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HEAT-INACTIVATED HBsAg AS A VACCINE AGAINST HEPATITIS B

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Hepatitis B (HB) vaccine was prepared as follows: hepatitis B surface antigen (HBsAg) was purified from pooled plasma positive for HBsAg (subtype adw2), boiled for 90 s at 101°C and adsorbed to aluminum hydroxide; subsequently, 1 ml doses, containing 0.15 μg HBsAg, were filled off and pasteurized for 10 h at 65°C. Potency studies of the vaccine were done in rabbits, and tests for safety were carried out in three non-immune chimpanzees and four human volunteers. Neither hepatitis (B) nor autoimmune phenomena were observed in any of the vaccinated chimpanzees or humans, whereas all rabbits and chimpanzees and three of the four humans formed anti-HBs antibody.

hepatitis B HBsAg HB vaccine

INTRODUCTION

As about 120 million persons of the world's population are estimated to be infected with hepatitis B virus (HBV), HBV infections are a worldwide problem [32]. The chronic carriers of HBV form a massive reservoir of infection for their environment, especially in regard to perinatal transmission of HBV [16]. For the carriers themselves, the presence of hepatitis B surface antigen (HBsAg) is often associated with the development of chronic liver disease and probably also of primary carcinoma of the liver. Although acute hepatitis B has a low-case fatality rate, an appreciable proportion, especially the subclinical cases, progresses into chronic liver disease, finally resulting in liver cirrhosis and death.

Some progress has been made in halting the spread of the virus, especially by the prevention of post-transfusion hepatitis B as a result of the screening of blood donors for the presence of HBsAg [26]. Also, hepatitis B immunoglobulin (HBIg) has proved to be valuable as pre- and post-exposure prophylaxis [13], but its protective effect is of limited

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duration, and individuals repeatedly exposed to HBV would require repeated administration of HBIg [9, 23]. Active vaccination should be the final answer for the prevention of HBV infections.

As HBV cannot be propagated in tissue cultures, the only available source of viral antigen at present is the plasma of chronic carriers of HBsAg. Krugman et al. [10] showed that a diluted HBV-containing serum, when heated at 98°C for 1 min, possessed the characteristics of live attenuated vaccine and gave partial protection against a challenge with live HBV in humans. Heating for 10 h at 60°C was ineffective for the neutralization of HBV in high concentrations [27, 29]. Subsequent studies [1, 6, 11, 12, 19–21] showed that vaccines consisting of purified HBsAg, inactivated by formaldehyde, were non-infectious when tested in a limited number of chimpanzees and humans, and gave effective protection against a challenge with live HBV. A vaccine was also prepared from a mixture of polypeptides of HBsAg; this vaccine proved to be non-infectious in chimpanzees and induced protective immunity against challenges with virulent HBV [7]. Because HB vaccines may be contaminated with human host proteins, it was feared that autoimmune disease might result from vaccination with these vaccines [33], but so far this fear has not been substantiated.

The successful cloning of HBV-DNA in *E. coli* by recombinant methodology offers the prospect of obtaining large amounts of pure and well-characterized HBV antigens for vaccine preparation [28].

The aim of our study was to prepare a HB vaccine from purified HBsAg and to evaluate this vaccine in experimental animals and human volunteers for its potency and safety. Heat was chosen as the method of inactivation for the vaccine, because pasteurization of human albumin solutions for 10 h at 60°C is effective in neutralizing the infectivity of HBV at low concentrations, whereas boiling for 1 min sufficiently inactivated HBV at high concentrations [10].

