn overlays the subcutaneous tissue. Researchers have shown that injection of a vaccine into the skin, and in particular the dermis, stimulates an immune response, which may also be associated with a number of additional advantages.

The conventional technique of intradermal injection, the "mantoux procedure", comprises steps of cleaning the skin, and then stretching with one hand, and with the bevel of a narrow gauge needle (26-31 gauge) facing upwards the needle is inserted at an angle of between 10-15°. Once the bevel of the needle is inserted, the barrel of the needle is lowered and further advanced whilst providing a slight pressure to elevate it under the skin. The liquid is then injected very slowly thereby forming a bleb or bump on the skin surface, followed by slow withdrawal of the needle.

More recently, devices that are specifically designed to administer liquid agents into or across the skin have been described, for example the devices described in WO 99/34850 and EP 1092444.

Particularly suitable devices for use with the intradermal vaccines described herein include short needle devices such as those described in US 4,886,499, US 5,190,521, US 5,328,483, US 5,527,288, US 4,270,537, US 5,015,235, US 5,141,496, US 5,417,662. Intradermal vaccines may also be administered by devices which limit the effective penetration length of a needle into the skin, such as those described in WO99/34850, incorporated herein by reference, and functional equivalents thereof. Also suitable are jet injection devices which deliver liquid vaccines to the dermis via a liquid jet injector or via a needle which pierces the stratum corneum and produces a jet which reaches the dermis. Jet injection devices are described for example in US 5,480,381, US 5,599,302, US 5,334,144, US 5,993,412, US 5,649,912, US 5,569,189, US 5,704,911, US 5,383,851, US 5,893,397, US 5,466,220, US 5,339,163, US 5,312,335, US 5,503,627, US 5,064,413, US 5,520,639, US 4,596,556US 4,790,824, US 4,941,880, US 4,940,460, WO 97/37705 and WO 97/13537. Also suitable are ballistic powder/particle delivery devices which use compressed gas to accelerate vaccine in powder form through the outer layers of the skin to the dermis. Additionally, conventional syringes may be used in the classical mantoux method of intradermal administration. However, the use of conventional syringes requires highly skilled operators and thus devices which are capable of accurate delivery without a highly skilled user are preferred.

The content of antigens in the intradermal vaccines of the present invention may be similar to conventional doses as found in intramuscular vaccines. Accordingly, the protein antigens present in the intradermal vaccines may in the range 1-100μg, preferably 5-50μg. Likewise, the amount of hepatitis B antigen in each vaccine dose is generally expected to comprise 0.1-100 μg of antigen, preferably 0.1-50 μg, preferably 0.1-10 μg, and may be between 1 and 5 μg. The standard dose of hepatitis B surface antigen in Engerix-B™ (GlaxoSmithKline Biologicals) is 10 μg for infants, children and adolescents
and 20 µg for adults. These amounts of antigen may be used in a low liquid volume vaccine. However, it is a feature of skin or intradermal vaccines that the formulations may be "low dose". Accordingly the protein antigens in "low dose" vaccines are preferably present in as little as 0.1 to 10 µg, preferably 0.1 to 5 µg per dose.

Similarly, the amount of hepatitis A antigen in a combination vaccine as described herein is generally expected to comprise a reduced amount of antigen compared to conventional intramuscular hepatitis A vaccines such as the GlaxoSmithKline Biologicals vaccine, Havrix™, although this is not essential and standard amounts may be used. The viral antigen content for the HAV 175 strain is determined by ELISA and expressed in ELISA Units or EU and conventional doses of Havrix™ are 720 EU for children and 1440 EU for adults. Suitable intradermal doses for HAV 175 in the intradermal vaccines described herein are between 10 EU and 1000 EU, for example about 180 EU or about 360 EU or about 720 EU (for adult doses).

As used herein, the term "intradermal delivery" means delivery of the vaccine to the region of the dermis in the skin. However, the vaccine will not necessarily be located exclusively in the dermis. The dermis is the layer in the skin located between about 1.0 and about 2.0 mm from the surface in human skin, but there is a certain amount of variation between individuals and in different parts of the body. In general, it can be expected to reach the dermis by going 1.5 mm below the surface of the skin. The dermis is located between the stratum corneum and the epidermis at the surface and the subcutaneous layer below. Depending on the mode of delivery, the vaccine may ultimately be located solely or primarily within the dermis, or it may ultimately be distributed within the epidermis and the dermis.


The present invention is illustrated by but not limited to the following examples, wherein:

Figure 1 illustrates the thiomersal free production process for Engerix B ™;

Figure 2 illustrates SDS-PAGE analysis of bulk antigen lots; and
Figure 3 illustrates residual yeast proteins in bulk antigen lots produced by a thiomersal free process.

Figure 4 illustrates Anti-HBs Ig antibody response 14 days post II

Figure 5 illustrates Anti-HBs Ig antibody response 14 days post III

Figure 6 illustrates IFN-gamma production 14 days post III

Example 1:
Production Process for Hepatitis B surface antigen in the presence of thiomersal
The Hepatitis B surface antigen (HBsAg) of SB Biologicals hepatitis B monovalent vaccine (Engerix B™) is expressed as a recombinant protein in Saccharomyces cerevisiae (see Harford et. al. loc. cit.). The 24 kD protein is produced intracellularly and accumulated in the recombinant yeast cells. At the end of the fermentation the yeast cells are harvested and disrupted in the presence of a mild surfactant such as Tween 20 to liberate the desired protein. Subsequently the cell homogenate, containing the soluble surface antigen particles, is prepurified in a series of precipitations and then concentrated via ultrafiltration.

Further purification of the recombinant antigen is performed in subsequent chromatographic separations. In a first step the crude antigen concentrate is subjected to gel permeation chromatography on Sepharose 4B medium. Thiomersal is present in the elution buffer at the 4B gel permeation chromatography step. The elution buffer has the following composition: 10mM Tris, 5% ethylene glycol, pH 7.0, 50mg/L thiomersal.

Thiomersal is included in this buffer to control bioburden. Most of this thiomersal is removed during the subsequent purification steps including ion exchange chromatography, ultracentrifugation and desalting (gel permeation) so that purified bulk antigen preparations prepared by the original process contain about 1.2µg and less than 2µg of thiomersal per 20µg of protein.

An Ion-Exchange chromatography step is performed using a DEAE-matrix and this pool is then subjected to a Cesium-gradient ultracentrifugation on 4 pre-established layers of different Cesium chloride concentrations. The antigen particles are separated from contaminating cell constituents according to their density in the gradient and eluted at the end of the centrifugation process. Cesium chloride is then removed from this pool by a second gel permeation on Sepharose gel.

When HBsAg is prepared by the process containing thiomersal in the 4B gel permeation buffer, protein concentrations of over 30mg/ml are recovered in the pooled HBsAg containing fractions from the CsCl gradient, corresponding to an equivalent concentration of HBsAg as assayed by the AUSZYME kit from Abbott Laboratories.
The CsCl ultracentrifugation step usefully eliminates residual lipids, DNA and minor protein contaminants from the HBsAg preparation. It is performed by zonal centrifugation in a Ti 15 rotor from Beckman Instruments, Fullerton, California at a speed of 30,000 rpm for about 40 to 60 hours. The sample to be purified is applied to layers of CsCl solution with final concentrations of 0.75, 1.5, 2.5 and 3.25 M CsCl. At the end of centrifugation the gradient is eluted into fractions. Fractions containing HBsAg may be identified by UV absorbance at 280 nm or by testing dilutions of the fractions with the AUSZYME kit. The HBsAg band is at a density of 1.17 to 1.23 g/cm³.

