PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 175, 314–319 (1984)

Vaccine against Human Hepatitis B Virus Prepared from Antigen Derived from Human Hepatoma Cells in Culture (41801)

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Abstract. Vaccine against human hepatitis B was prepared using antigen derived from hepatitis B carrier hepatoma cells grown in the interstices of a Diaflo hollow filter unit. Hepatitis B surface antigen (HBsAg) produced by these cells was purified by immune affinity chromatography, digestion with DNase and pepsin, and Sephadex G-150 separation. The Formalin-treated antigen was formulated in 20-μg dose on alum adjuvant with thimerosal added as a preservative. This cell culture vaccine was as potent as human plasma-derived vaccine as measured in a mouse potency assay. The vaccine proved safe in tests in chimpanzees and in human subjects who were in late stages of cancer of the central nervous system and who were receiving therapy for their condition. None of five subjects who received the vaccine developed untoward clinical reactions. Two of the subjects who received all three doses of vaccine developed antibody against HBsAg. Three persons, two given only the primary doses and one who was given all three doses but was lost to follow-up, demonstrated no response. The slow and relatively low antibody responses to the vaccine were similar to those in other immunosuppressed persons who were given vaccine of human plasma origin.

Human hepatitis B virus cannot be grown at present in cell culture. Current vaccines (1–5) against hepatitis B virus employ surface antigen that is obtained from the plasmas of human carriers of hepatitis B virus infection. The surface antigen stimulates antibody against the virus and prevents infection and illness caused by the agent. Available supply of suitable carrier plasma and the need to apply highly technical procedures to purify antigen and to render it safe limit the amount of plasma-derived vaccine that can be made and impose cost restrictions on its use.

Future alternatives to human plasma include the use of surface antigen produced by recombinant technology or synthetic short-length peptides that are the important immunodeterminants of viral antigen. Another alternative is the use of Alexander's hepatoma cell line (6) that carries the hepatitis B virus genome and that secretes hepatitis B surface antigen into the cell culture fluid.

Studies in our laboratories (7) have led to the development of an artificial capillary system for growing Alexander cells that produces fluids with high content of hepatitis B surface antigen. The purified antigen is similar in size, polypeptide composition, and immunoreactivity to the hepatitis B surface antigen from human plasma origin. A vaccine prepared from this cell culture antigen has been found to be potent in stimulating antibody against hepatitis B virus in animals and man. The findings are presented here.

Materials and Methods. *Cell culture.* Hepatoma cell culture line PLC/PRF/5 isolated by Alexander *et al.* (6) was obtained from Dr. R. J. Daemer of the National Institutes of Health. The cells were grown in the extracapillary space of a Diaflo cartridge (Amicon Corp., Lexington, Mass.) with hollow fibers of 100,000-mol wt cutoff that was connected to a 30-liter New Brunswick fermenter. Eagle's minimum essential medium that was supplemented with 10% calf serum, 50 mg/liter of neomycin, and 2 m*M* L-glutamine was circulated through the capillaries at 37°C. After 18 days the temperature of incubation was reduced to 32°C and 0.1 m*M* caffeine was added to the medium. Fluid was harvested three times per week from the cell side of the capillary units and stored frozen until used to prepare vaccine.

Vaccine preparation. Antigen was purified

0037-9727/84 $1.50

from the culture fluid by immune affinity chromatography as described in McAleer *et al.* (7). Briefly, the procedure used a Sepharose 4B column to which goat antibody against HBsAg was attached. The eluted antigen was concentrated by ultrafiltration and further purification was achieved by digestion with pancreatic DNase at 10 μg/ml for 2 hr at 20–25°C and at pH 2 with pepsin at 5.5 μg/ml for 18 hours at 37°C followed by molecular sieve chromatography using Sephadex G-150. Following treatment with 1:4000 Formalin at 36°C for 72 hr, the antigen was adsorbed to aluminum hydroxide. Formaldehyde was removed by centrifuging the alum-adsorbed antigen and resuspending that precipitate in physiological saline to give a final product containing 20 μg/ml of HBsAg and 0.5 mg/ml of aluminum. Thimerosal at a dilution of 1:20,000 was added as a preservative. Additional details of cell culture and vaccine preparation are given in McAleer *et al.* (7).