MATERIALS AND TECHNIQUES

Preparation of HB vaccine

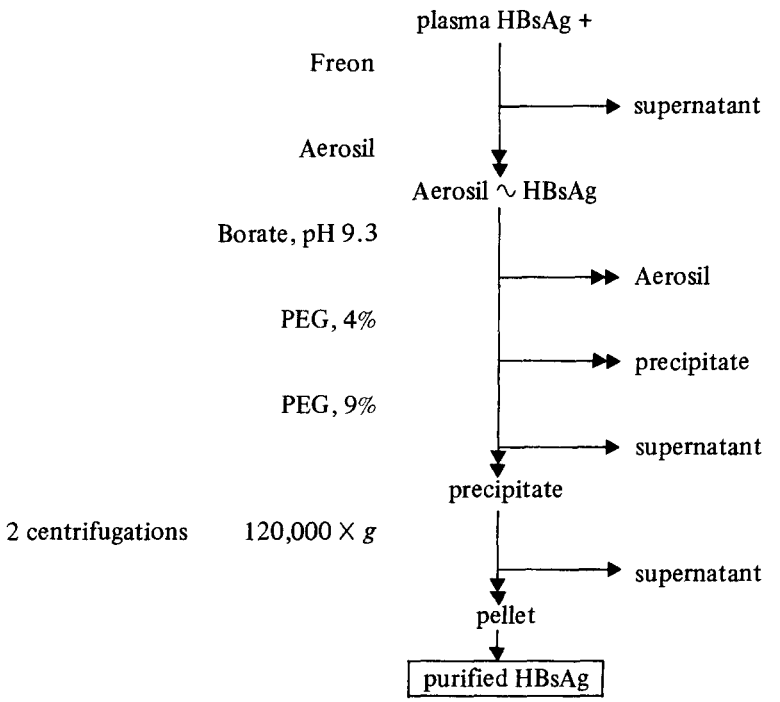
Source of material

Plasma was obtained from 16 unpaid blood donors, who had been proven to be persistent carriers of HBsAg. The HBsAg titer of the pool (2500 ml) was 1 : 640 by passive hemagglutination inhibition (PHAI) [22] and by competitive solid-phase radioimmunoassay (RIA) [3], i.e., the pool contained about 1.5 μg HBsAg/ml according to the provisional standard of the Bureau of Biologics of the F.D.A. (U.S.A.) [31]. Fourteen of the 16 donors were of HBsAg subtype adw2; the subtype of the two others could not be determined. Neither the individual donors nor the plasma pool were tested for HBeAg, anti-HBe antibody or DNA polymerase.

Purification of HBsAg

HBsAg was purified from the plasma pool according to Scheme 1. Plasma was mixed with Freon 113 (Dow Chemicals) in a ratio of 4 : 1 for 30 min at room temperature to clarify the plasma from lipid-like material. After centrifugation, the clarified supernatant plasma was incubated for 16 h at room temperature with 4% (w/v) Aerosil (Degussa, Frankfurt, F.R.G.) which resulted in the adsorption of HBsAg and other plasma proteins [2]. The non-adsorbed plasma proteins were removed by centrifugation for 15 min at 1500 × *g* and subsequent washing in phosphate-buffered saline (PBS-A: 140 mM NaCl, 10 mM phosphate, pH 7.4). HBsAg was eluted by incubating the Aerosil for 1 h at 37°C with 0.015 M borate solution, pH 9.3 (volume = 0.5 × starting volume). After centrifugation and separation, the eluate was neutralized with 0.1 N HCl. After the addition of polyethylene glycol (PEG) 6000 (Merck, Cincinnati, OH) to a final concentration of 40 g/l, the supernatant was separated from the precipitate by centrifugation. The precipitate was discarded. To the supernatant, PEG 6000 was added to a final concentration of 90 g/l. The precipitate was washed and then suspended in PBS-B (= PBS-A, to which was added 0.1% NaN_3, 0.01% EDTA; volume = 0.08 × starting volume, pH 7.8). The suspended precipitate was pelleted twice by ultracentrifugation for 4 h at 120,000 × *g*. Finally, the pellet was resuspended in PBS-A (volume = 0.4 × starting volume).

In the final solution, HBsAg was demonstrable in a titer of 1 : 64 (PHAI and RIA).

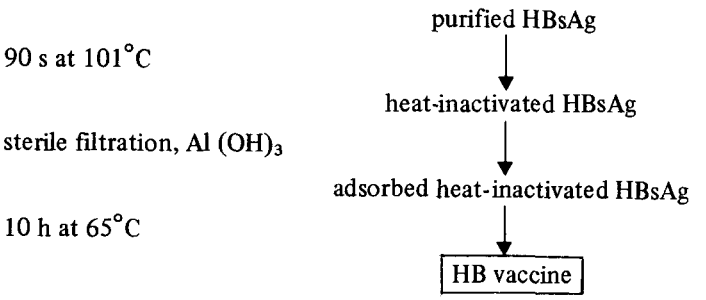


Scheme 1. Purification of HBsAg.