The solution containing the purified HBsAg is sterile filtered before being used to make a vaccine formulation.

Purification from the yeast cell lysate is complex as the antigen is produced intracellularly and a series of separation techniques designed to eliminate different types of (yeast) contaminants are necessary to obtain pure bulk antigen. The steps of purification are important, as the product to be purified is a lipoprotein particle containing multiple copies of the surface antigen polypeptide and this structure must be maintained throughout the purification process. It is a particularity of this process that it yields surface antigen particles which are fully immunogenic without the need for further chemical treatment to enhance immunogenicity (compare EPO135435).

The details of the production process are further described in European Patent 0199698.

**Example 2:**

**Production and characterization of yeast-derived HBsAg by a thiomersal free process.**

1. **Production and purification of yeast-derived HBsAg**

1.1 **Outline of the production process**

Hepatitis B surface antigen may be produced by fermentation of an appropriate strain of *Saccharomyces cerevisiae*, for example that described in Harford et. al. (loc. cit.).

At the end of large-scale fermentation of the recombinant yeast strain, the cells are harvested and broken open in the presence of a mild surfactant such as Tween 20. The surface antigen is then isolated by a multistep extraction and purification procedure exactly as described above in Example 1 up to the step of the first gel permeation on Sepharose 4B.

1.2 **Thiomersal-free purification process**

In the thiomersal free process the following two changes have been introduced compared to the process described in Example 1.
1. The elution buffer at the 4B gel permeation chromatography step no longer contains thiomersal.

2. Cysteine (2mM final concentration) is added to the eluate pool from the anion exchange chromatography step.

It was found that omission of thiomersal from the 4B gel permeation buffer may result in precipitation of the HBsAg particles during the CsCl density gradient centrifugation step with loss of product and aggregation or clumping of the recovered antigen.

Addition of cysteine at 2 mM final concentration to the eluate pool from the preceding anion exchange chromatography step prevents precipitation and loss of antigen during CsCl density centrifugation.

Cysteine is a preferred substance for this treatment as it is a naturally occurring amino acid and can be removed at the subsequent desalting step on a gel permeation column using Sepharose 4BCLFF as the column matrix.

There are no other changes in the manufacturing process compared to the process described in Example 1.

The thiomersal free process yields bulk antigen of a purity and with properties comparable to antigen from the process of Example 1.

1.2a

The thiomersal added to the 4B buffer at 50µg/ml is thought to decompose and the resulting ethyl mercury may attach covalently to free sulphhydryl groups on cysteine residues of the protein. The protein contains 14 cysteine residues of which 7 are located between positions 101 and 150.

This region of the protein is believed to be located at the surface of the particle and contain the major antigenic region of HBsAg including the immunodominant a region and the recognition site for the RF1 monoclonal antibody (Waters J et al, Postgrad. Med. J., 1987:63 (Suppl. 2): 51-56.and Ashton-Rickardt and Murray J. Med. Virology, 1989: 29: 196). Antigen purified with thiomersal present in the 4B gel permeation buffer contains about 0.5-0.6µg mercury at the end of the purification process. This mercury is not fully removed by simple dialysis.

In one experiment, 0.56µg Mercury per 20µg protein was measured on bulk antigen preparation. This preparation was dialysed for 16 hours at room temperature against 150mM NaCl, 10mM NaPO4 buffer pH 6.9. At the end of dialysis, a concentration of 0.33µg Hg per 20µg protein was measured.
In contrast, dialysis in the presence of a reducing agent such as L-cysteine at 0.1 to 5.0 mg/ml, DTT at 50 mM or 2-mercaptoethanol at 0.5 M, followed by a second dialysis to remove the reducing agent, results in reduction of the mercury content of the antigen preparation to less than 0.025μg Mercury per 20μg protein. This is the lowest limit of detection of the method.

The mercury content was determined by absorption spectrophotometry. The antigen is diluted in a solution containing 0.01 % w/v of potassium bichromate (K₂Cr₂O₇) and 5% v/v of nitric acid. Standard solutions are prepared with thiomersal as the mercury source. The atomic absorption of sample and standard solutions is measured after vaporisation in a vapour generator, with a mercury-specific cathode at 253.7 nm. Atomic absorption of the dilution liquid is measured as blank. The mercury content of the sample is calculated via the calibration curves obtained from the standard solutions. Results are expressed as μg of mercury per 20μg of protein.

1.3 Production of thiomersal free bulk antigen
The process steps for purification of bulk antigen are shown in Figure 1.

1.4 Composition of vaccine formulated without thiomersal.
A typical quantitative composition for a hepatitis B vaccine without preservative and formulated from antigen prepared by the thiomersal free process is provided in Table 1.

**Table 1:**

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Amount per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active constituent – Protein of which at least 95% is HbsAg</td>
<td>20 μg</td>
</tr>
<tr>
<td>Aluminium hydroxide (adsorbent) (expressed as Al₂O₃)</td>
<td>0.95 mg</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>9.0 mg (maximum)</td>
</tr>
<tr>
<td>Disodium phosphate dihydrate</td>
<td>0.98 mg</td>
</tr>
<tr>
<td>Sodium dihydrogen phosphate dihydrate</td>
<td>0.71 mg</td>
</tr>
<tr>
<td>Water for injection q.s. ad</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

The composition may be varied by the addition of 3D-MPL and/or other adjuvants.

2. Characterization of bulk antigen and vaccine produced by the thiomersal free process

2.1. Tests and assays on purified bulk antigen

2.1.1 Basis of comparison
Three lots of bulk antigen were prepared by the thiomersal free process according to this example (Example 1.2) and are identified as HEF001, HEF002 and HEF003. These were compared to a lot of bulk antigen (HEP2055) prepared by the previous process (as described in Example 1) in the presence of thiomersal.

2.1.2 Tests and assays on bulk antigen
The three bulk antigen lots produced by the thiomersal free process were tested and the results are summarised in Table 2.

Protein content was measured by the method of Lowry et al (J. Biol. Chem. 1951:193:265).

Endotoxin content was measured by a Limulus gel clotting technique using a commercially available kit from Cape Cod Associates, 704 Main St., Falmouth, MA 02540, USA. The reagent is standardized against the US Pharm. Endotoxin Reference Standard.

Tween 20 was measured by the method of Huddleston and Allred (J. Amer. Oil Chemist Soc., 1965:42:983).

HBsAg content was measured by the commercially available AusZYME kit from Abbott Laboratories, One Abbott Park Road, Abbott Park, IL 60064, USA. Assay procedure B of the manufacturer was employed. A batch of bulk antigen purified by the process containing thiomersal was used as a standard to establish the dose response curve.


Lipids were measured using a commercially available kit (Merkotest Total Lipids 3321) from E.Merck, B.P. 4119, Darmstadt D-6100, Germany.

DNA content was measured by the Threshold method using apparatus and reagents available from Molecular Devices Corp., Gutenbergstraße 10, Ismaning, Munich, Germany.

The values found in the tests and assays are in the range seen for bulk antigen lots manufactured using thiomersal in the elution buffer of the Sepharose 4B gel permeation step, with the exception of the antigenic activity by ELISA. The values for this measurement for the three HEF preparations are higher (1.63-2.25) than that found for the bulk antigen lot HEP2055 which has a ELISA/protein ratio of 1.13. The ELISA/protein ratios measured by the AUSZYME kit for thiomersal containing batches of bulk antigen are generally about 1.0 and within the range 0.8 - 1.2 and very rarely exceed 1.4.

