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STUDIES ON DELAYED HYPERSENSITIVITY TO HEPATITIS B ANTIGEN IN CHIMPANZEES

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SUMMARY

Twenty-eight chimpanzees were divided into six groups according to their history of previous immunization or exposure to hepatitis B antigen (HBAg) and studied for delayed hypersensitivity (DH) to HBAg. Purified HBAg derived from a normal human carrier was used for *in vivo* skin testing and *in vitro* leucocyte migration inhibition tests. Of seventeen chimpanzees immunized with HBAg in Freund's complete adjuvant (FCA), nine exhibited positive DH reactions to HBAg with good correlation between the *in vivo* and *in vitro* responses. Of the seventeen chimpanzees, fourteen also exhibited positive DH reactions to purified protein derivative of tuberculin (PPD) with marked erythema and induration; the other three exhibited only erythema with no induration. None of the seventeen animals exhibited any immediate reactivity to either HBAg or PPD. Intradermal injection of HBAg-negative human serum failed to elicit DH reactions in four animals who showed positive skin test with purified HBAg; the DH response was thus probably HBAg-specific. Nineteen chimpanzees, including six unimmunized animals, three chronic carriers of HBAg and two which had been injected with HBAg without FCA, failed to show DH response to HBAg. Thus, DH to HBAg was observed only in animals hyperimmunized with HBAg in FCA.

INTRODUCTION

Despite historical failures, the intimate association between hepatitis B antigen (HBAg, previously called HAA, SH or Au) and hepatitis B virus has stimulated renewed efforts to propagate the infectious agent in tissue culture and in laboratory animals. As a result, inapparent infection has been induced in some nonhuman primates (Hillis, 1961; Hepatitis Surveillance Report, 1967; Maynard, Berquist & Krushak, 1972; Prince, 1972; London

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et al., 1972). Of these, chimpanzees have proved to be the most useful model since biochemical and histopathological abnormalities resembling human viral hepatitis occur in these animals during infection with hepatitis B virus (Hillis, 1961; Hepatitis Surveillance Report, 1967; Maynard *et al.*, 1972; Prince, 1972). Furthermore, the HBAg chronic carrier state resembling that in man has been observed in many animals (Prince, 1971; Hirschman *et al.*, 1969), and has been induced in one chimpanzee by transfusing plasma from an HBAg carrier chimpanzee (Prince, 1972). Our interest in delayed hypersensitivity (DH) to HBAg in chimpanzee grew from the above observations. Investigation of DH to HBAg in man may be useful in the elucidation of the immunopathological mechanisms and the underlying basis for the chronic HBAg carrier state. Use of *in vitro* correlates of DH requires demonstration of a correlation between the results of these and of delayed skin test hypersensitivity reactions. The group of chimpanzees with known history of previous immunization, provided an opportunity to determine whether such a correlation can be demonstrated in a primate species. It does not appear desirable at the present time to perform *in vivo* skin testing with HBAg in man due to the hazard of infection.

MATERIALS AND METHODS

Antigens

HBAg subtype *ad* (Le Bouvier, 1971) was isolated from the plasma of a normal healthy carrier by a combination of isopycnic banding and rate zonal centrifugation in caesium chloride gradients (Bond & Hall, 1972). The first peak containing 20 nm particles, devoid of human plasma proteins as detected by double diffusion in gels, was used as purified HBAg for all experimental work (Vyas *et al.*, 1972). The purified HBAg gave positive DH skin reaction in guinea-pigs previously sensitized against HBAg, but failed to show such a reaction in guinea-pigs sensitized with normal human serum (Ibrahim, Adelberg & Vyas 1972). Purified protein derivative of tuberculin (PPD), without preservatives was obtained from the Central Veterinary Laboratory, Ministry of Agriculture Fisheries and Food, New Haw, Weybridge, Surrey, England, and used as a control antigen for DH reactions. HBAg-negative serum was obtained from a normal healthy donor.

Chimpanzees

Both male and female chimpanzees between the ages of 3 and 10 years and weighing 35 to 160 lb were used in this experiment. The chimpanzees were housed in separate cages in a special facility at the Sterling Forest Laboratory for Experimental Medicine and Surgery in Primates of the New York University Medical Center. Twenty-two chimpanzees, previously immunized with HBAg (by A.M.P.) and six unimmunized chimpanzees were made available for this study. Based on given history of immunization, the chimpanzees were divided into six groups (Table 1). Sernylan (0·25 mg per kg body weight) was used intramuscularly to anaesthetize the animals before skin test and withdrawal of 50 ml heparinized blood.

Skin tests

Test for DH was carried out by intradermal injection of 100 μg purified HBAg suspended in 0·1 ml of normal saline on one side above the upper lip. A similar injection of PPD 100 μg/0·1 ml was given on the other side of the upper lip. The skin tests were read at 6–8 hr for immediate reactivity and at 24 hr for the DH response. The cutaneous reactivity was

quantitated by measuring the diameter of erythema in millimetres and estimating induration by measuring skin fold thickness in millimetres at the test site minus the normal skin fold thickness (approximately 8 mm). Skin tests were repeated on each animal after an interval of 1 week.

