INFECTION AND IMMUNITY, Jan. 1975, p. 137-141

Vol. 11, No. 1
Printed in U.S.A.

Immune Response to Hepatitis B Surface Antigen

ALI B. IBRAHIM, GIRISH N. VYAS,* AND HERBERT A. PERKINS

University of California School of Medicine, San Francisco, California 94143, and Irwin Memorial Blood Bank, San Francisco, California 94118*

Received for publication 19 August 1974

A total of 69 persons were investigated for assessment of cell-mediated and humoral immunity to hepatitis B surface antigen (HBsAg). Three groups, each consisting of 20 normal persons, 20 HBsAg carriers, and 20 convalescent hepatitis B patients, were studied for HBsAg, anti-HBs, and leukocyte migration inhibition with purified HBsAg. Sequential sampling of an additional group of nine acute hepatitis B patients defined the cellular and humoral immune response to HBsAg. The antigen was eliminated rapidly by mounting of cell-mediated immune response detectable for a limited period, followed by antibody response in relatively few patients more than 3 months after clearance of circulating HBsAg.

With the recognition of hepatitis B surface antigen (HBsAg, originally designated Australia antigen; 2) as an immunological marker of hepatitis B virus, a better understanding of the natural history and the epidemiology of hepatitis B results from application of more sensitive and specific serologic methods for the detection of HBsAg and anti-HBs (1, 3, 5, 14, 17, 20, 23, 25). The immunological mechanisms in a spectrum of clinical manifestation of hepatitis B virus infection could be studied by assessing the cellular and humoral immune response to HBsAg. Although the results of recently published work with single patient samples have enhanced our understanding of the immunological mechanisms in hepatitis B, certain disparate results may in part be due to technical differences, to the nature of the antigen used in the assays, to limitation of the patient population studied, to failure of obtaining serial samples, and to lack of simultaneous testing of cellular and humoral immunity (4, 6, 7, 8, 9, 13, 16, 18, 24). In this study, cell-mediated immune response was measured in vitro by leukocyte migration inhibition by purified and immunochemically characterized HBsAg, and humoral immune response was measured by a sensitive and specific hemagglutination assay for antibody. In the first phase of the investigation, a point study of 20 convalescent hepatitis B patients, 20 asymptomatic HBsAg carriers, and 20 controls was carried out. The findings led to an investigation of sequential specimens from nine patients giving results that appear to define the cellular and humoral immune response to HBsAg during the course of and after hepatitis B virus infection.

MATERIALS AND METHODS

Selection of patients. Three groups were examined in the first stage of our study (Table 1). Groups A and B each consisted of 20 age- and sex-matched, healthy, adult voluntary blood donors in an excellent state of health. Routine examination of their sera for serum glutamic pyruvic transaminase, Immunoglobin G (IgG), IgA, IgM, and hemolytic complement ($C'H_{50}$) levels revealed them to be within normal limits established for our clinical laboratory. Subjects in group A were negative for HBsAg and anti-HBs, whereas subjects in group B were consistently positive for HBsAg and negative for anti-HBs over a period of at least 3 months. Group C consisted of patients convalescing from an acute episode of typical hepatitis B who had a loss of HBsAg from their serum during a period of 2 to 12 weeks before their immunological evaluation. A majority of the patients were attending the hepatitis clinic at the San Francisco General Hospital. None of the patients gave a history of transfusion or parenteral exposure to drugs.