2.1.3 SDS-PAGE gel analysis
The bulk antigen preparations were assayed by SDS-PAGE analysis in reducing conditions and Coomassie blue staining. All samples showed a major band at 24K with traces of a dimer protein. The samples were judged to be of high purity (> 99 % pure) as assessed by the absence of visible bands of contaminating proteins.

Samples (1μg) of the bulk antigen preparations were assayed by SDS-PAGE in reducing and non-reducing conditions and silver staining (Figure 2). In reducing conditions the samples showed an intense band migrating at 24K with traces of dimer and multimeric forms. The gel patterns are indistinguishable from that of HEP2055 as comparator. The samples were also run in non-reducing conditions. In these conditions less of the material migrates at 24K and the amount of polypeptide migrating at dimeric and multimeric positions is increased. The thiomersal free bulk antigen lots appear to have a somewhat higher degree of polymerisation than the comparator HEP2055 lot.

The identity of the 24K polypeptide revealed by Coomassie blue or silver staining was confirmed by Western blotting with rabbit polyclonal antibodies raised against plasma HBsAg. The bulk antigen preparations show a major band at 24K together with dimeric and trimeric forms. The technique reveals minor traces of breakdown products of the surface antigen protein. There are no differences between the bulk antigen prepared by the thiomersal free process and the HEP2055 lot.

The presence of residual yeast proteins was assayed by SDS-PAGE analysis in reducing conditions and Western blotting with rabbit polyclonal antiserum raised against yeast proteins (Figure 3). The technique is qualitative and does not permit quantitation of the impurities.

A constant band pattern is shown over the three bulk antigen lots prepared by the thiomersal free process and the HEP2055 lot with one exception.

A heavily staining band present at ± 23K in the HEP2055 bulk antigen is virtually absent in the 3 HEF preparations. The Western blotting shows that the thiomersal free purification process results in a purer antigen product.

**Table 2: Results of tests and assays on purified, thiomersal free bulk antigen**

<table>
<thead>
<tr>
<th>TEST</th>
<th>RESULT</th>
<th>HEF001</th>
<th>HEF002</th>
<th>HEF003</th>
<th>HEP2055</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH</td>
<td></td>
<td>6.8</td>
<td>6.8</td>
<td>6.8</td>
<td>6.8</td>
</tr>
<tr>
<td>Protein content by Lowry</td>
<td></td>
<td>1312 μg/ml</td>
<td>888 μg/ml</td>
<td>913 μg/ml</td>
<td>995 μg/ml</td>
</tr>
<tr>
<td>Endotoxin content</td>
<td></td>
<td>&lt; 0.25 EU</td>
<td>&lt; 0.25 EU</td>
<td>&lt; 0.25 EU</td>
<td>&lt; 0.25 EU</td>
</tr>
<tr>
<td>Tween 20 content</td>
<td></td>
<td>7.1 μg</td>
<td>6.6 μg</td>
<td>7.4 μg</td>
<td>5.8 μg</td>
</tr>
<tr>
<td>Antigenic activity by</td>
<td></td>
<td>2957 μg/ml</td>
<td>1505 μg/ml</td>
<td>1486 μg/ml</td>
<td>1128 μg/ml</td>
</tr>
<tr>
<td>TEST</td>
<td>RESULT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------------------------</td>
<td>--------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HEF001</td>
<td>HEF002</td>
<td>HEF003</td>
<td>HEP2055</td>
<td></td>
</tr>
<tr>
<td>ELISA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ELISA / protein ratio</td>
<td>2.25</td>
<td>1.69</td>
<td>1.63</td>
<td>1.13</td>
<td></td>
</tr>
<tr>
<td>Polysaccharide content</td>
<td>0.33 µg</td>
<td>0.35 µg</td>
<td>0.33 µg</td>
<td>0.34 µg</td>
<td></td>
</tr>
<tr>
<td>Lipid content</td>
<td>13.7 µg</td>
<td>12.8 µg</td>
<td>12.9 µg</td>
<td>11.8 µg</td>
<td></td>
</tr>
<tr>
<td>DNA content by Threshold</td>
<td>&lt; 1 pg</td>
<td>&lt; 1 pg</td>
<td>&lt; 1 pg</td>
<td>&lt; 1 pg</td>
<td></td>
</tr>
</tbody>
</table>

2.1.4 Other biochemical tests and assays

2.1.4.1 DNA content

The DNA content of the 3 bulk antigen lots was measured by the Threshold method (Molecular Devices Corp). The amounts measured were less than 10 pg DNA per 20 µg protein (Table 2); the same level of DNA content seen with bulk antigen produced by the current approved process.

2.1.4.2 Amino acid composition

The amino acid composition of the three HEF bulk antigen lots was determined after acid hydrolysis with 6N HCl by chromatography of the amino acids on an ion exchange column with post column ninhydrin detection. Proline and tryptophan were not determined. The results are given in Table 3.

The compositions found are in good agreement with that determined on HEP2055 and with the expected composition derived from the DNA sequence. Although the number of glycine residues measured for HEP2055 is close to the expected composition, a value of 16 to 17 residues is more usually measured for bulk antigen preparations. The mean number of cysteine residues found is the expected 14, showing that no extra cysteines are bound to the particle as a result of the treatment at the CsCl gradient step.

2.1.4.3 Quantification of free cysteine

The quantity of free cysteine present in bulk antigen preparations obtained according to the method described was measured after oxidation of the particles with performic acid without prior acid hydrolysis. Oxidised free cysteine residues were separated on an ion exchange column with post column detection by ninhydrin. The limit of detection of cysteine by this method is 1 µg per ml.

No free cysteine could be measured in the 3 HEF antigen preparations when tested at the initial protein concentrations given in Table 2.

The technique measures both free cysteine residues present in the buffer and cysteine residues which are attached to the HBsAg protein by disulphide bonding but which do not form part of the polypeptide sequence.
2.1.4.4 N-terminal sequence analysis
The presence of possible protein contaminants and degradation products in the three bulk antigen lots produced by the modified process was assessed by N-terminal sequence analysis based on Edman degradation. The N-terminal sequence MENITS... of the HBsAg protein was detected with no interference from other sequences. The N-terminal methionine was also confirmed to be 60-75 %blocked by acetylation, as observed previously for HBsAg polypeptide produced by the routine process.