In vitro assays. Radioimmune (RIA) assays (Abbott Laboratories) were used to measure HBsAg (Ausria II test), antibody against HBsAg (anti-HBs, Ausab test), and antibody against hepatitis B core antigen (anti-HBc, Corab test). DNA was measured by the method of Kapuscinski and Skoczylas (8), DNA polymerase according to Kaplan *et al.* (9), DNase by the method of Kunitz (10), bovine serum albumin by the complement-fixation assay of Osler *et al.* (11), and blood group substances according to the U.S. Code of Federal Regulations (21CFR680.22). Serum aspartate amino transferase (AST), serum alanine amino transferase (ALT), and pepsin were assayed following the manufacturer's instruction (Sigma–Frankel No. 505, Boehringer-Mannheim and Wortherington, respectively).

Infectivity for chimpanzees. Although PLC/PRF/5 cells are known not to produce infectious hepatitis B virus (12), tests were nevertheless carried out in chimpanzees on the aqueous vaccine before adsorption to alum. In the test, four chimpanzees weighing 30 to 50 kg each were selected for study based on negative findings in tests for HBsAg, anti-HBs, anti-HBc, elevation of transaminases, liver histopathology, and tuberculin reactions. Each of two animals was injected intravenously with 1.0 ml and the other two animals with 10.0 ml of aqueous unadsorbed vaccine. The chimpanzees were bled at weekly intervals during the 24-week followup period and sera were tested for presence of HBsAg, anti-HBs, anti-HBc, and for amounts of AST and ALT. Liver biopsies were taken monthly for histopathologic observations followed by sectioning and staining with H&E. The sections were examined by Dr. A. Phelps of these laboratories.

Tests in mice. An extinction assay for potency in mice (13) was performed, compared with that of a standard vaccine prepared for human hepatitis B plasma. In the test, groups of 10 5-week-old ICR/Ha mice propagated in these laboratories were given a single 1-ml injection intraperitoneally of serial fourfold dilutions of cell culture or human plasma vaccine in alum diluent. The mice were bled individually and tested for serum antibody level 4 weeks later. The amount of antigen needed to seroconvert 50% of the animals (ED_{50}) for each preparation was calculated by probit analysis (14) and the geometric mean antibody titer responses to the vaccines were determined.

Results. *Properties of the antigen.* The purified antigen, prior to adsorption to alum, was shown to be free of detectable DNA, DNA polymerase, DNase, pepsin, bovine serum albumin, and blood group substances, and was shown to be nonpyrogenic in rabbits. The physical and immunologic properties of the purified antigen were indistinguishable from the plasma-derived antigen. The content of antigen as measured by radioimmune assay was the same as that measured by the Lowry method (15) and the product consisted of essentially pure antigen. The UV spectrum was typical of purified HBsAg with an absorption maximum of 280 nm and an extinction coefficient ($\epsilon^{1\%}$) of 52. Electron microscopy, as shown in Fig. 1, revealed a uniform field of 22-nm particles.

Immunizing potency for mice. Table I shows the seroconversion rates and geometric mean titers for the cell culture vaccine compared with that for vaccine of human plasma origin. The ED_{50} values were essentially identical and the mean titer values for antibody were roughly the same.

