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PATENT SPECIFICATION (11) 1 508 878

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(54) HEPATITIS A ANTIGEN

(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 A (infectious hepatitis) antigen, to a method for its preparation and to its use in an assay for hepatitis A and for the preparation of hepatitis A vaccine.

Hepatitis A is a liver disease which, while not commonly fatal, can involve many weeks of debilitating illness. It is usually spread by direct contact with an infected individual or by contaminated drinking water or food. Studies of hepatitis A have been hampered by the lack of a simple specific assay for antibody against hepatitis A virus. The development of such an assay has not heretofore been possible because no preparations have previously been available which contain hepatitis A antigen in such quantities as to enable the performance of complement fixation, immune adherence or other serologic assays. The only assays previously available were neutralization testing in marmosets (Provost et al., Proc. Soc. Exp. Biol. Med., *142*, 1257, 1973) and immune electron microscopy (Feinstone et al., Science, 8 November 1973) with faecal extracts. These methods were cumbersome, expensive and not applicable to routine testing.

The present invention provides a composition consisting essentially of hepatitis A antigen and a physiological saline solution buffered at a pH in the range 6.0 to 7.8, the antigen having a buoyant density in CsCl of from 1.32 g/cm³ to 1.36 g/cm³. The antigen is preferably of a particle size of 27 mμ and the saline solution desirably contains hepatitis A antigen having a buoyant density in CsCl of from 1.32 g/cm³ to 1.36 g/cm³ in a concentration effective to give a measurable reaction with hepatitis A antibody in an immune adherence serological assay and is phosphate buffered to a pH of about 7.2.

Also in accordance with the present invention, a vaccine to prevent hepatitis A disease is prepared by inactivating or attenuating an active unattenuated antigen contained in such a composition.

The present invention also provides a vaccine to prevent hepatitis A disease consisting essentially of such a composition in which the hepatitis A antigen is inactivated or attenuated and the quantity of inactivated or attenuated hepatitis A antigen is effective to induce immunity to hepatitis A virus and a method of preventing hepatitis A disease in a susceptible mammalian species other than human comprising administering an effective amount of such a vaccine.

The hepatitis A (infectious hepatitis) antigen of the present invention may be obtained from the liver of a non-human primate, e.g. a marmoset, such as a *Saguinus mystax* marmoset infected intravenously with hepatitis A virus. The liver of the primate is removed subsequently at a time when the serum glutamic oxaloacetic transaminase and serum isocitric dehydrogenase enzymes are elevated, which generally occurs at from 14 to 40 days after inoculation. The liver is perfused with physiological saline at a pH of from 6.0 to 7.8, for example, phosphate buffered saline solution containing 0.005M sodium phosphate and 0.143 M NaCl, pH 7.2. The liver is then disrupted to release subcellular constituents, e.g. by grinding, and mixed with physiological saline to give a final 10% suspension by weight. The antigen consists of the supernate obtained after clarification at low speed centrifugation, e.g. at from 1,000 to 2,000 rpm for a short period of time, e.g. for from 5 minutes to 15 minutes. The antigen in this form is applicable to performance of tests by complement fixation and immune adherence for hepatitis A antibody. The intigen in this form contains about 10^9 of 27 mμ hepatitis A virus particles per cm³.

The clarified supernate containing the antigen can be purified and fractionated by density gradient techniques, such as isopycnic and/or rate zonal methods. The density gradient separation, either isopycnic, or zonal, may be performed in media known to the art, e.g., CsCl, NaBr, Na tartrate, sucrose and other materials of this type. The various fractions obtained from the separation are assayed against hepatitis A antibody for presence of hepatitis A antigen and fractions containing maximal hepatitis A antigen are selected. When carrying out the separation by buoyant density using CsCl as the medium, hepatitis A antigen is recovered maximally from the fraction having a buoyant density of from 1.32 to 1.36 cm^3. The antigen in this form also contains about 10^9 of 27 $m\mu$ hepatitis A virus particles per cm^3.

The antigen of the present invention is utilizable in an immunological assay for hepatitis A antibody. This assay is described in the specification of our copending application No. 50062/75 (Serial No. 1,508,879).