Compared with the original quantity of proteins in the plasma pool, HBsAg had now been purified by a factor of about 200. The electron microscope revealed exclusively spherical 22-nm particles; liver-specific protein (LSP) was not detected in this preparation.

Inactivation and final preparation of HB vaccine

HB vaccine was prepared according to Scheme 2. The purified HBsAg solution was boiled at 101°C in portions of 50 ml for 90 s. The temperature between the start and the end of the heating period (12 min) was recorded continuously. All portions were pooled and filtered through membrane filters (0.2 μm pore size). Aluminum hydroxide was added to a final concentration of 1.6 g/l. The final solution was filled into 1 ml ampoules which were then pasteurized for 10 h at 65°C.



Scheme 2. Preparation of HB vaccine.

Characterization of HB vaccine

After boiling (but before adsorption to aluminum hydroxide), the HBsAg titer dropped to 1 : 32, i.e., about 0.15 μg HBsAg/ml; the total protein content was 15 μg/ml as determined spectrophotometrically at 280 nm. Neither human serum proteins nor LSP were detected in this preparation. The final product was sterile and passed the pyrogen and general safety tests according to the pharmacopeia.

Techniques

Serological tests for HBV markers

The serum samples of all animals and humans were tested for the presence of HBsAg by Ausria II-125 (Abbott). HBsAg was quantitated by passive hemagglutination inhibition (PHAI) [22] and competitive solid-phase radioimmunoassay (RIA) [3]. The subtype of HBsAg was determined by the Ouchterlony technique (kindly performed by Dr. A.M. Couroucé, Centre National de Transfusion Sanguine, Paris).

The presence of anti-HBs was determined by passive hemagglutination (PHA) [22] and RIA (Ausab, Abbott) techniques. Anti-HBs titrations (PHA) in different serum samples of one animal or one human were carried out on one day. As reference, HBIg concentrate

(Central Laboratory of The Netherlands Red Cross Blood Transfusion Service) was used. This preparation contains 100 I.U./ml, corresponding to a titer of 1 : 32,000 in PHA. To determine whether the antibodies were directed against the common antigen 'a' of HBsAg or against subtype-specific determinants, all sera positive for anti-HBs were tested by PHA on red cells coated with either HBsAg subtype adw2 or ayw3.

In the anti-HBs-positive serum samples of rabbits and chimpanzees, the presence of antibodies against other subtypes was determined by absorbing the sera with HBsAg adw2 and HBsAg ayw3. Subsequently, these absorbed sera were examined, by using red cells coated with ayw1, ayw2, ayw3, ayw4, adw2 and adw4 (kindly performed by Dr. A.M. Couroucé, Paris). Human sera, positive for anti-HBs, were treated with dithiothreitol (DTT) [17] to inactivate the serological activity of the IgM antibodies and to differentiate between IgM and IgG anti-HBs. As a control the above HBIg concentrate was used, in which anti-HBs was present as IgG only.

Anti-hepatitis-B core (anti-HBc) tests were carried out by solid-phase radioimmunoassay techniques [5, 30] (kindly performed by Prof. G.N. Vyas, Univ. of California, San Francisco, CA; Prof. J. Desmyter, Rega Institute, Louvain, Belgium; and Dr. W.H. Gerlich, Hygiene Institut der Univ. Göttingen, F.R.G.).

Liver-specific protein (LSP), anti-LSP and antibodies against liver-cell membrane antigens (LMAg)

Determinations of LSP of the purified HBsAg solution before and after boiling were performed with the PHA and Ouchterlony techniques with four hetero-anti-LSP antisera prepared in rabbits and rats. Tests for anti-LSP were also done with these techniques by using different fractions of purified LSP prepared from livers of humans and rabbits [14]. Detection of antibodies against LMAg was carried out with an indirect immunofluorescence technique on isolated human and rabbit hepatocytes [8]. (The tests for LSP, anti-LSP and anti-LMAg were kindly performed by Prof. K.H. Meyer zum Büschenfelde, Klinik Charlottenburg, Freie Univ., Berlin, F.R.G.).