Purified HBsAg. Large volumes of ACD plasma of an asymptomatic healthy carrier of subtype *adw* were derived by plasmapheresis and stored at −20 C until used for purification of HBsAg by zonal ultracentrifugation. The purification was carried out by two successive runs of isopycnic banding in cesium chloride gradients followed by a rate sedimentation separation of the 20-nm particles from the filamentous forms and larger Dane particles. Immunochemical characterization of the purified HBsAg particles devoid of human plasma proteins has been described previously (22). The purified HBsAg not only consisted of the 20-nm particles of HBsAg, but also lacked the deoxyribonucleic acid (DNA) polymerase activity associated with the core of the Dane particles (11). The purified HBsAg (20-nm particles at a concentration of 1 mg/ml in saline) was kept frozen in small samples and used for in vitro assays throughout the 2-year period of the investigation. Before use, the

TABLE 1. *Results of cell-mediated and humoral immunity to HBsAg*

Test	Patients		
	A (Normal controls)	B (Chronic carriers)	C (Convalescents from hepatitis B)
No. tested	20	20	20
HBsAg-positive	0	20	2[a]
Anti-HBs	0	0	3 (C_1 (1:4), C_7 (1:4), C_8 (1:32))
Positive LMI	0	0	3 (C_2 (0.69), C_3 (0.52), C_6 (0.67))
Repeat LMI			2 (C_3 (0.99), C_6 (1.13))

[a] These two samples were negative in counter electrophoresis and hemagglutination inhibition but were positive in radioimmunoassay performed retrospectively.

antigen was diluted so that a 0.1-ml sample contained 30 μg of HBsAg.

Leukocyte migration inhibition assays. A 40-ml sample of blood was collected with a disposable plastic syringe containing 0.4 ml of phenol-free heparin, and the erythrocytes were allowed to sediment at 37 C. Leukocyte-rich supernatant plasma was aspirated, and the cells were used for an in vitro leukocyte migration inhibition (LMI) test in the presence and absence of HBsAg. The LMI assay, in duplicate, was performed by the method of Rosenberg and David (19). A predetermined optimal dose of 30 μg of HBsAg in medium 199 with Hanks salts (Grand Island Biological Co. Grand Island, N.Y.) with 10% normal horse serum (Microbiological Associates, Bethesda Md.) was used. Each chamber contained only one capillary tube. After incubation at 37 C for 24 h, the area of migration in each test was projected with an EPOI-LP-6 profile projector, traced on paper, and measured by planimetry. The migration index was derived from the average area of migration in the presence and absence of HBsAg. A migration index of less than 0.8 was considered significant. All positive LMI tests were repeated at an interval of 2 to 3 weeks.

Serological methods. Presence of HBsAg was detected by counterelectrophoresis (10) and by hemagglutination inhibition (21). When counterelectrophoresis and hemagglutination inhibition were negative, a solid-phase sandwich radioimmunoassay (15) kit was used for confirmation (AUSRIA, Abbott Laboratories, Chicago Ill.). Tests for antibodies to HBs were carried out by titration of sera with inert indicator erythrocytes coated with purified HBsAg (21). Titers less than 1:4 were not taken into consideration unless the titer showed a definite rise in a subsequent specimen.

RESULTS

The results of cell-mediated immunity to HBsAg assessed by LMI assay and humoral immune response assessed by antibody titration with HBsAg-coated cells are depicted in Table 1. Cell-mediated immunity was not demonstrable in any of the 20 controls or in the 20 chronic carriers of HBsAg. However, the LMI was positive in subjects C_2, C_3, and C_6, who were convalescing from hepatitis B. Subject C_2 was not available for repeat testing, but the repeat LMI tests in the other two subjects turned negative within a 2- to 3-week period. It was noteworthy that subjects C_2 and C_3 were negative for HBsAg when tested by hemagglutination inhibition but positive in radioimmunoassay at the time their LMI indexes were 0.69 and 0.52, respectively. The distribution of LMI indexes in each group are graphically presented in Fig. 1. Three subjects, C_1, C_7, and C_8, showed anti-HBsAg 11 to 12 weeks after conversion to an HBsAg negative test. Repeat LMI tests 3 weeks later on C_3 and C_6 were negative. Thus, the results in Table 1 indicate the transient nature of LMI and late occurrence of antibodies in a minority of patients in group C. Simultaneous occurrence of anti-HBs and HBsAg or LMI was never observed in any subject.

