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Development and Chimpanzee Testing of a Vaccine against Human Hepatitis B (39288)

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This paper describes the development and evaluation in chimpanzees of a vaccine against hepatitis B or "serum hepatitis." Certain of the findings were presented earlier (1).

The virus of hepatitis B does not multiply significantly in cell culture and there has been no source of virus for vaccine preparation. The discovery (2, 3) in the blood of certain human subjects of Australia antigen (now known as hepatitis B virus surface antigen HB_sAg) and the demonstration (4) of the relationship of that antigen to human hepatitis B virus opened the way to development of a vaccine against hepatitis B. Krugman *et al.* (5) prepared a vaccine in which boiled diluted plasma from a human carrier of Australia antigen stimulated antibody in human subjects and protected against the disease. We have prepared vaccine using highly purified Australia antigen that was treated with formalin, tested for safety and potency, and evaluated for protective efficacy in chimpanzees.

Materials and methods. Hepatitis B vaccine preparation (lot 559). Plasma pools were collected from overtly healthy human donors with hepatitis B antigenemia. Approximately 89% of the plasma was from hepatitis B type *ad* and 11% from type *ay* donors. Separation of Australia antigen from plasma consisted principally of two cycles of isopycnic banding and rate zonal separation (6) to yield a partially purified product. Further chemical procedures yielded highly purified hepatitis B antigen for vaccine. The purified antigen was adjusted to a concentration of 20 μg/ml protein (Lowry) and was treated for 72 hr at 36° with 1:4000 formalin. The treated material was the vaccine.

Ordinary tests for safety and sterility. The vaccine was tested by procedures that are consistent, where pertinent, with the existing standards of the Bureau of Biologics, Food and Drug Administration for vaccines (7). Additional tests for hepatitis A virus were carried out in white moustached marmosets (*Saguinus mystax;* 8). Tests for presence of extraneous human blood proteins were done by polyacrylamide gel electrophoresis analysis (9, 10) and for blood group substances by standard procedures using Ortho diagnostic reagents. Tests for pyrogenic activity were carried out in rabbits.

In vitro assays. Tests for hepatitis B surface antigen (HB_sAg) were by the Ausria I or Ausria II method (Abbott Laboratories); by the passive hemagglutination test (PHA) (Virgo Reagents Tests, Electro-Nucleonics Laboratories Inc.); or by complement-fixation (CF) according to Purcell *et al.* (11). Tests for antibody against hepatitis B surface antigen (anti-HB_s) were by the Ausab solid phase radioimmune assay (Abbott Laboratories) or by the PHA assay (Ausure, Electro-Nucleonics; see Table II). Tests for complement-fixing antibody against hepatitis B core antigen (anti-HB_c) were according to Hoofnagle *et al.* (12) and were kindly performed for us by Drs. J. H. Hoofnagle and A. W. Schulman. The assay for hepatitis A virus antibody was by the immune adherence test (IA; 13). The assays for SGOT and for SGPT were performed by the Sigma-Frankel method (Sigma Technical Bulletin No. 505, Sigma Chemical Co.).

Tests for live hepatitis virus in chimpanzees given lot 559 vaccine. Chimpanzees are susceptible to human hepatitis B virus (14, 15). Animals weighing 20–40 lb were iso-

lated 4 to 6 weeks prior to inoculation and were selected for absence of hepatitis B surface antigen and antibody, for absence of elevation in transaminases, for absence of hepatitis histopathology, and for negative tuberculin reaction. Four chimpanzees meeting the above criteria were each given 1.0 ml of the vaccine intravenously. Six similar chimpanzees were each given 1.0 ml of 1:1000 dilution of plasma from a case of human hepatitis B obtained from Dr. L. Barker, Bureau of Biologics, Food and Drug Administration, and containing about 1000 chimpanzee infectious doses per milliliter. The animals were bled just prior to injection, on the first, fourth, and seventh days after injection, and at weekly intervals thereafter for a total of 40 weeks. Monthly liver biopsies were made for histopathologic examination for hepatitis. The sera were assayed as described in the text and shown in Fig. 2 and Table III.