Other serological tests

The presence of antibodies against smooth muscle, mitochondria and nuclear antigens was investigated by an indirect immunofluorescence technique [4]. The absence of human serum proteins from the purified HBsAg solution as well as the absence of antibodies against human serum proteins from the serum samples of immunized animals and humans was demonstrated by immunoelectrophoresis.

Light microscopy and immunofluorescence tests on liver tissue

Liver tissue specimens, obtained by biopsy with a Menghini needle, were divided into two pieces. One piece was fixed in buffered formaldehyde 4% (pH 7.2) and embedded in paraplast. Sections of 4 μm were stained with hematoxylin-eosin, periodic acid-Schiff, Gomorri reticulin, Von Gieson elastin and Perls. The other piece was snap-frozen in liquid nitrogen and stored at -190°C until used. Then 4 μm sections were cut in the

cryostat and incubated with a rabbit anti-HBs antibody labeled with fluorescein isothiocyanate (FITC) [24].

The presence of hepatitis-B core antigen (HBcAg) was studied by using a human anti-HBc serum as first layer and an FITC-labeled anti-human IgG (Dakopatts, Denmark) as second layer. As a control, sections were incubated with normal serum as first layer, onto which the FITC-labeled anti-human IgG was applied subsequently.

The presence of IgG, IgM and C3 was investigated with FITC-labeled rabbit antisera against human IgG, IgM and C3 (Dakopatts, Denmark) [25]. All specimens were coded before examination.

Liver function tests (LFT's)

The levels of glutamic pyruvic transaminase (GPT), glutamic oxaloacetic transaminase (GOT) [15] and gamma-glutamyl transferase (γGT) [18] were determined in all serum samples of chimpanzees and humans. In man (males), the upper normal value was 40 units/l for the GPT and GOT and 28 units/l for the γGT. The upper limit of normal values for these LFT's for chimpanzees was established by examining in duplicate 75 serum samples of 20 adult non-immunized chimpanzees taken over a period of 10 months. The mean of these measurements plus twice the standard deviation was considered to be the upper normal value, i.e. for the GPT 53 units/l, for the GOT 35 units/l and for the γGT 24 units/l. Hepatitis was considered to be present in humans as well as in chimpanzees when the value of the LFT's amounted to 2.5 times the upper normal value.

Potency testing

Three pairs of rabbits were injected intramuscularly (i.m.) with 1, 0.1 and 0.01 doses of alum-adsorbed HB vaccine, and boosted with the same dose after 31 and 66 days. Blood samples were obtained every 7–10 days up to 100 days. Similar experiments were repeated after the HB vaccine had been stored for one year. Finally, the potency was established in three chimpanzees and four humans (see below).

Safety testing

Chimpanzee studies

Three male adult chimpanzees, weighing 48, 56 and 66 kg, were selected for tests on the safety of the vaccine based on the following criteria: absence (in two tests) from the serum of HBsAg, anti-HBs antibody, anti-HBc antibody, (auto)antibodies against smooth muscle, nuclear antigens, mitochondria, liver-specific protein (LSP), liver-cell membrane antigen (LMAg) as well as the absence of abnormal liver function tests (LFT's) and histological abnormalities of liver tissue, indicating hepatitis. The animals were anesthesized with ketaminehydrochloride (Parke-Davis) at a dosage of 10 mg/kg i.m. One chimpanzee (Ufford) received intravenously (i.v.) only one dose of aqueous HB vaccine, not adsorbed to aluminum hydroxide. The other two (Simon and Nico) each received 2 doses of the

aqueous vaccine i.v. and 10 doses alum-adsorbed vaccine i.m. The animals were bled just before the injection and at 1–2 week intervals afterwards. After 34 weeks, all three animals were boosted 5 times at monthly intervals with one dose i.m. The total follow-up period was 21 months.