During the second stage of our study, six patients with hepatitis B were followed up as closely as possible from the time of onset of clinical disease and diagnostic detection of HBsAg at intervals as detailed in Table 2. Three additional patients, 7, 8, and 9, from the hepatitis clinic were not available for full study; however, their results also were consistent with the fact the LMI test becomes transiently positive 4 to 8 weeks after onset of acute disease, concomitant with clearing of the antigen from the circulation. All patients recovered from the acute epidose of hepatitis without clinical, sero-

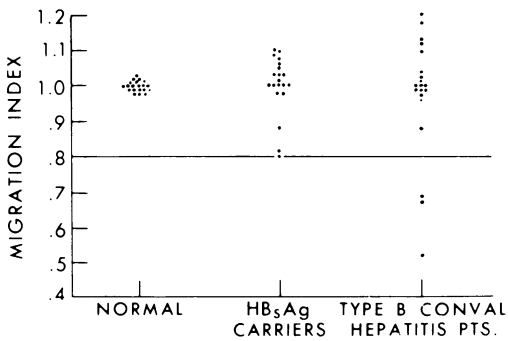

FIG. 1. *Results of the leukocyte migration inhibition test with HBsAg. Conval pts, convalescent patients.*

TABLE 2. *Results of a study of sequential specimens of patients with hepatitis B*

Patient no.	Clinical onset	Dates tested	Migration index	HBsAg	Anti-HBs	SGPT[a] units	Total mg of bilirubin/ 100 ml
1	30 Apr. '72	19 May	1.10	+	–	297	1.8
		31 May	1.03	+	–	745	4.0
		29 June	0.05	$+_w$[b]	–	30	1.5
		20 July	0.81	–	–	18	1.6
		16 Feb.	NT[c]	–	1:32	12	1.5
2	20 Oct. '72	23 Oct.	NT	$+_w$	–	910	9.6
		31 Oct.	NT	+	–	960	7.2
		8 Nov.	NT	+	–	192	4.5
		13 Nov.	1.00	+	–	96	2.8
		28 Nov.	0.43	+	–	37	2.3
		11 Jan.	1.04	–	–	19	1.0
3	10 Dec. '72	17 Dec.	NT	+	–	1350	1.1
		4 Jan.	1.03	+	–	1280	5.4
		17 Jan.	1.20	+	–	1680	18.0
		12 Feb.	1.07	+	–	160	3.1
		12 Mar.	0.64	–	–	34	0.35
		8 Aug.	1.03	–	–	23	0.12
4	27 Nov. '72	14 Dec.	NT	+	–	40	2.4
		17 Jan.	1.00	+	–	1590	2.5
		28 Feb.	1.04	–	–	45	1.2
		8 Aug.	NT	–	1:4	NT	NT
5	30 Oct. '72	29 Nov.	1.20	+	–	220	4.6
		12 Dec.	0.78	+	–	15	1.2
		27 Dec.	1.01	–	–	16	1.1
		3 Oct.	NT	–	–	NT	NT
6	Feb. '73	16 Mar.	1.08	+	–	225	18
		10 Apr.	0.67	–	–	62	3.1
		24 Apr.	0.98	–	–	34	2.0
		17 May	1.03	–	–	23	1.1
		8 Oct.	NT	–	1:16	NT	1.1
7	First HBsAg (-ve)	9 July	0.52	$+_w$	–	21	0.6
	2 July '73	2 Aug.	0.99	NT	–	NT	1.2
8	First HBsAg (-ve) date	22 June	0.67	–	–	15	0.9
	15 June '73	6 July	1.13	–	–	NT	NT
9	First HBsAg (-ve) date	24 Apr.	0.69	$+_w$	–	39	0.7
	16 Apr. '73						

[a] Serum glutamic pyruvic transminase.

[b] $+_w$, Weak positive, i.e., negative in counterelectrophoresis and hemagglutination inhibition but positive in radioimmunoassay.