Tests for potency of the vaccine. Groups of 14 initially seronegative guinea pigs, weighing 350 to 400 g, received graded doses of vaccine in 1.0 ml volume by the subcutaneous route on Days 0, 14, and 56. The animals were bled just prior to each dose of vaccine and 28 days after the third injection. Seronegative grivet *(Cercopithecus aethiops)* monkeys weighing 4 to 5 lb were given graded doses of vaccine as above on Days 0, 28, and 56. Bleedings were as above. The sera were tested for hepatitis B antibodies.

Tests for protective efficacy of the vaccine in chimpanzees. Six chimpanzees, qualified as described above, were each given three 20-μg doses of lot 559 hepatitis B vaccine in 1 ml volume subcutaneously at 4-week intervals. The animals were bled weekly thereafter for 16 weeks and at the end of that time, they were challenged by intravenous injection with approximately 1000 chimpanzee infectious doses of the same human hepatitis B challenge virus used in the tests for safety described above. At the same time, five chimpanzees that had not been vaccinated were given the challenge virus. The animals were bled at weekly intervals for 24 weeks and were tested for development of hepatitis B antigenemia, for transaminases, and for development of antibody against hepatitis B surface and core antigen.

All the tests in chimpanzees were carried out in animals, held in isolation in facilities, at the Albany Medical College, International Center for Environmental Safety (ICES) in Alamogordo, New Mexico, under the general supervision of Dr. Kenneth F. Soike.

Results. Tests for safety in chimpanzees. The findings in the four chimpanzees given vaccine intravenously and in the five control animals given live human hepatitis B virus are shown in Table I. All of the control animals developed hepatitis based on two or more indicators. Four of the five animals showed hepatitis B surface antigen in their blood (antigenemia) and two of the five showed transaminase elevations. Two of the chimpanzees developed antibody against the core (probable nucleocapsid) of the virus and four developed antibody against surface antigen. Two of the five showed positive histopathologic findings for hepatitis. By contrast, none of the animals given the vaccine was positive in any of the tests for hepatitis B, indicating lack of presence of infectious virus. The six champanzees given vaccine subcutaneously in the efficacy trials (see Table III) were also monitored for indication of live hepatitis B virus, as described below. None of the chimpanzees given the vaccine intravenously or subcutaneously developed hepatitis A antibody or showed an elevation in hepatitis A antibody titer, ruling out the presence of hepatitis A virus in the vaccine.

Tests for potency. It is seen in Table II that as little as 2 μg of antigen induced antibody in half the guinea pigs after a single dose of vaccine and that 83% (10/12) of the animals developed antibody when three 0.5-μg doses of vaccine were given. The antibody responses in grivet monkeys were less than in guinea pigs. Sixty-seven percent (4/6) of the animals developed antibody after three 2.0-μg doses of vaccine.

Tests for protective efficacy of the vaccine in chimpanzees. Six chimpanzees were given three 20-μg doses of the vaccine subcutaneously at monthly intervals. Figure 1 shows that all but one (836) of the animals developed detectable antibody against hepatitis B

TABLE I. ASSAY FOR INFECTIVITY (SAFETY) OF LOT 559 FORMALIN-TREATED HEPATITIS B VACCINE GIVEN INTRAVENOUSLY INTO SUSCEPTIBLE CHIMPANZEES THAT WERE OBSERVED FOR 40 WEEKS AFTER INJECTION.

Material injected	Chimpanzee	Findings during 40-week observation period[a]				
		Antigenemia (HB_sAg)	Enzyme elevation (SGPT)	Antibody: Surface anti-HB_s	Antibody: Core anti-HB_c	Hepatitis histopathology
Control						
Human hepatitis B live virus given intravenously (1000 chimpanzee infectious doses)	804	+[b]	+	+	+	+
	805	+	+	0	+	+
	806	+	0	+	0	0
	702	+	0	+	0	0
	748	0	0	+	0	0
Test						
Lot 559 vaccine given intravenously, 20 μg/dose	815	0[b]	0	0	0	0
	816	0	0	0	0	0
	817	0	0	0	0	0
	763	0	0	0	0	0

[a] HB_sAg by Ausria II test; anti-HB_s by Ausab test; anti-HB_c test by Drs. Hoofnagle and Schulman; histopathology by Dr. M. J. Iatropoulos (ICES).
[b] Positive (+) at some time(s) during the 40-week period or negative (0) during the entire period.