Table 3: Amino acid composition of HBsAg

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>HEF001</th>
<th>HEF002</th>
<th>HEF003</th>
<th>Mean comp.</th>
<th>HEP2055</th>
<th>Expected comp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>11.3</td>
<td>11.3</td>
<td>11.3</td>
<td>11.3</td>
<td>11.5</td>
<td>10</td>
</tr>
<tr>
<td>Thr</td>
<td>17.5</td>
<td>17.4</td>
<td>17.2</td>
<td>17.4</td>
<td>17.8</td>
<td>17</td>
</tr>
<tr>
<td>Ser</td>
<td>21.4</td>
<td>21.6</td>
<td>21.4</td>
<td>21.5</td>
<td>20.9</td>
<td>23</td>
</tr>
<tr>
<td>Glu</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>11.0</td>
<td>10.5</td>
<td>9</td>
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<tr>
<td>Pro</td>
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<td>nd</td>
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<tr>
<td>Gly</td>
<td>17.1</td>
<td>16.8</td>
<td>16.7</td>
<td>16.9</td>
<td>14.6</td>
<td>14</td>
</tr>
<tr>
<td>Ala</td>
<td>7.5</td>
<td>7.4</td>
<td>7.4</td>
<td>7.4</td>
<td>7.2</td>
<td>6</td>
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<tr>
<td>Cys</td>
<td>12.3</td>
<td>14.95</td>
<td>14.9</td>
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<td>14</td>
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<tr>
<td>Val</td>
<td>10.9</td>
<td>11</td>
<td>10.9</td>
<td>10.9</td>
<td>10.7</td>
<td>11</td>
</tr>
<tr>
<td>Met</td>
<td>6.8</td>
<td>6.7</td>
<td>7.1</td>
<td>6.9</td>
<td>7.1</td>
<td>6</td>
</tr>
<tr>
<td>Ile</td>
<td>12.3</td>
<td>12.4</td>
<td>12.5</td>
<td>12.4</td>
<td>12.2</td>
<td>16</td>
</tr>
<tr>
<td>Leu</td>
<td>26.3</td>
<td>26.6</td>
<td>26.2</td>
<td>26.4</td>
<td>26.7</td>
<td>33</td>
</tr>
<tr>
<td>Tyr</td>
<td>6.8</td>
<td>6.8</td>
<td>6.8</td>
<td>6.8</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Phe</td>
<td>13.8</td>
<td>13.9</td>
<td>13.8</td>
<td>13.8</td>
<td>13.9</td>
<td>15</td>
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<tr>
<td>His</td>
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<td>2.8</td>
<td>3.3</td>
<td>3.0</td>
<td>3.3</td>
<td>1</td>
</tr>
<tr>
<td>Lys</td>
<td>4</td>
<td>4</td>
<td>3.9</td>
<td>4.0</td>
<td>4.2</td>
<td>3</td>
</tr>
<tr>
<td>Arg</td>
<td>5.7</td>
<td>5.8</td>
<td>5.7</td>
<td>5.7</td>
<td>6.1</td>
<td>5</td>
</tr>
<tr>
<td>Trp</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>13</td>
</tr>
</tbody>
</table>

2.1.4.5 Laser light scattering analysis
Particle size comparisons were made by laser light scattering between the HBsAg particles produced using the modified process and the HEP2055 reference lot (Table 4). The mean molecular weights determined show good consistency between the preparations.

Table 4: HBsAg particle molecular weights by laser light scattering

<table>
<thead>
<tr>
<th>Antigen lot</th>
<th>MW (Daltons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEF001</td>
<td>3.07 x 10^6</td>
</tr>
<tr>
<td>HEF002</td>
<td>2.76 x 10^6</td>
</tr>
</tbody>
</table>
HEF003  $2.76 \times 10^6$
HEP2055  $3.34 \times 10^8$

2.1.4.6 Electron microscopy

The bulk antigen preparations were examined by electron microscopy after fixation and staining with uranyl acetate.

The particles observed were similar in all the samples and conformed to the ± 20 nm subspherical or cobblestone-like particles typical of HBsAg. The particles observed in the 3 HEF lots were indistinguishable from HEP2055.

2.1.5 Immunological analyses

2.1.5.1 Reactivity with RF1 monoclonal antibody

The three bulk antigen preparations were tested for their reactivity with the RF1 monoclonal antibody by ELISA inhibition assay. The RF1 monoclonal antibody has been shown to protect chimpanzees against challenge with HBV and is considered to recognize a protective conformational epitope on the HBsAg particle (Waters JA, 1987, J.Post Grad.Medical, 63: 51-56; Iwarson S et al, 1985, J.Med. Virol., 16: 89-96).

The RF1 hybridoma may be propagated in the peritoneal cavity of BalbC mice or in tissue culture.

Ascitic fluid diluted at 1/50000 in saturation buffer (PBS containing 1% BSA, 0.1% Tween 20) was mixed 1:1 with various dilutions in PBS of the HBsAg samples to be tested (final concentrations ranging between 100 µg and 0.05µg/ml).

Mixtures were incubated in Nunc Immunoplates (96U) for 1 hr at 37°C before being transferred for 1 hr at 37°C onto plates coated with a standard preparation of HBsAg. The standard HBsAg preparation was a lot of bulk antigen (Hep 286) purified by the thiomersal containing process. After a washing step with PBS containing 0.1% Tween 20, biotin-conjugated sheep anti-mouse IgG diluted 1/1000 in saturation buffer was added to and incubated for 1 hr at 37°C. After a washing step, streptavidin-biotinylated peroxidase complex diluted 1/1000 in saturation buffer was added to the same wells and incubated for 30 min at 37°C. Plates were washed and incubated with a solution of OPDA 0.04%, H$_2$O$_2$ 0.03% in 0.1M citrate buffer pH 4.5 for 20 min at room temperature. The reaction was stopped with 2N H$_2$SO$_4$ and the optical densities (O.D.) were measured at 490/630 nm and plotted graphically.

The IC50, defined as the concentration of antigen (inhibitor concentration) that inhibits 50% of the antibody binding to coated HBsAg was calculated using a 4 parameters equation and expressed in ng/ml.
A series of HEP antigen lots including HEP2055 were also tested, together with the Herpes simplex gD antigen as negative control. The assay measures the ability of each test antigen to inhibit binding of RF1 to a standard antigen preparation (HEP286) bound to microtitre plates.

Table 5 gives the concentrations of each antigen found to inhibit 50% of RF1 binding to the fixed antigen.

**Table 5: Inhibition of binding of RF1 monoclonal antibody to HbsAg**

<table>
<thead>
<tr>
<th>Bulk antigen</th>
<th>IC50 (ng/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEP286</td>
<td>3834</td>
</tr>
<tr>
<td>HEP673</td>
<td>3437</td>
</tr>
<tr>
<td>HEP720</td>
<td>3150</td>
</tr>
<tr>
<td>HEP2055</td>
<td>2384</td>
</tr>
<tr>
<td>HEF001</td>
<td>468</td>
</tr>
<tr>
<td>HEF002</td>
<td>574</td>
</tr>
<tr>
<td>HEF003</td>
<td>540</td>
</tr>
</tbody>
</table>

*IC50 = antigen concentration (ng/ml) inhibiting 50% of RF1 binding to fixed antigen

The results show that 4 to 7 fold less HEF antigen is required to inhibit RF1 binding (Table 5). This shows that antigen prepared by the modified process has an increased presentation of the RF1 epitope compared to HEP bulk antigen.

The same type of inhibition assay was performed using human sera from Engerix B™ vaccinees instead of the RF1 mAb and did not reveal differences between the HEP antigen lots and the HEF antigens.

2.1.5.2. Affinity of binding to monoclonal RF1

The kinetic parameters of RF1 monoclonal antibody binding to the 3 HEF antigen lots and to HEP2055 were measured by surface plasmon resonance using a Biacore 2000 apparatus from Amersham Pharmacia Biotech, Amersham Place, Little Chalfont, Bucks, UK.

The kinetic parameters measured were:

- $k_a$: the association rate constant (M$^{-1}$S$^{-1}$)
- $k_d$: the dissociation rate constant (S$^{-1}$)
- $K_a$: the equilibrium or affinity constant (M$^{-1}$)

\[
\frac{k_a}{k_d}
\]
where $K_a =$

The values found are given in Table 6.

<table>
<thead>
<tr>
<th>Bulk antigen</th>
<th>$k_a$ (x 10$^{-3}$)</th>
<th>$k_d$ (x 10$^5$)</th>
<th>$K_a$ (x 10$^{-7}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEF001</td>
<td>6.81</td>
<td>3.21</td>
<td>21.97</td>
</tr>
<tr>
<td>HEF002</td>
<td>6.89</td>
<td>3.73</td>
<td>18.83</td>
</tr>
<tr>
<td>HEF003</td>
<td>7.39</td>
<td>4.67</td>
<td>15.80</td>
</tr>
<tr>
<td>HEP2055</td>
<td>3.31</td>
<td>6.30</td>
<td>5.31</td>
</tr>
</tbody>
</table>

The three HEF antigen lots gave similar association / dissociation constants and binding affinity values. In contrast HEP2055 has a weaker affinity for binding to RF1. This is consistent with the results from the ELISA inhibition assay which showed that antigen prepared by the thiomersal free process had an increased presentation of the RF1 epitope.

