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PATENT SPECIFICATION

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(72) Inventors ALEXANDER URSZU BERTLAND II
ALFRED A. TYTELL
GEORGE PETER LAMPSON and
EUGENE BERNARD BUYNAK

(54) HEPATITIS B ANTIGEN AND METHOD OF PRODUCING IT

(71) We, MERCK & CO INC., a corporation duly organized and existing under the laws of the State of New Jersey, United States of America, of Rahway, New Jersey, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to hepatitis B antigen.

Hepatitis B is one of two types of viral hepatitis which results in a systemic infection with the principal pathologic changes occurring in the liver. This disease affects mainly adults and is maintained chiefly by transfer of infection from long term carriers of the virus. Usual methods of spread are by blood transfusion, contaminated needles and syringes, through skin breached by cuts or scratches, by unsterilized dental instruments, by saliva, by venereal contact or by exposure to aerosolized infected blood.

The incubation period of type B hepatitis is relatively long: from 6 weeks to 6 months may elapse between infection and the onset of clinical symptoms. The illness usually begins with fatigue and anorexia, sometimes accompanied by myalgia and abdominal discomfort. Later jaundice, dark urine, light stools and tender hepatomegaly may appear. In some cases, the onset may be rapid, with appearance of jaundice early in association with fever, chills and leukocytosis. In other cases, jaundice may never be recognized and the patient may be aware only of a "flu-like" illness. It is estimated that the majority of hepatitis infections result in a mild, anicteric illness.

Although qualitatively similar to viral hepatitis A, the disease is readily diagnosed by the appearance of the Australia antigen particles, now designated by HB_sAg (surface antigen), in the blood or other clinical specimens (saliva, urine, bile, faeces). There occurs in the blood of infected individuals a relatively large population (10^{14}—10^{15}/ml.) of spherical particles. The particles are 18—22 nm in diameter and have the same antigenic determinants as the surface of the 42 nm Dane particle which may be the virus of hepatitis B, now designated HBV.

U.S. Patent 3,735,004 indicates that boiled infectious serum was used to vaccinate children with resulting antibody to HB_sAg (anti-HB_s) and that such serum provided protection against challenge. The dose of boiled serum used was equivalent to about 1×10^{12} HB_s particles. The recipients of such serum did not develop clinical or biochemical disease. This serum, however, is not suitable for use as a vaccine, because of its impure nature, its lack of reproducibility and its non-quantitation.

In accordance with the present invention, there is provided a method of purifying hepatitis B antigen from a partially purified concentrate obtained by cooling plasma obtained from donors, removing the resulting cryo-precipitate by light centrifugation and concentrating the remaining clarified plasma, which contains hepatitis B antigen, comprising treating the concentrate with a quantity of pepsin effective to digest proteinaceous matter at a pH within the range wherein pepsin is enzymatically active, treating the concentrate with a quantity of urea effective to dissociate proteinaceous matter, and removing pepsin, pepsin degradation products and urea, and optionally adding formaldehyde in a concentration effective to inactivate viruses.

The resulting product, which constitutes another embodiment of the present invention, is highly purified, reproducible, characterizable hepatitis B antigen separated from extraneous undesirable proteins and/or antigen, is useful as a vaccine. The product of the present invention has a particle size diameter of from about 18 to about 22 nm, an $E_{278nm}^{1\%}$ of from about 52 to about 54 and a UV spectrum substantially as shown in the single Figure of the accompanying drawing.

In a practical method of carrying out the method of the present invention, the starting material is plasma obtained from donors, e.g., by plasmaphoresis. The level of antigen may be measured in known manner by radioimmune assay, passive hemagglutination or complement fixation. The plasma is cooled and the cryoprecipitate which forms is removed by light centrifugation. The remaining clarified plasma containing HB_sAg is concentrated by one or more techniques, e.g. isopycnic banding, rate zonal banding or precipitation, e.g. by means of polyethylene glycol, ammonium sulfate or sodium sulfate. This partially purified concentrate is the starting material for the present invention.