TABLE II. POTENCY TESTS IN GUINEA PIGS AND IN GRIVET MONKEYS OF LOT 559 FORMALIN-TREATED HEPATITIS B VACCINE.

Vaccine (μg/Dose)	Hepatitis B antibody response after: Dose 1		Dose 2		Dose 3	
	Seroconversion rate	Mean titer	Seroconversion rate	Mean titer	Seroconversion rate	Mean titer
Guinea pig test[a]						
20	12/14[b]	127	10/11	107	10/10	75
4	7/12	9	7/8	36	8/8	52
2	7/14	3	5/11	7	10/10	46
1	4/13	2	5/8	8	8/8	37
0.5	2/14	1	3/14	2	10/12	20
Grivet monkey test[c]						
20	2/6[b]	3.2	2/6	2.5	5/6	22.6
2	0/6	<8	1/6	2.0	4/6	9.0
0.5	0/6	<8	1/6	1.6	2/6	3.6

[a] Doses given subcutaneously on Days 0, 14, and 56.
[b] Antibodies in guinea pig sera determined by Ausure test and by Ausab test in grivet monkey sera.
[c] Doses given subcutaneously on Days 0, 28, and 56.

surface antigen; the titers ranged from 32 to 208 estimated Ausab units (as defined by Abbott Laboratories).

The tests of the bloods from these animals taken at weekly intervals during the 16-week period before challenge served as an additional assay for safety of the vaccine. None of the animals developed hepatitis B antigenemia and none showed increase in transaminase. Further, none of the animals developed antibody against the core antigen of hepatitis B that can result only from infection with live hepatitis B virus.

At the end of the sixteenth week, the six vaccinated chimpanzees and five unvaccinated control animals were challenged with approximately 1000 chimpanzee infectious doses of live hepatitis B virus given intravenously. The animals were followed for 24 weeks for development of hepatitis B virus infection.

Figure 2 shows the findings in a single vaccinated chimpanzee (834) and in an unvaccinated control animal (769). The vaccinated animal showed no indication of hepatitis B infection, as revealed in tests for

TABLE III. FINDINGS IN TESTS FOR PROTECTIVE EFFICACY OF LOT 559 HEPATITIS B VACCINE IN CHIMPANZEES ON CHALLENGE INTRAVENOUSLY WITH HUMAN HEPATITIS B VIRUS AND OBSERVATION FOR 24 WEEKS.

Chimpanzees			Findings after challenge with live virus							
			Antigenemia (HB_sAg)		Enzyme elevation (SGPT)		Antibody development			
							Anti-HB_sAg		Anti-HB_cAg (CF)	
Group	Animal	Anti-HB_sAg antibody prechallenge, yes (+) or no (0)	Week of onset	Weeks duration	Week of onset	Weeks duration	Week of onset	Weeks duration	Week of onset	Weeks duration
Vaccinated animals, 3 doses, subcutaneously	832	+	Neg.		Neg.		Neg.[a]		Neg.	
	833	+	Neg.		Neg.		Neg.		Neg.	
	834	+	Neg.		Neg.		Neg.		Neg.	
	835	+	Neg.		Neg.		Neg.		Neg.	
	837	+	Neg.		Neg.		Neg.		Neg.	
	836	0 (failure)	Neg.		Neg.		18	6	Neg.	
Unvaccinated controls	818	0	10	4	Neg.		13	11	16	
	823	0	8	4	Neg.		12	12	12	
	831	0	11	5	Neg.		18	16	16	
	769	0	13	11	21	2	Neg.		22	2
	768	0	19	5	Neg.		Neg.		Neg.	

[a] No increase in antibody titer.