2.2. Test and assays on vaccine formulated with antigen produced by the modified process

The three HEF antigen lots were adsorbed onto aluminium hydroxide and formulated as vaccine according to the composition as shown in Table 1. The presentation is the adult dose in vials (20 µg antigen protein in 1ml). The lots are identified as DENS001A4, DENS002A4 and DENS003A4.

Vaccine potency was measured by an in-vitro antigen content assay using the Abbott Laboratories AUSZYME ELISA kit and a classical lot of vaccine formulated with 50 µg/ml thiomersal as standard. Vaccine potency was measured using method B as described in PharmaEuropa Special Issue Bio97-2 (December 1997). The three HEF lots give high values for antigen content, nearly twice the stated content of 20 µg antigen protein.

2.2.1 Reactivity of thiomersal free "DENS" vaccine with RF1 monoclonal antibody

The antigenicity of the adsorbed vaccine was further tested in an inhibition assay with RF1 monoclonal antibody. The assay measures the ability of the vaccine sample to inhibit RF1 binding to fixed bulk antigen (HEP286).

Ascitic fluid diluted at 1/50000 in saturation buffer (PBS containing 1% BSA, 0.1% Tween 20) was mixed 1:1 with various dilutions in PBS of the vaccine samples to be tested (concentration ranging between 20µg and 0.05µg/ml).
Mixtures were incubated in Nunc Immunoplates (96U) for 2 hr at 37°C with agitation before being transferred onto HBsAg coated plates. The HBsAg preparation used for coating was a lot of bulk antigen (Hep 286) purified by the thioesal containing process. These plates are then incubated for 2 hr at 37°C with agitation. After a washing step with PBS containing 0.1% Tween 20, biotin-conjugated sheep anti-mouse IgG diluted 1/1000 in saturation buffer was added and incubated for 1 hr at 37°C. After a washing step, streptavidin-biotinylated peroxidase complex diluted 1/1000 in saturation buffer was added to the wells and incubated for 30 min at 37°C. Plates were washed and incubated for 20 min at room temperature with a solution containing OPDA 0.04%, H₂O₂ 0.03% in 0.1M citrate buffer pH 4.5. The reaction was stopped with 2N H₂SO₄ and optical densities (O.D.) were measured at 490/630 nm and plotted graphically.

The IC₅₀, defined as the concentration of antigen (inhibitor concentration) that inhibits 50% of the antibody binding to coated HBsAg was calculated using a 4 parameters equation and expressed in ng/ml.

Vaccine prepared from bulk antigen produced by the modified process was compared to Engerix B™ vaccine formulated from classical HEP bulk antigen and without thiomersal as preservative.

The assays were run in triplicate.

The results are given in Table 7 and show that about half the quantity of DENS vaccine is required to achieve 50% inhibition of RF1 binding as compared to preservative free Engerix B™ vaccine. This reflects an increased presentation of the RF1 epitope on the HEF/DENS antigen and is consistent with the tests done with RF1 antibody on the purified bulk antigen.

<table>
<thead>
<tr>
<th>Vaccine lot</th>
<th>IC-50 (ng/ml) (1)</th>
<th></th>
<th></th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>DENS001A4</td>
<td>913</td>
<td>662</td>
<td>603</td>
<td>726</td>
</tr>
<tr>
<td>DENS002A4</td>
<td>888</td>
<td>715</td>
<td>521</td>
<td>708</td>
</tr>
<tr>
<td>DENS003A4</td>
<td>817</td>
<td>685</td>
<td>582</td>
<td>695</td>
</tr>
<tr>
<td>ENG5100A2</td>
<td>1606</td>
<td>1514</td>
<td>1481</td>
<td>1534</td>
</tr>
<tr>
<td>ENG3199B9</td>
<td>1329</td>
<td>1170</td>
<td>1286</td>
<td>1262</td>
</tr>
<tr>
<td>ENG3328A9</td>
<td>1417</td>
<td>1194</td>
<td>1334</td>
<td>1315</td>
</tr>
</tbody>
</table>

(1) concentration of vaccine inhibiting 50% of RF1 antibody binding to fixed antigen
2.2.2 Immunogenicity of DENS vaccine in mice

A study was performed in Balb/C mice in order to compare the immunogenicity of the three DENS consistency lots to Engerix B™ produced according to the current antigen manufacturing process and formulated with thiomersal.

The following lots were tested:

- # DENS001A4
- # DENS002A4
- # DENS003A4
- # ENG2953A4/Q as reference

Briefly, groups of 12 mice were immunised intramuscularly twice at 2 weeks interval with vaccine doses corresponding to 1/10 (2 µg) or 1/50 (0.4 µg) of the adult human dose. Antibody response to HBsAg and the isotypic profile induced by vaccination were monitored from sera taken at day 28.

**EXPERIMENTAL DESIGN**

Groups of 12 Balb/C mice were immunised intramuscularly in both legs (2x50µl) on days 0 and 15 with the following vaccine doses:

**Table 8: Groups and vaccine dose**

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccine</th>
<th>Volume</th>
<th>Antigen dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DENS001A4</td>
<td>100µl</td>
<td>2µg</td>
</tr>
<tr>
<td>2</td>
<td>Diluted 5X in PO4/NaCl</td>
<td>100µl</td>
<td>0.4µg</td>
</tr>
<tr>
<td>3</td>
<td>DENS002A4</td>
<td>100µl</td>
<td>2µg</td>
</tr>
<tr>
<td>4</td>
<td>Diluted 5X in PO4/NaCl</td>
<td>100µl</td>
<td>0.4µg</td>
</tr>
<tr>
<td>5</td>
<td>DENS003A4</td>
<td>100µl</td>
<td>2µg</td>
</tr>
<tr>
<td>6</td>
<td>Diluted 5X in PO4/NaCl</td>
<td>100µl</td>
<td>0.4µg</td>
</tr>
<tr>
<td>7</td>
<td>ENG2953A4/Q</td>
<td>100µl</td>
<td>2µg</td>
</tr>
<tr>
<td>8</td>
<td>Diluted 5X in PO4/NaCl</td>
<td>100µl</td>
<td>0.4µg</td>
</tr>
</tbody>
</table>

On days 15 (2 weeks post I) and 28 (2 weeks post II) blood was taken from the retroorbital sinus.

For the design of this experiment (4 formulations x 2 doses with 12 mice per group), the power was estimated a priori with the PASS statistical program. The PASS (Power and Sample Size) statistical programme was obtained from NCSS, 329 North 1000 East, Kaysville, Utah 84037. For the 2 way analysis of variance, a 2.5 fold difference of GMT between formulations with an alpha error of 5% should be detected with a power > 90%.
RESULTS

Serology:
Humoral responses (Total Ig and isotypes) were measured by ELISA assay using HBsAg (Hep286) as coating antigen and biotin conjugated anti-mouse antibodies to reveal anti-HBs antibody binding. Only post II sera were analysed.

Table 9 shows the mean and GMT anti-HBs Ig antibody responses measured on individual sera at 2 weeks post II.