In vitro leucocyte migration inhibition tests

The capillary migration inhibition test (Rosenberg & David, 1971) was performed on peripheral blood leucocytes using approximately 100 μg of HBAg or PPD in medium 199 with Hanks's salts (Grand Island Biological Company) containing 10% normal horse serum

TABLE 1. Grouping of chimpanzees according to history of previous immunization to HBAg

Group number	Chimpanzee number	Treatment
I	20, 31, 116	Chimpanzees numbers 20 and 116 were spontaneous carriers of HBAg type *ad*. The carrier state was induced in chimpanzee number 31 by transfusion of HBAg type *ad*-positive chimpanzee plasma
II	12, 37, 39, 40, 41, 45, 65	Injected with HBAg-positive chimpanzee plasma in FCA and pooled human purified HBAg type *ad* and *ay* in FCA
III	33, 43, 46, 47, 53, 54, 61	Injected with human purified HBAg type *ad* or *ay* in FCA
IV	25, 27, 29	Transfused with HBAg-positive chimpanzee plasma and injected with human purified HBAg type *ad* and *ay* in FCA
V	23, 38	Transfused only with HBAg-positive chimpanzee plasma
VI	49, 50, 55, 60, 63, 73	Unimmunized normal controls

(Microbiological Associates, Bethesda, Maryland). Leucocyte migration tests were performed in duplicate at each time with one capillary in each chamber containing antigen; similarly, duplicate tests were performed in the absence of antigen. After incubation at 37°C for 24 hr, the area of migration in each test, projected by an EPOI LP-6 profile projector (Ehrenreich Photo-Optical Industries, Garden City, New York), was traced on paper and measured by planimetry. The migration index was derived from the average area of migration in the presence and in the absence of antigen. Agreement between the duplicate capillaries was usually within $\pm 6\%$ of the average area of migration. An index of 0·80 or less was considered significant. The procedure was repeated on each animal after an interval of 1 week.

Haemagglutination assay

Tests for humoral immune response to HBAg was performed by haemagglutination assay for anti-HBAg after absorption of the defibrinated chimpanzee plasma, using haemagglutination assay (Vyas & Shulman, 1970). HBAg was detected by inhibition of haemagglutination (Vyas & Shulman, 1970).

TABLE 2. Results of *in vivo* and *in vitro* testing of delayed hypersensitivity (DH) to hepatitis B antigen (HBAg) and PPD in chimpanzees

Group number	Chimpanzee number	24-hr skin reaction to HBAg E†/I‡		Migration index in presence of HBAg		24-hr skin reaction to PPD E†/I‡		Migration index in presence of PPD	Anti-HBAg
		I test	II test	I test	II test	I test	II test		
I*	20	0/0	0/0	0·89	0·98	0/0	0/0	NT	0
	31	0/0	0/0	1·01	1·12	0/0	0/0	NT	0
	116	0/0	0/0	0·97	0·89	0/0	0/0	NT	0
II	12§,††	15/4	20/5	1·02	0·75	22/5	20/5	NT	3200
	37	0	0	1·01	1·03	20/5	15/4	NT	16
	39**	15/4	15/4	0·63	0·72	20/4	15/4	0·61	1600
	40**	20/5	20/4	0·71	0·67	18/5	15/4	NT	400
	41¶	0/0	15/4	1·00	0·99	15/3	20/5	NT	3200
	45	0/0	0/0	1·30	1·21	15/0	10/0	NT	12,800
	65	0/0	0/0	1·02	1·11	20/4	20/5	NT	1600
III	33††	0/0	0/0	1·01	0·99	20/5	20/4	NT	32
	43**	15/3	15/4	0·67	0·67	20/3	15/4	0·55	6400
	46	15/4	15/4	0·72	0·65	15/4	15/4	NT	6400
	47	0/0	0/0	1·02	1·11	20/4	15/4	NT	400
	53**	18/3	18/4	0·68	0·68	20/4	20/4	NT	6400
	54	15/4	15/4	0·66	0·75	20/4	15/4	NT	6400
	61	0/0	20/4	1·03	0·80	20/4	20/4	NT	6400

IV	25††	0/0	0/0	1·11	1·00	15/0	10/0	NT	128
	27	0/0	0/0	0·98	1·00	12/0	15/0	NT	1600
	29§,††	20/5	15/4	1·01	0·80	15/5	22/6	NT	3200
V	23	0/0	0/0	1·10	1·03	0/0	0/0	NT	0
	38	0/0	0/0	1·02	1·00	0/0	0/0	NT	64
VI	49	0/0	0/0	1·10	1·03	0/0	0/0	NT	0
	50	0/0	0/0	1·00	1·02	0/0	0/0	1·03	0
	55	0/0	0/0	1·12	1·10	0/0	0/0	0·98	0
	60	0/0	0/0	0·97	0·89	0/0	0/0	NT	0
	63	0/0	0/0	1·00	1·01	0/0	0/0	NT	0
	73	0/0	0/0	1·02	1·03	0/0	0/0	NT	0