In isopycnic banding the partially purified concentrate is contacted with a liquid medium having a density gradient therein which includes the density of the specific antigen required. The liquid medium is then subjected to ultracentrifugation to attain an equilibrium distribution of the serum components through the density gradient, according to their individual densities. Successive fractions of the medium are then displaced and those containing the desired antigen are then separated. The application of this technique to the purification of Australia antigen is described in German Patent Specification 2,049,515 and United States Patent Specification 3,636,191.

In rate zonal banding the partially purified concentrate is subjected to ultracentrifugation in contact with a liquid medium having a density gradient therein, but this time using the rate zonal technique, i.e. at a rate and for a period such that equilibrium is not attained, the HB_sAg and other residual serum components being distributed through the medium according to their sedimentation coefficients in the medium.

The liquid media used in the process may be of any density gradient in the appropriate ranges. Suitable solutes for such solutions include, e.g., sucrose, potassium bromide, cesium chloride, sodium bromide and potassium tartrate. The isopycnic banding step is conveniently carried out in a centrifuge, for example, Electronucleonics-K, by filling the stationary rotor with saline solution, then successively displacing the saline solution upwards with aliquot portions of a liquid medium solution of increasing density until a step gradient is formed.

The plasma is introduced at the top of the rotor displacing some of the highest density solution from the bottom. The centrifuge is brought up to speed through a programmed speed control system which prevents mixing during the initial reorientation phase. When equilibrium is attained and the product is in its proper density position, the rotor is slowed down through the same system to prevent mixing upon reorientation to the original configuration. Then the gradient is drained from below and the proper density cut collected. A similar technique is used in the rate zonal banding. The proper density cut from this banding is the partially purified concentrate of hepatitis B antigen.

The protein concentration of the partially purified concentrate is then adjusted to form about 40 to about 200 micrograms per ml. by addition of sterile, pyrogen-free phosphate buffered saline (PBS). The solution is then acidified to from about pH 2 to about pH 4, preferably with HCl at about 25°C.

A solution of purified pepsin in water, preferably prepared from crystalline pepsin, is added so that the solution contains about 1 microgram of pepsin per from about 40 to about 500 micrograms of protein. The solution is incubated until digestion of the extraneous protein by the pepsin is complete, typically in from about 8 to about 24 hours at a temperature of about 30°C. to about 38°C. The solution is then brought to about pH 7 by addition of a base, e.g. NaOH. The pepsin digested material is concentrated by ultrafiltration at about 5°C. and urea is added (preferably highly purified crystalline grade) until the final concentration is effective to dissociate proteinaceous matter, typically from about 4M to about 8M. The mixture is then incubated at elevated temperature, typically at from about 30°C. to about 38°C. for from about 16 to about 24 hours to effect further purification.

The incubated mixture is clarified by filtration and chromatographed on a column to remove urea, pepsin and remnants of pepsin digestion. The column is eluted with a sterile, pyrogen-free physiologically acceptable buffer which is compatable with the column, e.g. PBS, a mixture of NaH_2PO_4 and NaH_2PO_4 or tris. Fractions determined to contain HB_sAg by known methods, e.g. radio-immune assay, complement fixation and ultraviolet spectrophotometry, are pooled and diluted to a level of about 20 micrograms HB_sAg per ml. with sterile, pyrogen-free physiologically acceptable buffer, (some examples of which have been given above). The diluted pooled fractions are sterile filtered. To the filtered batch formaldehyde is optionally added at a concentration known to inactivate viruses, e.g. from about 50 to about 200 micrograms per ml. The mixture is then incubated at from about 30°C. to about 38°C. for from about 50 to about 100 hours. A sterile solution of a physiologically acceptable neutralizing agent, e.g. $NaHSO_3$, is added to substantially neutralize the formaldehyde. The material is dispensed aspecially into glass vials and stored for future use.

The purified hepatitis B surface antigen of the present invention is a highly purified, reproducible product and characterized in hav-

ing a particle size diameter of from about 18 to about 22 nm, an $E_{278nm}^{1\%}$ typically from about 52 to about 54, and a UV spectrum as shown in Figure 1.

The accompanying drawings is an ultraviolet spectrum of the purified Hepatitis B antigen of the present invention.