Comparable antibody responses are induced by the DENS and classical hepatitis B formulations: GMT ranging between 2304 and 3976 EU/ml for the DENS lots compared to 2882 EU/ml for SB Biologicals hepatitis B monovalent vaccine (Engerix B™) at the 2 µg dose, and GMT ranging between 696 and 1182 EU/ml for the DENS lots compared to 627 EU/ml for SB Biologicals hepatitis B monovalent vaccine (Engerix B™) at the 0.4 µg dose.

- As expected a clear antigen dose range effect is observed for all formulations at the 2 µg and 0.4 µg doses with a 3 to 6 fold difference in GMTs.
- Four non responder mice (titers < 50EU/ml) were observed without clear links to the antigen doses or lots used for the injection (Groups 1, 2, 3 and 8; one mouse per group). Based on statistical analysis (Grubbs Test) these mice were discarded from further analysis.

Table 9  Antibody response in mice at day 28 (2 weeks post II)

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccine</th>
<th>Dose</th>
<th>Number</th>
<th>ELISA TITERS (Ig)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>1</td>
<td>DENS001A4</td>
<td>2 µg</td>
<td>11</td>
<td>3466</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0.4 µg</td>
<td>11</td>
<td>1283</td>
</tr>
<tr>
<td>3</td>
<td>DENS002A4</td>
<td>2 µg</td>
<td>11</td>
<td>2436</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>0.4 µg</td>
<td>12</td>
<td>984</td>
</tr>
<tr>
<td>5</td>
<td>DENS003A4</td>
<td>2 µg</td>
<td>12</td>
<td>4583</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>0.4 µg</td>
<td>12</td>
<td>997</td>
</tr>
<tr>
<td>7</td>
<td>ENG2953A4/Q</td>
<td>2 µg</td>
<td>12</td>
<td>3999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.4 µg</td>
<td>11</td>
<td>737</td>
</tr>
</tbody>
</table>

Statistical analysis:
A 2 way-analysis of variance was performed on the anti-HBs titers after log transformation of post II data, using the vaccines (4 lots) and antigen doses (2µg and 0.4µg) as factors. This analysis confirmed that a statistically significant difference was observed between the two antigen doses (p value < 0.001) and did not show any significant difference between the vaccine lots (p value = 0.2674). As previously mentioned the power was
estimated a priori and the design of the experiment was such that a 2.5 fold difference of GMT with a alpha error of 5% could be detected between formulations with a power >90%.

5

**Isotypic profile:**
Table 10 shows the isotypic repartition (IgG1, IgG2a and IgG2b) calculated from an analysis on pooled sera at post II.

- As expected, a clear TH2 response is induced by these alum based vaccines as mainly IgG1 antibodies are observed.
No difference is observed between the DENS lots or SB Biologicals hepatitis B monovalent vaccine in term of isotypic profile.

15

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccine</th>
<th>Dose</th>
<th>IgG1</th>
<th>IgG2a</th>
<th>IgG2b</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DENS001A4</td>
<td>2 μg</td>
<td>91</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.4 μg</td>
<td>87</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>DENS002A4</td>
<td>2 μg</td>
<td>97</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.4 μg</td>
<td>87</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>DENS003A4</td>
<td>2 μg</td>
<td>98</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.4 μg</td>
<td>93</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>ENG2953A4/Q</td>
<td>2 μg</td>
<td>88</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.4 μg</td>
<td>88</td>
<td>9</td>
<td>3</td>
</tr>
</tbody>
</table>

**Table 10** Repartition of IgG isotypes in pooled day 28 sera

**Example 3:**

**Formulation of combined vaccines**

The bulk antigen of the invention is particularly suitable for formulation in a combined vaccine comprising IPV.

25 Stability studies performed on initial lots of a combined DTPa-HBV-IPV vaccine indicated a decline in potency of the IPV component, particularly of type 1 poliomyelitis antigen, when using an in vitro immunoassay (determination of D-antigen content by ELISA) and an in vivo rat potency test. No potency loss was observed for type 3. For type 2, the potency loss was within the expected range (not more than 10 % loss per year of storage).
Studies were initiated to determine the cause of this loss of potency in the combined DTPa-HBV-IPV vaccine. From the observation that the stability of IPV in SB Biologicals' DTPa-IPV vaccine is satisfactory (not more than 10% antigen content loss per year of storage), it was concluded that the HBV component was likely to be responsible for the instability of IPV in the DTPa-HBV-IPV vaccine.

The HBV component used in the initial DTPa-HBV-IPV formulation is the purified r-DNA, yeast-derived HBsAg also used for the manufacture of SB Biologicals hepatitis B monovalent vaccine and prepared as described in Example 1.

As a first attempt to determine which element in the HBV component was deleterious to IPV, the HBsAg bulk was analysed for the presence of thiomersal. It has been previously found (Davisson et al., 1956, J. Lab. Clin. Med 47: 8-19) that thiomersal used as preservative in DTP vaccines "was detrimental to the poliomyelitis virus" in a DTP-IPV combination. This observation was considered by vaccine manufacturers who have replaced thiomersal with other preservatives to formulate their IPV-containing vaccines. More recently, the effect of thiomersal on IPV potency under conditions of long-term storage at +4 °C was reinvestigated. The loss of potency of type 1 polio virus antigen to undetectable levels after 4-6 months was reported (Sawyer, L.A. et al. 1994, Vaccine 12: 851-856).

Using atomic adsorption spectroscopy, approximately 0.5µg of mercury (Hg) per 20µg of HBsAg was detected in antigen purified according to Example 1.

This amount of mercury (as thiomersal and ethylmercury chloride, the thiomersal degradation product) can reduce to undetectable levels the ELISA response for D-antigen type 1 content in an IPV bulk concentrate incubated at 37°C for 7 days.

A method was established to release mercury present in the HBsAg bulk. It was postulated that mercury could be bound to thiol groups on the HBsAg particle and could therefore be released in the presence of reducing agents. After experimentation with other reducing agents, L-Cysteine was selected as the agent for release of mercury from the HBsAg particle. After dialysis of HBsAg bulk against saline solution containing 5.7mM L-Cysteine, no mercury was detected in the retentate (detection limit of the testing method: 25ng Hg/20µg HBsAg). The dialysed antigen was mixed with IPV bulk concentrate and the stability of the type 1 virus was assessed by measuring the D-antigen content after incubation at 37°C for 7 days. The IPV bulk concentrate non-mixed and mixed with HBsAg not treated with cysteine were used as controls. The reference ELISA titre was obtained on the samples stored at +2 °C to +8 °C for 7 days.

The results are summarised in Table 11:

<table>
<thead>
<tr>
<th>Sample</th>
<th>D-antigen content (type 1)</th>
<th>Loss</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7 days/4 °C</td>
<td>7 days/37 °C</td>
</tr>
</tbody>
</table>

25
<table>
<thead>
<tr>
<th>IPV (non-mixed)</th>
<th>31.6</th>
<th>24.2</th>
<th>23 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPV+HBsAg not treated</td>
<td>31.1</td>
<td>18.1</td>
<td>42 %</td>
</tr>
<tr>
<td>IPV + HBsAg-cysteine-treated</td>
<td>31.4</td>
<td>27.6</td>
<td>12 %</td>
</tr>
<tr>
<td>IPV + thiomersal (1 µg/ml)</td>
<td>30.5</td>
<td>11.0</td>
<td>74 %</td>
</tr>
</tbody>
</table>

(9) expressed in D-antigen units (DU)

The data obtained on these laboratory preparations clearly demonstrate that the stability of the type 1 polio virus is significantly improved if HBsAg is treated with cysteine to remove residual mercury prior to mixing with IPV.