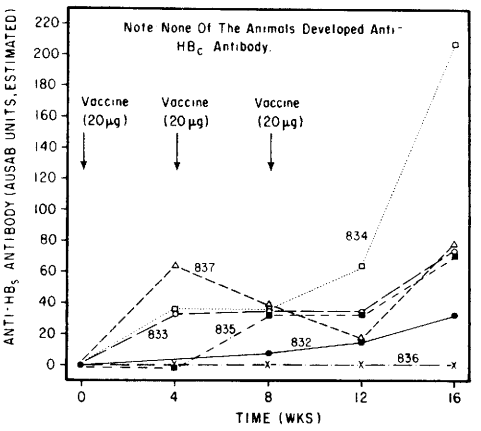

FIG. 1. Hepatitis B antibody response, according to time, in six chimpanzees given three doses, subcutaneously, of 20 μg each (1 ml) of lot 559 vaccine at monthly intervals.

antigenemia, for development of antibody against hepatitis B core antigen, and for biochemical and pathologic evidences of liver involvement. By contrast, the unvaccinated control animal showed hepatitis B antigenemia, developed antibody against hepatitis B virus core antigen, and developed elevated levels of SGOT and SGPT.

The findings in the total number of animals in the test and control groups are given in Table III. The five unvaccinated control animals, all initially devoid of antibody against hepatitis B surface antigen, developed hepatitis with hepatitis B virus antigenemia. One animal showed an elevation in transaminase. This animal and three others (818, 823, and 831) developed antibody against hepatitis B core antigen. Three of the animals developed antibody against the hepatitis B virus surface antigen. By contrast, none of the six vaccinated animals displayed hepatitis B antigenemia, none developed enzyme elevation, and none developed complement-fixing antibody against hepatitis B core antigen.

Discussion. The infectious hepatitis B virus is believed to be the Dane particle (16). The Dane particle, which is 42 nm in diameter, consists of an inner core (nucleocapsid, HB_cAg) and an outer shell of glycoproteins on the virus surface designated HB_sAg. The Australia antigen, measuring 22 nm in diameter and found to circulate in abundance in the sera of certain persons who are infected with hepatitis B virus, is believed to be the hepatitis B virus surface antigen pro-

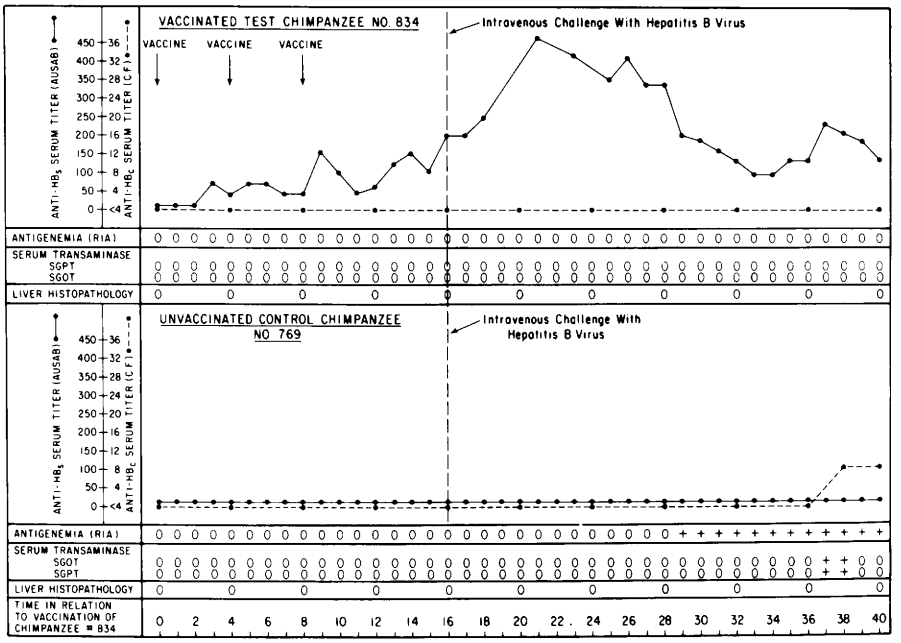

FIG. 2. Chart showing dynamics of viral, pathologic, and serologic responses in a vaccinated and a nonvaccinated control chimpanzee prior to and following intravenous challenge with human hepatitis B virus.

duced by the virus. Highly purified surface antigen comprised the vaccine.