The following Example 1 illustrates the present invention without, however limiting it. Examples 2 to 5 show the effect of compositions of the present invention. The word 'Sephadex' is a trade mark.

EXAMPLE 1

The rotor of a centrifuge, Electronucleonics K, is filled with 8,400 ml of phosphate buffer. After running the rotor up to 10,000 rpm to degas the system, the following step gradient is pumped into the bottom of the stationary rotor:

1. 2,400 ml of 10% NaBr, ρ=1.08
2. 1,000 ml of 20% NaBr, ρ=1.17
3. 1,500 ml of 30% NaBr, ρ=1.28
4. 3,500 ml of 40% NaBr, ρ=1.41

Plasma containing Australia antigen, 1,750 ml is pumped into the top of the stationary rotor displacing 1,750 ml of 40% NaBr from the bottom of the rotor. The rotor is accelerated to 30,000 rpm and run at this speed for 4 hours. The rotor is then stopped and 1,750 ml of 40% NaBr are pumped into the bottom of the rotor forcing the plasma out the top. An additional 1,750 ml of fresh plasma containing Australia antigen are pumped into the top of the rotor displacing an equal volume of 40% NaBr out the bottom of the rotor. The rotor is then run at 30,000 rpm for 18 hours. After stopping the rotor the HB_sAg rich material in the 1.21—1.24 density region, 1,000 ml, is collected and dialyzed against phosphate buffer, degassed. The rotor is then filled with phosphate buffer, degassed as above, and the following step gradient pumped into the bottom of the stationary rotor:

1. 2,400 ml of 5% sucrose, ρ=1.02
2. 1,750 ml of 15% sucrose, ρ=1.06
3. 1,750 ml of 25% sucrose, ρ=1.10
4. 2,500 ml of 50% sucrose, ρ=1.23

The HB_sAg rich material from the NaBr isopycnic banding step, 1,000 ml, is pumped into the rotor top displacing 1,000 ml. of 50% sucrose out the rotor bottom. The rotor is then run at 28,000 rpm for 18 hours. After stopping the rotor the HB_sAg rich material in the 1.135—1.165 density region, 1,000 ml, is collected. This product is then diluted to 40 micrograms protein per milliliter to yield 56 liters of material. The diluent is phosphate buffered saline. The 56-liter batch of zonal centrifuge partially purified hepatitis B antigen (HB_sAg) at 40μg/ml is acidified to pH 2 at room temperature by the addition of one normal hydrochloric acid with stirring. 56 ml of a 1 mg/ml solution of crystalline pepsin in distilled water is added. The final concentration of pepsin was 1 μg/ml. The HB_sAg batch is incubated 16 hours at 37°C. and neutralized to pH 7 by the addition of one normal sodium hydroxide.

The pepsin-digested batch is concentrated 295—fold by ultrafiltration using an Amicon apparatus equipped with an XM100A membrane. Pepsin degraded non-antigen protein passes through the membrane into the ultrafiltrate but HB_sAg particles are retained.

Solid urea is added to the concentrate to make a 4—8 molar urea solution which is then incubated another 16 hours at 37°C. The urea-treated HB_sAg concentrate is clarified by filtration through a fiberglass filter and chromatographed on Sephadex G-150. The antigen is eluted at the void volume and is free from urea, pepsin and other protein impurities. The fraction containing purified HB_sAg is diluted to a use level and sterile filtered. Formalin is added to a concentration of 90—100 μg/ml for 72 hours at 36°C to further insure against the possibility of the presence of infectious viruses. At the end of formalin treatment, excess formaldehyde is neutralized with sodium bisulfite. The purified product has an $E_1\%$ of 52.3 compared to an $E_1\%$ of 23.8 for the zonal centrifugation starting material. The purified product consists of spherical particles having a diameter of from 18—22 nm and UV spectrum as shown in the accompanying drawing.

A summary of purification, biological and physical properties are shown in the following table.