The data presented above also show a loss of D-antigen content of 23 % for the reference IPV preparation after incubation for 7 days at 37 °C. This confirms the inherent instability of the type 1 Mahoney polio virus, as previously reported (Sawyer, L.A. et al. (1994), Vaccine 12: 851-856).

Although commercial lots of DTPa-HBV-IPV and DTPa-HBV-IPV/Hib vaccines have been prepared using a dialysis process with 5.7 mM L-Cysteine to remove residual mercury and preserve the stability of IPV, the dialysis process is not suited to large scale production and involves a series of supplementary steps to prepare thiomersal or mercury free HBsAg.

In contrast, the HBsAg of the present invention, prepared without thiomersal, may be directly used in formulations of combined vaccines especially those containing IPV.

4. **Summary**

The previously used process for purification of yeast-derived surface antigen contains a gel permeation step where the mercury containing anti-microbial compound thiomersal is included in the elution buffer to control bioburden.

The thiomersal is not completely cleared during the subsequent steps of the process so that about 1.2 µg thiomersal per 20 µg protein is present in the purified bulk antigen.

In order to produce a completely thiomersal (mercury) free bulk antigen the purification process has been altered at two steps.

- Thiomersal is omitted from the elution buffer at the 4B gel permeation step.
- Cysteine (2 mM final concentration) is added to the eluate pool from the anion exchange chromatography step. This prevents precipitation of antigen during CsCl density gradient centrifugation.

There are no other changes to the production process.

The bulk antigen produced by the modified process has been characterized. Physico-chemical tests and assays show that the thiomersal free antigen is indistinguishable in its...
properties from antigen produced by the previously used process. The antigen particles have the same constituents.
The identity and integrity of the HBsAg polypeptide is unaffected by the modified process as judged by SDS-PAGE analysis, Western blotting using polyclonal anti-HBsAg antibodies, N-terminal sequence analysis and amino acid composition. Electron microscopy and laser light scattering analysis show that the particles are of the typical form and size expected for yeast-derived HBsAg. Analysis by Western blotting with anti-yeast protein serum shows that the antigen produced by the thiomersal free process has a similar pattern of contaminating yeast proteins. However, the amount of a contaminating band migrating at 23K is greatly reduced in the 3 HBsAg lots produced using the modified process.

Immunological analyses show that the thiomersal free particles have an increased antigenicity.

The particles are more reactive with the Abbott AUSZYME kit (containing a mixture of monoclonal antibodies) giving ELISA/protein ratios of 1.6 to 2.25. This increased antigenicity is also shown with the protective RF1 monoclonal antibody. About 4 to 7 fold less thiomersal free antigen is required to inhibit RF1 binding to a standard fixed antigen. The thiomersal free and classical antigen inhibition of binding curves fall into two distinct families. This difference is also shown by measurements of the binding affinity constant for RF1 using surface plasmon resonance. The binding affinities of the thiomersal free preparations are 3 to 4 fold higher compared to the lot of classical bulk antigen.

The bulk antigen preparations were formulated as vaccine by adsorption onto aluminium hydroxide and without preservative.

Testing for in vitro potency using the Abbott AUSZYME ELISA kit and thiomersal containing SB Biologicals hepatitis B monovalent vaccine as standard showed that high in vitro potency values were obtained. The antigen content measured by this test was nearly double the stated value of 20μg protein per ml.

An increased reactivity of vaccine prepared from thiomersal free antigen was also seen in an inhibition assay with RF1 monoclonal antibody for binding to fixed antigen. About half the quantity of thiomersal free vaccine was required to give 50% inhibition of RF1 binding to fixed antigen as compared to antigen purified by the previously used process and formulated without preservative.

This increased antigenicity of the thiomersal free vaccine with respect to RF1 is consistent with the results from the in vitro potency test (antigen content) and with the RF1 antibody tests performed on the bulk antigen preparations.

A mouse immunogenicity test was performed using priming and booster vaccinations two weeks apart and doses of 2 and 0.4 μg antigen. Mice were bled on day 28, 14 days after the booster. The sera were analysed for antibody titre and isotype composition. A clear antigen dose effect was observed for the two doses administered but there was no statistically significant difference in the response in terms of antibody titres (GMT) between thiomersal free and preservative free vaccines.
No substantial differences were observed in the isotype profiles.

**Example 4**

The objective of this experiment was to evaluate the immunogenicity of HBs thio free based formulations delivered intradermally (ID) in rats. The experiment was designed to investigate the feasibility and immunogenicity of a dose range of HBs/ formulated with a constant quantity of Al(OH)₃ administered ID (using regular needles) compared to intramuscular (IM) administration.

The humoral response (anti-HBs Ig titers) was analysed after each immunisation, while the cellular responses (proliferation and cytokines production) were only investigated post III.

**EXPERIMENTAL DESIGN**

<table>
<thead>
<tr>
<th>Group</th>
<th>Antigen(s) + dosage</th>
<th>Formulation</th>
<th>Delivery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HBs 20μg/Al(OH)₃ 50μg</td>
<td>HBs thio free</td>
<td>ID</td>
</tr>
<tr>
<td>2</td>
<td>HBs 2μg/Al(OH)₃ 50μg</td>
<td>HBs thio free</td>
<td>ID</td>
</tr>
<tr>
<td>3</td>
<td>HBs 0.2μg/Al(OH)₃ 50μg</td>
<td>HBs thio free</td>
<td>ID</td>
</tr>
<tr>
<td>4</td>
<td>HBs 20μg/Al(OH)₃ 50μg</td>
<td>HBs thio free</td>
<td>IM</td>
</tr>
<tr>
<td>5</td>
<td>HBs 2μg/Al(OH)₃ 50μg</td>
<td>HBs thio free</td>
<td>IM</td>
</tr>
<tr>
<td>6</td>
<td>HBs 0.2μg/Al(OH)₃ 50μg</td>
<td>HBs thio free</td>
<td>IM</td>
</tr>
<tr>
<td>7</td>
<td>HBs 0.2μg/Al(OH)₃ 50μg</td>
<td>HBs classical</td>
<td>IM</td>
</tr>
</tbody>
</table>

Inbred Lewis rats were used in groups of 10 animals. The Immunisation schedule was 0, 14, 28 days.

The following readouts were taken:

<table>
<thead>
<tr>
<th>Readout</th>
<th>Timepoint</th>
<th>Sample-type</th>
<th>v/f</th>
<th>Analysis method</th>
</tr>
</thead>
</table>
Anti-HBs  14 Post I  serum  Po  Elisa

5  Anti-HBs  14 Post II  serum  In  Elisa

CMI  14 Post III  spleens  Po  Production of Cytokines in vitro (CMI) + proliferation

10  Anti-HBs  14 Post III  serum  In  ELISA

In= Individual / Po= Pool

15  Sample analysis

Anti-HBs serology (Ig response)

Quantitation of antibody was performed by ELISA using HBs (Hep 800 as coating antigen). Antigen and antibody solutions were used at 50 µl per well. Antigen was diluted to a final concentration of 1 µg/ml in PBS and was adsorbed overnight at 4°C onto the wells of 96 well microtiter plates (Maxisorb Immuno-plate, Nunc, Denmark). The plates were then incubated for 1 hr at 37°C with PBS containing 1% bovine serum albumin and 0.1% Tween 20 (saturation buffer). Sera dilution performed in the saturation buffer were added to the coated plates and incubated for 1 hr 30 min at 37°C. The plates were washed four times with PBS 0.1% Tween 20 and biotin-conjugated anti-mouse Ig (from Prozan) diluted 1/10000 in saturation buffer was added to each well and incubated for 1 hr 30 min at 37°C. After a washing step, avidin-horseradish peroxidase complex (Dako, UK) diluted 1/10000 in saturation buffer was added for an additional 30 min at 37°C.