Liver biopsies were obtained before the injection of the vaccine and at 15, 24, 34, 38 and 42 weeks thereafter. As controls, two adult chimpanzees, matched for age and weight, were injected i.m. each with 10 doses of 1.6 mg aluminum hydroxide solution without HBsAg. Serum samples (n = 14) and liver biopsies (n = 14) of each of the two animals were obtained over a period of one year.

Also as controls, liver biopsies (n = 32) of the above-mentioned 20 non-immunized adult chimpanzees were collected over a period of 10 months.

Human studies

After it had been shown that the HB vaccine was safe in chimpanzees, four male volunteers (Nos. 0100, 0103, 0109, 0111) were selected on the following criteria: low risk of contracting HBV infections, no prior hepatitis and, as shown by 2 different tests, absence from the serum of HBsAg, anti-HBs antibody, anti-HBc antibody, autoantibodies as mentioned above and abnormal LFT's. With his informed consent each volunteer received one dose of the alum-adsorbed vaccine (stored for more than 3 years at 4°C) i.m. and was boosted another three times at about monthly intervals. At 12 months, three of the four volunteers had a fifth injection of one dose; one of them (no. 0111) received the fifth injection at 22 months.

Samples of serum were obtained from all volunteers at 2–3 week intervals for 30 weeks and thereafter at 1–3 month intervals. The total follow-up period was 2 years.

RESULTS

Immunogenicity studies

Potency studies in rabbits

As shown in Table 1, the two rabbits injected with 1 ml of the HB vaccine formed anti-HBs after 2 weeks; in all three pairs, anti-HBs antibody was demonstrable after the first booster. After the second boost, the anti-HBs titer was 256–51 200, depending on the injected dose. All serum samples that were anti-HBs-positive in the PHA technique were also positive with the RIA technique and vice versa, except for one serum sample (see Table 1). After the vaccine had been stored for one year at 4°C, the same experiments were repeated in six rabbits with similar results. It was established that the anti-HBs antibodies of all rabbits were directed against the common antigen 'a' of HBsAg. In a number of the anti-HBs-positive sera, antibodies against sub-antigens 'd' and 'x(g)' of HBsAg were demonstrated after absorption (see Techniques).

TABLE 1

Potency study of HB vaccine in rabbits

Rabbit no.	Amount of HB vaccine (ml i.m.)	PHA anti-HBs titers on day					
		0	10	31[a]	38	66[a]	73
1	1	0	0[b]		5120		51,200
2		0	512		5120		25,600
3	0.1	0	0		32		3200
4		0	0		1280		51,200
5	0.01	0	0		16		1600
6		0	0		128		256

[a] Boost with the same dose.

[b] Positive in RIA (1.5 times the cut-off value).

Immunogenicity in chimpanzees

Fig. 1 shows that in the two chimpanzees (Simon and Nico) who had been injected with 2 doses of aqueous vaccine i.v. and 10 doses of alum-adsorbed vaccine i.m., anti-HBs was demonstrable exclusively by the RIA technique after 1 and 2 weeks. Until 2 weeks after the second injection (36 weeks after the first injection), the serum samples from both apes were only positive for anti-HBs antibody within the RIA technique (2–5 times the cut-off value). The third ape (Ufford), who had been given one dose of aqueous HB vaccine i.v., became positive for anti-HBs antibody one month after the second injection in the RIA technique only (value 1.5 times the cut-off value), whereas in the PHA technique, anti-HBs antibody was not demonstrated until one month after the third injection. All serum samples remained anti-HBs-positive during the follow-up period of 21 months. The anti-HBs-positive samples from all three chimpanzees had antibodies directed against the common antigen 'a' of HBsAg; chimpanzee Ufford had also formed anti-d antibody.

Immunogenicity in humans

Fig. 2 shows that, of the four human volunteers, No. 0109 had formed anti-HBs antibody after the second injection of HB vaccine, No. 0100 after the third injection, and No. 0111 after the fourth injection. The anti-HBs titer varied from 64 to 256 at 1–2 months after the fourth injection. All three volunteers showed a marked increase in anti-HBs titer 1–2 months after the fifth injection. One year after this last injection, the three individuals still had anti-HBs antibody in their sera.