	Zonal Centrifugation Product	Final Product
OD_{280nm}	0.095	0.115
OD_{260nm}	0.085	0.082
Volume (ml)	56,000	23,300
TOTAL OD_{280nm}	5,320	2,680
Lowry Protein (μg/ml)	40.0	22.0
Total Protein (mg)	2,240	513
% Protein Removal	—	77
CF Units per ml.	64	128
Total CF Units	3,584,000	2,982,000
% Yield in CF	—	83

	Zonal Centrifugation Product	Final Product
CF Units per μg/ml Protein	1.6	5.8
RIA Units/ml	2,000	8,000
TOTAL RIA Units	112,000,000	180,000,000
% Yield in RIA	—	160
RIA Units per μg Protein	50	364

EXAMPLE 2

When injected subcutaneously with a single dose (1.0 ml.) containing 20 micrograms of the hepatitis B surface antigen of the present invention, four out of a group of six chimpanzees developed antibodies. After two similar additional doses at four-week intervals, five of the six chimpanzees had developed antibodies.

EXAMPLE 3

Sixteen chimpanzees were divided into three groups. Group A (six chimpanzees) was inoculated intravenously with 1.0 ml. of BOB hepatitis B virus; Group B (four chimpanzees) was inoculated intravenously with 1.0 ml. of the hepatitis B surface antigen of the present invention; Group C (six chimpanzees) was the control group and received no inoculation. All chimpanzees of Group A had evidence of clinical hepatitis B infection (either antigenemia, enzyme elevations and/or antibody response) within forty weeks. None of the chimpanzees of Group B or C showed evidence of clinical hepatitis B infection over the same forty-week period.

EXAMPLE 4

Three groups of grivet monkeys (six monkeys in each group) were given 3 injections subcutaneously at four-week intervals with 1.0 ml. doses containing varying amounts of the antigen of the present invention. The following table shows the dosage administered to each group in each injection and the number of animals out of the total in that group showing antibody formation prior to the next injection, or within 4 weeks following the last injection.

Group	Vaccine Dose (μg/ml)	No. of Animals Showing Antibody Response		
		Week 0	Week 4	Week 8
A	20	2/6	2/6	5/6
B	2	0/6	1/6	4/6
C	0.5	0/6	1/6	2/6

Significantly, more than 50% of the animals showed an antibody response after three doses of vaccine at a doseage level of 2 micrograms.

EXAMPLE 5

Three groups of guinea pigs (14 in each group) were injected subcutaneously at 0, 14 and 56 day intervals with 1.0 ml. doses containing varying amounts of the antigen of the present invention. The following table shows the dosage administered to each group, each injection and the number of animals out of the total in that group showing antibody formation when tested on day 0, 28, 56 and 84.

Group	Vaccine Dose (μg/ml)	No. of Animals showing Antibody Response			
		Day 0	Day 28	Day 56	Day 84
A	20	0/14	12/14	10/11	10/10
B	2	0/14	7/14	4/11	10/10
C	0.5	0/14	2/14	3/14	10/12

Significantly, over 80% of the guinea pigs showed antibody formation after three doses of vaccine at a dosage level of 0.5 microgram.

WHAT WE CLAIM IS:—

1. A method of purifying hepatitis B antigen from a partially purified concentrate obtained by cooling plasma obtained from donors, removing the resulting cryoprecipitate by light centifugation and concentrating the remaining clarified plasma, which contains hepatitis B antigen, comprising treating the concentrate with a quantity of pepsin effective to digest proteinaceous matter at a pH within the range wherein pepsin is enzymatically active, treating the concentrate with a quantity of urea effective to dissociate proteinaceous matter, and removing pepsin, pepsin degradation products and urea, and optionally adding formaldehyde in a concentration effective to inactivate viruses.

2. A method according to Claim 1, in which

	Zonal Centrifugation Product	Final Product
CF Units per μg/ml Protein	1.6	5.8
RIA Units/ml	2,000	8,000
TOTAL RIA Units	112,000,000	180,000,000
% Yield in RIA	—	160
RIA Units per μg Protein	50	364

EXAMPLE 2

When injected subcutaneously with a single dose (1.0 ml.) containing 20 micrograms of the hepatitis B surface antigen of the present invention, four out of a group of six chimpanzees developed antibodies. After two similar additional doses at four-week intervals, five of the six chimpanzees had developed antibodies.