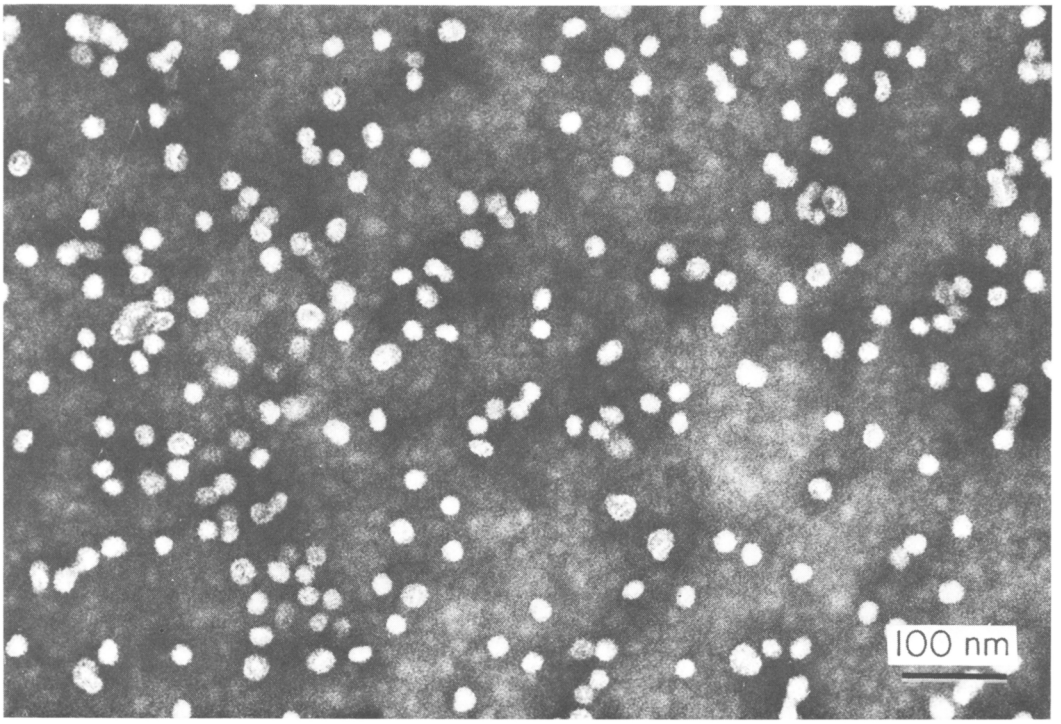

FIG. 1. Electron micrograph of HBsAg particles produced in cell culture.

Safety findings in chimpanzees. All four chimpanzees used in the tests presented negative findings for HBsAg, anti-HBs, and anti-HBc. All tests for HBsAg and for anti-HBc remained negative for the 24-week observation period and there was no significant elevation in transaminases. Two of the four animals (one which had received a 1-ml dose, one a 10-ml dose) developed antibody against the surface antigen, as commonly noted in tests of plasma-derived vaccine. The liver histopathologic findings were normal prior to injection of vaccine and remained normal during the 24-week observation period.

TABLE I. MOUSE POTENCY OF HBsAg VACCINE PREPARED IN CELL CULTURE COMPARED WITH THAT PREPARED FROM INFECTIOUS HUMAN HEPATITIS B PLASMA

	Cell culture vaccine		Plasma vaccine lot 799-2	
Vaccine (μg)	No. mice positive/total	G.M.[a] titer	No. mice positive/total	G.M.[a] titer
10	8/10	1431	8/10	1729
2.5	8/10	504	9/10	1204
0.625	7/10	74	4/10	8
0.156	0/9	<8	0/10	<8
ED_{50}[b]	0.79 mcg		0.81 mcg	

[a] Geometric mean titer.
[b] Dose required to seroconvert 50% of mice.

Tests in man. Five patients who attended the Neuro-Oncology clinic at the Hospital of the University of Pennsylvania and who were negative for all human hepatitis B markers were included in the study. All persons in the study were receiving periodic therapy for advanced neoplastic disease (chemotherapy, radiation, and steroids) and all gave informed written consent. Two of the five subjects received all three doses of vaccine at 0, 1, and 6 months. The remaining received one, two, or three doses of vaccine and were lost to further study because of ill health or change of residence. It was intended that blood samples would be taken prior to and 1, 2, 3, 6, and 7

TABLE II. FINDINGS IN TWO CANCER PATIENTS WHO RECEIVED CELL CULTURE HEPATITIS B VACCINE AT TIME 0, 1, AND 6 MONTHS

		Titer[a]					
			Months after vaccination				
Observation	Patient	Prevaccine[b]	1	2	3	6	7
Anti-HBs	717-4	<8	<8	8	16	16	36
	-6	<8	<8	<8	ND	<8	36
HBsAg	717-4	—	—	—	—	—	—
	-6	—	—	—	—	—	—
Anti-HBc	717-4	—	—	—	—	—	—
	-6	—	—	—	—	—	—
AST	717-4	18	25	16	24	16	14
	-6	9	7	8	ND	9	6
ALT	717-4	19	23	24	21	22	18
	-6	24	20	20	ND	17	12

[a] Titer is expressed in units. ND, no determination; —, below the limit of detection.
[b] Seven days prior to starting vaccination.

months after vaccination but these specimens were available only from two subjects who received all three doses of vaccine.