The results of the PHA and RIA techniques were similar in all samples studied. The anti-HBs antibodies of these three volunteers were all directed against the common antigen 'a' of HBsAg. By measuring the anti-HBs titer before and after DTT treatment, it was found that presumably anti-HBs IgG followed anti-HBs IgM within one month. The

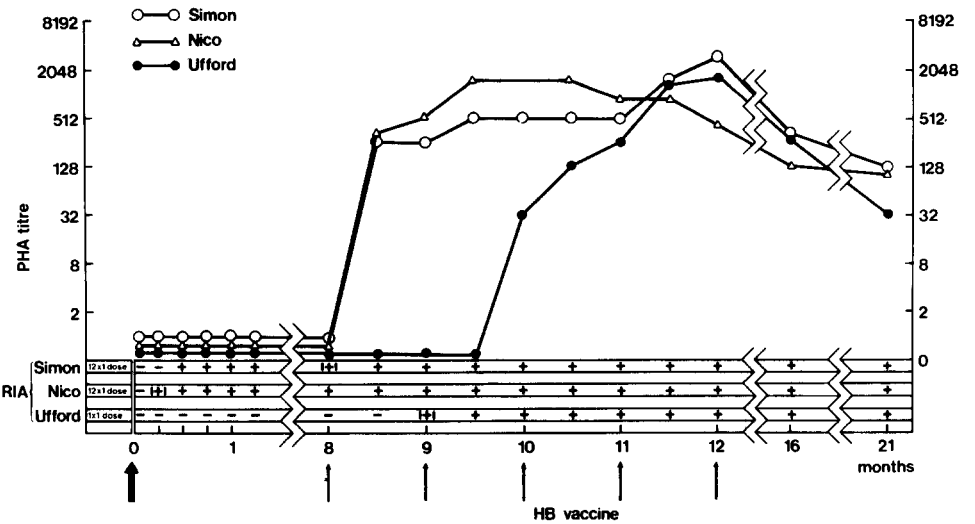


Fig. 1. Immunization of three chimpanzees with HB vaccine. Anti-HBs responses in three chimpanzees, as measured by PHA and RIA. Two animals (Simon and Nico) were injected with 10 ml alum-adsorbed HB vaccine i.m. and 2 ml aqueous vaccine i.v., whereas one animal (Ufford) received only 1 ml aqueous vaccine i.v. After 8 months, all animals were boosted another 5 times with 1 ml alum-adsorbed HB vaccine at monthly intervals.

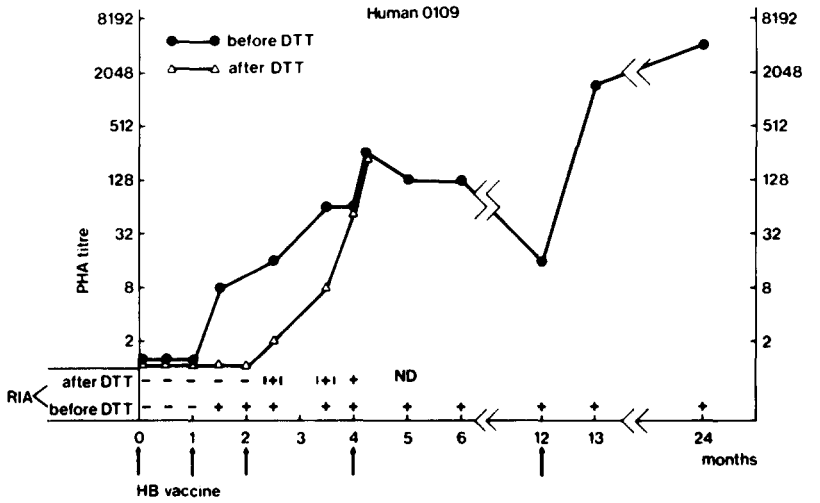
findings in the PHA and RIA techniques agreed well with each other; when, after DTT treatment of the serum, a lower anti-HBs titer was found by PHA, the counts per minute observed in the RIA were also markedly less (see Fig. 2).

In one volunteer (No. 0103), no anti-HBs was found even after five injections.