* Animals in this group were carriers of HBAg.
† Erythema diameter in millimetres.
‡ Induration—skin fold thickness at the test site in millimetres minus the normal skin fold thickness (approx. 8 mm).
§ Discrepancy between skin reaction and migration index was resolved in repeat test.
¶ Discrepancy between *in vivo* and *in vitro* DH response to HBAg.
** Chimpanzees who gave no delayed reaction to HBAg-negative human serum.
†† Chimpanzees who were injected with human serum proteins that gave delayed reaction to HBAg-negative human serum.
NT = not tested.

RESULTS

Ten of seventeen chimpanzees from Groups II, III, and IV exhibited erythema and induration characteristic of positive DH reaction at 24 hr (Table 2) following injection of the purified HBAg. None of the seventeen animals, however, showed any skin reaction 6–8 hr after injection of either HBAg or PPD. Correlation between *in vivo* and *in vitro* response to HBAg exhibiting positive skin test and significant inhibition of leucocyte migration was good in most of these animals. However, chimpanzees numbers 12, 29 and 41 exhibited an apparent discrepancy between *in vivo* and *in vitro* response to HBAg at least once out of two tests carried out at an interval of 1 week. Fourteen of seventeen chimpanzees from Groups II, III and IV also exhibited positive DH reactions to PPD with marked erythema and induration. The other three showed only erythema with no induration. Two animals exhibiting positive DH reaction to PPD (numbers 39 and 43) and two showing no reaction to PPD (numbers 50 and 55), exhibited good correlation with leucocyte migration inhibition test using PPD (Table 2). Chimpanzees numbers 39, 40, 43 and 53 who gave a positive DH reaction to HBAg, were also tested with 0·1 ml of a 1:30 dilution of HBAg-negative human serum. No reactions to human serum were observed. However, chimpanzees numbers 12, 25, 29 and 33 with known exposure to human serum proteins in CFA gave a positive DH reactions to 0·1 ml of 1:30 dilution of HBAg-negative human serum. Of these four animals, two (numbers 12 and 29) gave a positive DH reaction to HBAg with marked erythema and induration whereas the other two (numbers 25 and 33) did not give any such reaction to HBAg. The chimpanzees from Groups I, V and VI did not show DH reaction to either HBAg or PPD by both *in vivo* and *in vitro* tests.

All animals from Groups II, III, IV and V which had been injected with HBAg exhibited antibody to HBAg except for one animal in Group V (Table 2). The three chimpanzees in Group I showed HBAg, but no antibodies. The six chimpanzees in Group VI exhibited neither HBAg nor anti-HBAg.

DISCUSSION

The failure of HBAg to elicit DH skin reaction in guinea-pigs sensitized against normal human serum was considered adequate assurance of the purity of HBAg used for DH testing. Since skin testing with HBAg-negative human serum elicited DH reaction in four chimpanzees (numbers 12, 25, 29 and 33) previously sensitized with human serum proteins as well as purified human HBAg, but failed to elicit DH response in the four animals (numbers 39, 40, 43 and 53) sensitized to HBAg alone, the response observed with HBAg was considered specific. Additional animals were not tested with human serum to prevent unnecessary immunization with heterologous proteins. HBAg without FCA injected into chimpanzees failed to induce DH to HBAg. It is not clear why only nine out of seventeen chimpanzees immunized with HBAg in CFA exhibited *in vivo* and *in vitro* DH to HBAg. It is possible, however, that the route of injection, the relative amounts of HBAg and CFA in the preparation might have influenced the DH response to HBAg since the initial experiments were not designed to induce DH in these chimpanzees. It is important to note that the three chronic carriers of HBAg did not exhibit any DH response to HBAg. Since leucocyte migration inhibition in man is considered a good correlate of DH to several antigens in man (Soborg & Bendexin, 1967; Rosenberg & David, 1971), Ito *et al.* (1972) demonstrated *in vitro*

leucocyte migration inhibition to HBAg in man. However, *in vivo* and *in vitro* correlation of DH to HBAg has been reported only in guinea-pigs (Ibrahim *et al.*, 1972).

Chimpanzees injected with HBAg in FCA showed high antibody titres when compared with those injected with HBAg alone. However, no correlation between DH response and humoral antibody response was observed in chimpanzees. It was noteworthy that animals with low titre (number 40 with 1:400) to high titres (numbers 53 and 54 with 1:6400) of antibody gave positive DH reaction whereas other animals with similar or higher antibody titres (numbers 45 and 47) did not give any immediate or delayed reactions to HBAg. Therefore, it appears unlikely that the DH reactions to HBAg observed in chimpanzees were due to antigen–antibody complexes.

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