The hepatitis A antigen in any form whether derived from liver tissue or other tissues can be inactivated or attenuated for use as a vaccine against hepatitis A virus. Inactivation of infectivity may be achieved by treatment with formalin. The amount of formalin is effective to inactivate the infectivity of the antigen while retaining the immunogenicity such that the material is effective as a vaccine. Typically, formalin, 37% by weight formaldehyde solution, is diluted in from 1000 to 10,000 parts by weight of the antigen preparation and stirred at from 4°C. to 60°C. for 2 hours to 30 days, preferably the formalin is diluted in from 2000 to 6000 parts of the virus preparation at from 20°C. to 45°C. for from 2 days to 6 days, particularly at about 37°C. for about 3 days.

The vaccine of the present invention may be used to immunize against hepatitis A virus in susceptible mammalian species such as, e.g. marmosets and chimpanzees.

The following examples illustrate the present invention.

EXAMPLE 1

Isolation of Antigen

Human hepatitis A virus is used to inoculate intravenously a *S. mystax* marmoset according to the procedure described by Mascoli et al. Proc. Soc. Exp. Biol. Med. *142*, 276, (1973). The liver is removed 27 days after virus inoculation, at which time the serum glutamic oxaloacetic transaminase and serum isocitric dehydrogenase enzymes are elevated. The liver is perfused with phosphate buffered saline solution, pH 7.2, minced with scissors and ground in a mortar with sterile alundum and added to phosphate buffered saline to give a final 10% suspension by weight in the phosphate buffered saline. The antigen consists of 350 cc of supernate obtained after clarification by centrifugation at 1500 rpm for 10 minutes. The antigen at this stage contains greater than 10^9 of 27 $m\mu$ hepatitis A virus particles per cm^3. The clarified antigen is then applied to a caesium chloride gradient tube covering the density range of from 1.1 to 1.4 g/cm^3 and antigen having a buoyant density of from 1.32 g/cm^3 to 1.36 g/cm^3 is separated for use in the hepatitis A assay described in a previously mentioned copending application. This antigen contains about 10^9 of 27 $m\mu$ hepatitis A virus particles per cm^3.

EXAMPLE 2

Electron Microscopy of Infected Marmoset Liver

Small pieces of marmoset liver tissue, from animals infected with hepatitis A virus, are fixed in 1% by volume osmium tetroxide, dehydrated in an ethanol series and embedded in Epon 812. Sections are cut on an LKB Ultramicrotome III using a diamond knife. Sections are picked up on copper grids, stained in uranyl acetate, post-stained with lead citrate and examined in the electron microscope. The virus closely resembles the enteroviruses in size and shape. The diameter is 27 $m\mu$. The virus is present in the cytoplasm and tends to be localized in small vesicles that may be bound by multilayer membranes. The 27 $m\mu$ virus particles of the preparation are identified as hepatitis A virus by several criteria. Extracts of the liver are able to transmit hepatitis A infection to other marmosets. The virus is not present in normal marmoset livers. Identical 27 $m\mu$ virus particles are obtained from the blood of the infected marmoset. This preparation of 27 $m\mu$ virus particles is specifically neutralized by convalescent human hepatitis A serum but not by pre-illness serum.

EXAMPLE 3

Immune Electron Microscopy

Antigen, 0.05 ml., obtained as described in Example 1, is incubated with human convalescent hepatitis A sera, 0.02 ml. of a 1:20 dilution by weight. The mixture is incubated at 37°C. for one hour and then held at 4°C. for a period of three hours. A drop of the material is placed onto a carbon-coated, 300-mesh (U.S. standard) copper grid, and allowed to adsorb for 30 seconds. The grid is then stained for 2 minutes with 2% by volume aqueous phosphotungsic acid, pH 6.0 (adjusted with 1N KOH) and examined in a Philipps 300 electron microscope at 80KV. After reaction with hepatitis A antibody, characteristic halos of antibody molecules are seen to surround the numerous 27 $m\mu$ hepatitis A virus particles and to bind them into an immune complex.