Safety studies

Chimpanzees

None of the three chimpanzees showed signs of viral hepatitis B for the whole period of follow-up after vaccination: HBsAg or anti-HBc was demonstrable in none of the serum samples, and the LFT's remained within a range considered normal for chimpanzees. No sign of viral hepatitis was observed in the liver biopsy specimens, and neither HBsAg nor HBcAg was demonstrable. However, 15 weeks after the first injection, small focal-reactive lesions, consisting of some histiocytes, lymphocytes and polymorphonuclears localized in the liver lobules, were seen in the biopsy specimens of all three apes. The same lesions were seen in five of 28 biopsy specimens of the two control chimpanzees treated with aluminum suspensions without HBsAg and in 11 of 32 specimens of untreated control chimpanzees. With Perls's stain, deposits of iron in various quantities were found, showing a diffuse and granular blue staining of parenchymal cells, especially in the periportal areas. Sometimes, groups of histiocytes laden with iron-positive pigment granules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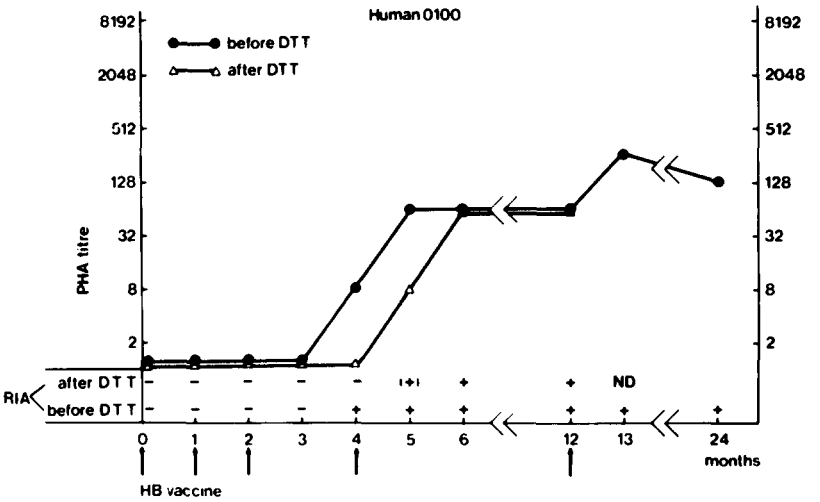
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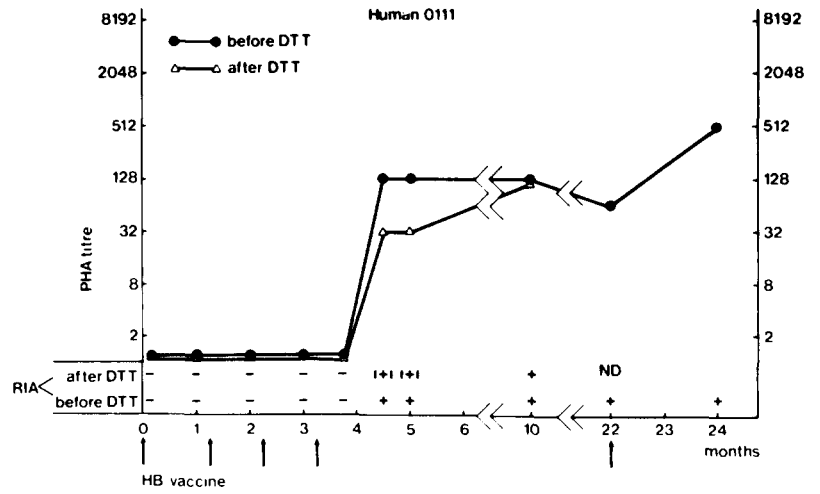
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were seen. Fibrosis was observed in none of these livers. The afore-described non-specific reactive lesions were exclusively found in the tissue specimens in which siderosis was observed; the more siderosis was present, the more non-specific reactive lesions were seen.

Humans

None of the four volunteers showed signs of viral hepatitis B for the whole period of follow-up. Neither HBsAg nor anti-HBc was demonstrable in the serum, and all LFT's remained normal. No local or generalized harmful symptoms were observed and all four subjects remained in perfect health.