As shown in Table II, the two persons who received all 3 doses of vaccine including the booster dose and who were bled at the appropriate time intervals were found to respond; the anti-HBs titer was 36 estimated Ausab units for both. None of the others who did not complete the study showed any serologic response in tests of the available serum specimens. The HBs and anti-HBcAg markers for hepatitis B infections remained negative in all five patients and there was no significant elevation in AST or ALT.

The five patients given vaccine presented only minimal or no objective or subjective indications of local or systemic reactions to the vaccine.

Discussion. These findings have demonstrated the practical feasibility for preparing human hepatitis B vaccine from antigen derived from Alexander cells employing Diaflo capillary filter units for cell propagation. Purity of the vaccine and its potency for mice were equivalent to those of vaccine of human plasma origin. Trials in man have been limited to date to patients with cancer. Cancer patients, who are on customary therapy and who are immunosuppressed, respond poorly to vaccine of human plasma origin (Dr. C. Stevens, personal communication) and also respond poorly to the Alexander cell vaccine as shown in the present study.

The use of antigen from hepatitis B carrier cells that are hepatoma-derived poses important questions of safety. Tabor *et al.* (12) have reported inability to detect live infectious hepatitis B virus in tests in chimpanzees. Experiments by others (16, 17) have failed to detect intact hepatitis B virus by electron microscopy and by sensitive serologic techniques. In addition, hepatitis Be antigen and hepatitis B virus-specific DNA polymerase, both of which are closely associated with intact hepatitis B virus, were not found in concentrated Alexander cell culture fluids. The hepatitis B vaccine antigen, tested prior to adsorption to alum, was similarly shown to be free of detectable infectious hepatitis B virus in the present experiments.

The process used to prepare hepatitis B vaccine from cell culture fluid was designed to exclude or minimize the amount of DNA that might be present in the initial culture fluid. The highly sensitive diaminophenylindole

(DAPI) reagent for measuring DNA permits detection of no less DNA than 1/400 the total amount of protein. In the present study, the initial harvest fluid from cell cultures contained about 3000 μg/ml of protein and the test for DNA was negative, indicating a theoretical maximum of 7.5 μg/ml of DNA. An estimate of reduction of DNA during the process of purification was measured using radiolabeled DNA from Alexander cells. It was found that there was a $10^{4.1}$-fold removal of DNA in the affinity chromatography step and $10^{1.9}$-fold removal in the enzyme treatment and molecular sieve chromatographic steps giving a total 10^6-fold reduction in DNA. Thus, an initial crude culture fluid containing 7.5 μg of DNA/ml would contain less than 0.0000075 μg of DNA/ml following purification. It may be noted that the hepatitis viral DNA makes up no more than one part in 100,000 of the hepatoma cell genome (18), reducing the theoretical amount of hepatitis B viral DNA to only one hundred thousandth that of the last figure. The DNase treatment is intended to reduce to small fragments any residual intact viral or cellular DNA that might be present. Autoradiography of DNase-treated samples of cellular DNA revealed that essentially all the DNA was reduced to fragments comprising less than 72 base pairs (bp). A segment of DNA, to be oncogenic, must be of substantial length. Graham *et al.* (19) found that the smallest fragment of adenovirus DNA that could transform primary rat kidney cells was 1500 bp long. They also found that 10^{11} molecules of DNA were required for transformation. The ras gene of Harvey murine sarcoma virus, an RNA oncogene, contains about 1100 nucleotides (20). It may be concluded that the purification and DNA-destructive processes aimed at DNA used to prepare the vaccine were of such nature as to delete any possible oncogenic DNA that might have been present in the starting fluid.

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Received September 2, 1983. P.S.E.B.M. 1984, Vol. 175.
Accepted November 11, 1983.