The conventional safety and sterility tests of the vaccine ruled out possible contamination with exogenous microbial and viral agents. The tests in marmosets (8) ruled out possible contamination with human hepatitis A virus. Presently, there is no highly sensitive method for detecting live hepatitis B virus, since it does not propagate in cell culture and chimpanzees represent the best means currently available for detecting possible presence of the agent. Live virus was found absent in tests in the four chimpanzees given the vaccine intravenously and in the six animals given three doses of the vaccine subcutaneously. In additional tests not reported here, none of the nine chimpanzees given 100-ml volumes intravenously of the present and two other lots of vaccine developed evidence of hepatitis B infection by the end of 20 weeks. The relative sensitivity of man as compared with chimpanzees for developing hepatitis B infection is not known and is not likely to be measured for obvious reasons. There are, however, other determinants for safety. The density gradient centrifugation used in the purification process effectively separates the small noninfectious 22-nm Australia antigen particles used to produce vaccine from the 42-nm infectious Dane particles. Further, the conditions used in purification, exclusive of the treatment with formalin, were found to be highly destructive of infectivity of all of a number of viruses tested. Thus, the procedures were shown to inactivate, totally, highly infectious samples of representative examples of a wide number of virus families. These included Vesicular Stomatitis Virus (Rhabdovirus), Newcastle Disease Virus (Myxovirus), Reovirus (Reovirus), Mengovirus (Picornavirus), Herpes Simplex 1 (Herpesvirus), Infectious Bronchitis Virus (Coronavirus), and Human Hepatitis A Virus (likely an Enterovirus-like Virus (17)).

The absence of blood serum proteins and blood group substances in the vaccine gave evidence of a high degree of purity of the product and freedom from contaminating human proteins.

The highly purified and formalin-treated hepatitis B vaccine proved very active in inducing antibody against hepatitis B antigen in guinea pigs, monkeys, and chimpanzees. A dose-response curve was obtained

for each animal species depending upon the amount of antigen given per dose and on the number of doses administered. Most, if not all, vaccinated animals develop antibody against the antigen after three doses of the vaccine given 2 weeks or 1 month apart.

Most importantly, the vaccine given in three doses was highly protective in preventing hepatitis in chimpanzees challenged by intravenous injection with the live hepatitis B virus in infected human plasma. All of the six vaccinated chimpanzees, five of which had developed detectable antibody against surface antigen following vaccine, were protected against development of antigenemia following challenge with the live virus and none showed complement-fixing antibody against core antigen. Antigenemia, aside from enzyme elevation, is considered to be the primary indication for infection with hepatitis B virus in these animals.

The demonstration of protection in chimpanzees against hepatitis B virus by administration of purified formalin-treated hepatitis B surface antigen is consistent with the protection afforded human subjects given crude boiled human plasma in early experiments carried out by Krugman *et al.* (5). The findings in the present study show promise for practical control of hepatitis B by highly purified viral antigen vaccine. Animal studies of purified hepatitis B vaccines along similar lines are being carried out by Purcell *et al.* (personal communication).

Summary. Highly purified hepatitis B virus surface antigen (Australia antigen) purified by physical and chemical procedures from infected human plasma was used to prepare hepatitis B vaccine. The purified antigen was treated with formalin and the vaccine was tested exhaustively for safety by ordinary procedures and additionally in marmosets (for live hepatitis A virus) and in chimpanzees (for live hepatitis B virus). The vaccine was highly potent, inducing antibody in guinea pigs, grivet monkeys, and chimpanzees given three doses of vaccine containing up to 20 μg of hepatitis B antigen per dose. A protective efficacy trial was carried out in chimpanzees that were given three doses of vaccine subcutaneously and then challenged intravenously with 1000 chimpanzee infectious doses of human hepatitis B virus. All of five unvaccinated control animals developed hepatitis B virus antigenemia following challenge and all of six vaccinated animals were protected, including one animal that had failed to develop detectable antibody following vaccination.

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