Absence of (auto) immune phenomena

Antibodies against LSP, LMAg, nuclear factors, smooth muscle and mitochondria or antibodies against human serum proteins were not observed in any of the serum samples of the immunized rabbits, chimpanzees or humans. No deposits of IgG, IgM or C3 were found in the liver biopsies of the chimpanzees.

DISCUSSION

This study shows that partly purified HBsAg, inactivated by heating and adsorbed to aluminum hydroxide, was immunogenic in rabbits, chimpanzees and three out of four humans. Although the humans formed anti-HBs antibody only after 2–4 injections, the antibodies remained demonstrable during the whole observation period of 2 years. As has been observed by others [12], a significant increase in titer was observed when the individuals were boosted 1–2 years after the first injection. Our study indicates that, in humans, anti-HBs IgM is followed by anti-HBs IgG within one month. Similar observations have been made in chimpanzees vaccinated with polypeptide HB vaccine [7].

It was established that the anti-HBs antibodies formed in experimental animals and humans were directed against the common antigen 'a' of HBsAg, and were therefore probably protective. Challenge experiments with live HBV could not be carried out owing to lack of facilities for isolation of infected chimpanzees at the time of this study.

In none of the vaccinated chimpanzees were signs of hepatitis B observed, which is in accord with all other HB vaccine studies in chimpanzees [1, 6, 7, 11, 12, 19–21]. Although no sign of hepatitis B was seen in the liver biopsy specimens of the vaccinated chimpanzees, non-specific reactive lesions, probably due to siderosis, were observed in the liver tissue of some of the vaccinated as well as of the non-vaccinated animals. The

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Fig. 2. Immunization of three humans with HB vaccine. Anti-HBs responses, as measured by PHA and RIA, in three of the four human volunteers injected 5 times i.m. with one dose of alum-adsorbed HB vaccine. By treating part of the serum samples with dithiothreitol (DTT), it was demonstrated that anti-HBs IgG followed anti-HBs IgM within one month. All three individuals showed a marked increase in anti-HBs titer after the last boost, given 1–2 years after the first injection.

levels of GPT in control chimpanzees were significantly higher than those observed in humans, whereas the levels of GOT and γGT were in the same range for both species. No clinical or sub-clinical hepatitis B was diagnosed in any of the four human volunteers who were at low risk of contracting HBV infection. Hilleman et al. [6] showed that formaldehyde-inactivated HB vaccine was safe in 66 sero-negative individuals and 66 anti-HBs-positive human individuals. Maupas et al. [12] evaluated formaldehyde-inactivated HB vaccine in humans, exposed to a high risk of contracting HBV infection, and found 27 (12%) of 217 vaccinated humans (ward staff and patients of hemodialysis centers) to be infected with live HBV after injection of the vaccine, whereas in the non-vaccinated control group, 39 (71%) of 55 individuals had signs of HBV infection. Also 14 other vaccinated persons, not included in the above number of vaccinated humans, became infected with live HBV within the first 2 months of the immunization program. It cannot be ruled out, although it is not likely, that the observed HBV infections in the vaccinated group of persons were caused by the HB vaccine itself, owing to insufficient inactivation of HBV by the formaldehyde treatment.

The fear that host antigens present in HB vaccines would cause the formation of autoimmune antibodies was not substantiated by our study. In none of the serum samples of the vaccinated rabbits, chimpanzees or humans were (auto)antibodies against human antigens found; similarly, no signs of autoantibody fixation were found in the liver tissue specimens of chimpanzees.

The different lots of HB vaccine, which are at present under evaluation, contain HBsAg at about 40–50 μg/ml (U.S.A., Japan) or 5 μg/ml (France) [31]. Our study indicates that a much smaller quantity (0.15 μg/ml) of HBsAg per dose of HB vaccine may suffice to induce immunity to HBV. This is important, as in the future HB vaccines may have to be used, especially in developing countries, for the prevention of chronic carriership of HBV, which means that these vaccines should be available in large quantities at low cost.

On the strength of the results reported in this article, a second and larger batch of HB vaccine to be ultimately used in humans at risk to be infected with HBV, is being prepared and evaluated with regard to safety, potency and protective effect. This new collaborative project is performed by the same partners and partially supported by The Netherlands Foundation for Preventive Medicine.

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