EXAMPLE 4

Complement Fixation Assay

Antigen obtained as in Example 1 but prior to CsCl buoyant density gradient separation is heated for 2 hours at 56°C. This material is serially diluted and tested against serial dilutions of human hepatitis A antibody in standard block titrations by the microtiter method of complement fixation assay. Two units of antigen thus defined are used to subsequently assay human sera for hepatitis A antibodies. Hepatitis A antibody thus detected is specific to hepatitis A convalescent patients. The hepatitis B patients do not show hepatitis A antibody responses. This complement fixation assay using crude hepatitis A antigen from infected marmoset liver is usable in diagnosis of human hepatitis A infection.

EXAMPLE 5

The procedure of Example 1 is repeated employing in lieu of *S. mystax* individuals from the following genera and species: *S. nigricollis, S. fuscicollis, S. oedipus, Callithrix jacchus, C. argentata, Cercopithecus aethiops, Pan troglodytes,* and *Anthropopithecus troglodytes.* In each case the antigen obtained is successfully used in the hepatitis A assay described in our previously mentioned copending application.

EXAMPLE 6

Both the clarified extract and the final product of Example 1 prepared under aseptic conditions are treated with 1:4000 by weight formalin at 37°C. for 72 hours. Excess of residual formalin is neutralized with sodium bisulfite. All treatments are performed under aseptic conditions. The product is stored at 4°C. Subcutaneous or intramuscular injection of 4 doses of 1 ml. given at 2 weeks intervals into *S. mystax* marmosets and guinea pigs induces circulating hepatitis A antibody in these animals. Further, the marmosets are rendered resistant to challenge with virulent doses of hepatitis A virus.

WHAT WE CLAIM IS:—

1. A composition consisting essentially of hepatitis A antigen and a physiological saline solution buffered at a pH in the range 6.0 to 7.8, the antigen having a buoyant density in CsCl of from 1.32 g/cm³ to 1.36 g/cm³.

2. A composition according to Claim 1, in which the hepatitis A antigen is of a particle size of 27 mμ.

3. A composition according to Claim 1 or 2, in which the physiological saline solution contains hepatitis A antigen having a buoyant density in CsCl of from 1.32 g/cm³ to 1.36 g/cm³ in a concentration effective to give a measurable reaction with hepatitis A antibody in an immune adherence serological assay.

4. A composition according to Claim 3, in which the physiological saline solution is phosphate-buffered.

5. A composition according to Claim 4, in which the pH is about 7.2.

6. A composition according to any one of Claims 3 to 5, in which the hepatitis A antigen is inactivated or attenuated.

7. A vaccine to prevent hepatitis A disease consisting essentially of a composition as claimed in Claim 6, in which the quantity of inactivated or attenuated hepatitis A antigen is effective to induce immunity to hepatitis A virus.

8. A method of preparing a vaccine to prevent hepatitis A disease comprising inactivating or attenuating an active unattenuated antigen contained in a composition according to any one of Claims 1 to 5.

9. A method according to Claim 8, in which the inactivation or attenuation is effected by formalin.

10. A method of preventing hepatitis A disease in a susceptible mammalian species other than human comprising administering an effective amount of a vaccine according to Claim 7.

11. A method of preparing a hepatitis A antigen, substantially as hereinbefore described in Example 1 or 5.

12. A method of attenuating a hepatitis A antigen, substantially as hereinbefore described in Example 6.

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2. A composition according to Claim 1, in which the hepatitis A antigen is of a particle size of 27 mμ.

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7. A vaccine to prevent hepatitis A disease consisting essentially of a composition as claimed in Claim 6, in which the quantity of inactivated or attenuated hepatitis A antigen is effective to induce immunity to hepatitis A virus.

8. A method of preparing a vaccine to prevent hepatitis A disease comprising inactivating or attenuating an active unattenuated antigen contained in a composition according to any one of Claims 1 to 5.

9. A method according to Claim 8, in which the inactivation or attenuation is effected by formalin.

10. A method of preventing hepatitis A disease in a susceptible mammalian species other than human comprising administering an effective amount of a vaccine according to Claim 7.

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