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Sixteen chimpanzees were divided into three groups. Group A (six chimpanzees) was inoculated intravenously with 1.0 ml. of BOB hepatitis B virus; Group B (four chimpanzees) was inoculated intravenously with 1.0 ml. of the hepatitis B surface antigen of the present invention; Group C (six chimpanzees) was the control group and received no inoculation. All chimpanzees of Group A had evidence of clinical hepatitis B infection (either antignemia, enzyme elevations and/or antibody response) within forty weeks. None of the chimpanzees of Group B or C showed evidence of clinical hepatitis B infection over the same forty-week period.

EXAMPLE 4

Three groups of grivet monkeys (six monkeys in each group) were given 3 injections subcutaneously at four-week intervals with 1.0 ml. doses containing varying amounts of the antigen of the present invention. The following table shows the dosage administered to each group in each injection and the number of animals out of the total in that group showing antibody formation prior to the next injection, or within 4 weeks following the last injection.

Group	Vaccine Dose (μg/ml)	No. of Animals Showing Antibody Response		
		Week 0	Week 4	Week 8
A	20	2/6	2/6	5/6
B	2	0/6	1/6	4/6
C	0.5	0/6	1/6	2/6

Significantly, more than 50% of the animals showed an antibody response after three doses of vaccine at a doseage level of 2 micrograms.

EXAMPLE 5

Three groups of guinea pigs (14 in each group) were injected subcutaneously at 0, 14 and 56 day intervals with 1.0 ml. doses containing varying amounts of the antigen of the present invention. The following table shows the dosage administered to each group, each injection and the number of animals out of the total in that group showing antibody formation when tested on day 0, 28, 56 and 84.

Group	Vaccine Dose (μg/ml)	No. of Animals showing Antibody Response			
		Day 0	Day 28	Day 56	Day 84
A	20	0/14	12/14	10/11	10/10
B	2	0/14	7/14	4/11	10/10
C	0.5	0/14	2/14	3/14	10/12

Significantly, over 80% of the guinea pigs showed antibody formation after three doses of vaccine at a dosage level of 0.5 microgram.

WHAT WE CLAIM IS:—

1. A method of purifying hepatitis B antigen from a partially purified concentrate obtained by cooling plasma obtained from donors, removing the resulting cryoprecipitate by light centifugation and concentrating the remaining clarified plasma, which contains hepatitis B antigen, comprising treating the concentrate with a quantity of pepsin effective to digest proteinaceous matter at a pH within the range wherein pepsin is enzymatically active, treating the concentrate with a quantity of urea effective to dissociate proteinaceous matter, and removing pepsin, pepsin degradation products and urea, and optionally adding formaldehyde in a concentration effective to inactivate viruses.

2. A method according to Claim 1, in which

* C L M S *

the pH of the concentrate is adjusted to about 2 with HCl.

3. A method according to Claim 1 or 2, in which the pepsin is added until the concentration is about 1 microgram of pepsin to from 40 to 500 micrograms of protein.

4. A method according to any one of Claims 1 to 3, in which the urea is added until its concentration is in the range 4*M* to 8*M*.

5. A method according to any one of Claims 1 to 4, in which formaldehyde is added following removal of pepsin, pepsin degradation products and urea.

6. A method according to Claim 5, in which the formaldehyde is added until its concentration is from 50 to 200 micrograms/ml.

7. A method according to Claim 5 or 6, in which the removal is effected by filtration and chromatography.

8. A method according to Claim 1 substantially as hereinbefore described in Example 1.

9. A purified hepatitis B surface antigen having a particle size diameter of from about 18 to about 22 nm, an $E_{278nm}^{1\%}$ of from about 52 to about 54 and a UV spectrum substantially as shown in the single Figure of the accompanying drawing.

10. An antigen according to Claim 9 having an $E_{278nm}^{1\%}$ of about 52.

11. An antigen according to Claim 9 when prepared by a method according to any one of Claims 1 to 8.

12. A vaccine effective in protecting against hepatitis B comprising an antigen according to Claim 9, 10 or 11 in a physiologically acceptable sterile, pyrogen-free buffer.

For the Applicants,
D. YOUNG & CO.,
Chartered Patent Agents,
9 & 10 Staple Inn,
London, WC1V 7RD.

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