Plates were washed as above and incubated for 20 min with a solution of o-phenylenediamine (Sigma) 0.04% H2O2 0.03% in 0.1% Tween 20 0.05M citrate buffer pH 4.5. The reaction was stopped with H2SO4 2N and read at 490/630 nm. ELISA titers were calculated from a reference by SoftmaxPro (using a four parameters equation) and expressed in EU/ml.

Cytokine production

Two weeks after the third immunisation, rats were sacrificed, spleens were removed aseptically and pooled (2 pool of 4 organs per group). Cell suspensions were prepared in RPMI 1640 medium (GIBCO) containing additives and 5% foetal calf serum. Cells were cultured at a final concentration of 5x10^6 cells/ml, in 1ml per flat-bottomed 24 wells-plates with different concentrations (10-0.1 µg/ml) of HBs antigen (Hepcyts 626). Supernatants were harvested 72 hrs later and frozen until tested for the presence of IFN_ and IL4 using commercial kits (OptEIA kit from Becton Dickinson).
Results

The results are shown in Figures 4-6.

Conclusions

Comparable antibody responses were achieved in post III by all HBs /Al(OH)3 formulations administered by IM and ID routes.

ID vaccination with 0.2μg HBs induced superior titers (2.2 fold) post II compared to IM vaccination.

Regarding the cellular response, groups vaccinated with highest antigen dose (20μg HBs) for example produced similar levels of IFN gamma in vitro.
CLAIMS

1. The use of a hepatitis B antigen free or substantially free of thiomersal, in the
   manufacture of a prophylactic or therapeutic hepatitis B vaccine for intradermal delivery.

2. The use according to claim 1 wherein the hepatitis B antigen is purified in the
   presence of a reducing agent having a free -SH group.

3. The use according to claim 1 or claim 2 wherein the vaccine comprises less than
   0.025 µg mercury per 20 µg hepatitis B antigen.

4. The use according to any of claims 1 to 3 wherein the hepatitis B antigen is
   prepared by subjecting crude hepatitis B antigen preparation to the following steps:
      (a) gel permeation chromatography;
      (b) ion–exchange chromatography; and
      (c) mixing with a reducing agent having a free –SH group

5. The use according to any preceding claim wherein the reducing agent is
   cysteine, glutathione, dithiothreitol or β-mercaptoethanol

6. The use according to claim 5 wherein the reducing agent is cysteine.

7. The use according to claim 6 wherein the cysteine is added to a final concentration
   of between 1 – 10mM.

8. The use according to claim 7 wherein the cysteine is added to a final concentration
   of about 2mM.

10. The use according to any of claims 1 to 9 wherein the hepatitis B antigen is
    purified in the absence of thiomersal.

11. The use according to any of claims 1 to 9 wherein the antigen is purified in the
    presence of thiomersal before treatment with the reducing agent.

12. A hepatitis B vaccine composition substantially free of thiomersal, in a dose
    volume suitable for intradermal delivery.

13. A hepatitis B vaccine composition according to claim 12 wherein the dose volume
    is 0.2 ml or less.

14. A hepatitis B vaccine composition according to claim 12 or claim 13 wherein the
    amount of hepatitis B antigen per dose is equal to the amount for a conventional hepatitis
    B vaccine, or less than or equal to half the amount for a conventional hepatitis B vaccine.
15. A hepatitis B vaccine according to claim 14 which comprises 5 μg or less of hepatitis B antigen for infants, children and adolescents and 10 μg or less for adults.

16. The use or vaccine according to any preceeding claim wherein the vaccine also comprises one or more of the antigens selected from the group consisting of: diphtheria toxoid (D), tetanus toxoid (T) acellular pertussis antigens (Pa), inactivated polio virus (IPV), haemophilus influenzae antigen (Hib), hepatitis A antigen, herpes simplex virus (HSV), chlamydia, GSB, HPV, streptococcus pneumoniae and neisseria antigens.

17. The use or vaccine according to any preceeding claim wherein the hepatitis B antigen is a surface antigen.

18. The use or vaccine according to claim 17 or claim wherein the vaccine is a combined hepatitis B plus hepatitis A vaccine.

19. The use or vaccine according to claim 18 wherein the vaccine is for administration to human subjects in a two dose schedule.

20. The use or vaccine according to claim 18 or 19 wherein the hepatitis A antigen is derived from the HM 175 strain.

21. The use or vaccine according to any preceeding claim wherein the vaccine further comprises an adjuvant.

22. The use or vaccine according to claim 20 wherein the vaccine comprises an aluminium salt.

23. The use or vaccine according to claim 21 wherein the hepatitis B antigen is adsorbed to aluminium hydroxide or aluminium phosphate.

24. The use of a hepatitis B antigen and a cysteine solution in the manufacture of an intradermal vaccine for the prophylaxis or therapy of hepatitis B virus infection.

25. A pharmaceutical kit comprising an intradermal administration device and a hepatitis B vaccine formulation substantially free of thiomersal.

26. The kit according to claim 25 wherein the hepatitis B vaccine is obtained from a method involving purification of antigen in the presence of a reducing agent having a free –SH group such as cysteine.

27. The kit according to claim 26 wherein the volume of a dose of vaccine is 0.05 to 0.2 ml.

28. The kit according to any of claims 25 to 27 wherein the amount of antigen is at a low dose compared to conventional vaccines.
Figure 1: Flow diagram of the thiomersal free production process for Engerix B™

- Fermentation
  - Yeast culture
  - Centrifugation
- Harvest
  - Concentrated yeast
  - Grinding in Bead Mill
  - Homogenate
- Extraction
  - PEG precipitation
  - Centrifugation
  - Supernatant
- Extraction
  - CsCl precipitation
  - Centrifugation
  - Supernatant
  - Ultrafiltration
  - Ultrafiltrate
  - 4B Gel permeation chromatography
- Purification
  - Eluate
  - Anion exchange chromatography
  - Eluate
  - Ultracentrifugation
  - Pooled fractions
  - Desalting gel permeation chromatography
  - Eluate
  - Sterile filtration
- formulation
Figure 2: SDS-PAGE analysis of bulk antigen lots: silver staining; 1 μg protein per sample.

Lane 1: HEF 001, reducing conditions
Lane 2: HEF002, reducing conditions
Lane 3: HEF003, reducing conditions
Lane 4: HEP2005, reducing conditions
Lane 5: Molecular weight markers
Lane 6: HEF001, non reduced
Lane 7: HEF002, non reduced
Lane 8: HEF003, non reduced
Lane 9: HEP2055, non reduced

The positions of the molecular weight markers are indicated by the black dots: 92500, 66200, 45000, 31000, 21500, 14400.
Figure 3: Residual yeast proteins in bulk antigen lots produced by the thiomersal free process: Western blotting with rabbit anti-yeast protein serum

Lane 1: Molecular weight markers (precoloured)
Lane 2: Low molecular weight markers (biotinylated)
Lane 3: HEF001, 20 mcg protein
Lane 4: HEF002, 20 mcg protein
Lane 5: HEF003, 20 mcg protein
Lane 6: HEP2055, 20 mcg protein

The positions of the low molecular weight markers are indicated by black dots: 92500, 66200, 45000, 31000, 21500, 14400.
Figure 4 Anti-HBs Ig antibody response 14 days post II
Figure 5 Anti-HBs Ig antibody response 14 days post III
Figure 6  IFN-gamma production 14 days post III