[c] NT, Not Tested.

logical (HBsAg), or biochemical (serum glutamic pyruvic transaminase levels) evidence of chronic liver disease. Antibody response was observed inconsistently and only after clearance of the antigen. It must be mentioned that the LMI value of 0.05 in patient 1 (Table 2) is unusually low, but it was considered a true positive because in duplicate tests the results were identical and the control tests without the antigen showed normal migration.

Based on the results in Tables 1 and 2, one can generalize the picture of immunological events in acute hepatitis B infection as shown in Fig. 2. In natural infection, the immune response to HBsAg appears to be divided into three phases. In the first phase, the clinical

hepatitis is observed with occurrence of HBsAg in the serum. In the second stage, the removal of HBsAg appears to be a cell-mediated event heralded by detection of positive LMI which reverts to negative status within a short span of 3 to 6 weeks. In the third stage, occurring several weeks to months after disappearance of HBsAg, antibody may or may not appear.

DISCUSSION

The immunological phenomena which permit chronic asymptomatic persistence of HBsAg in apparently healthy individuals have remained obscure, despite the hypothetical suggestion of immunological tolerance to HBsAg (6). Nielsen et al. (16) have investigated chronic carriers with and without liver disease and clearly concluded that a general immunodeficiency state is not a prerequisite for developing persistent antigenemia. The reports of T-cell deficiency in chronic carriers of HBsAg resulting in a decreased response to phytohemagglutinin stimulation continue to be controversial (4, 9, 16). Cell-mediated immune response has been demonstrated in normals and patients with previous history of hepatitis B by leukocyte migration inhibition assay by using HBsAg-positive human serum or liver homogenates from HBsAg-positive liver biopsies (7, 13, 24) and by purified antigen (8, 18). Frei et al. (8), in comparing purified, semipurified, and nonpurified HBsAg in LMI tests, concluded that the LMI studies should be performed with purified antigen. Since skin tests with HBsAg cannot be performed in man, delayed hypersensitivity and LMI have been correlated in chimpanzee model (12). Thus, LMI in man may be considered an adequate in vitro correlate of studying cell-mediated immunity to HBsAg.

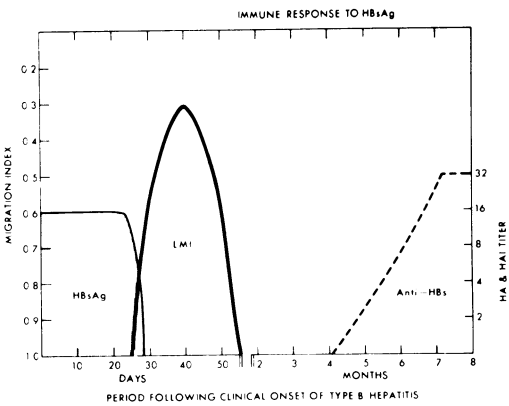

FIG. 2. *Immunological phenomena in patients with acute hepatitis B. HA, Titer of anti-HBs; HAI, inhibitory titer of HBsAg in hemagglutination test.*

There is generally an agreement in all reports that cell-mediated immunity to HBsAg can be demonstrated by the LMI technique during the period of convalescence. Although we could also demonstrate positive LMI reaction, we found it transiently in the group of eight patients followed serially and in two of the 20 convalescing patients. However, two groups have reported positive LMI during a period of several months of convalescence (8, 24). Further, the simultaneous occurrence of LMI and antibodies detected in convalescent patients by Reed et al. (18) differs from our findings that antibody never appeared until several weeks after disappearance of the LMI reaction. This discrepancy may be due to the purity of antigen used and/or to technical differences. We felt that clear definition of immune response to HBsAg can be best obtained by uniformly using 20-nm particles of purified HBsAg devoid of detectable plasma protein and deoxyribonucleic acid polymerase associated with the core of the Dane particles. To exclude effects of possible alloantibodies to leukocytes, we have avoided using homologóus human serum in the migration inhibition test, as opposed to other investigators (7, 13, 24). Our study is unique in using purified HBsAg in defining the natural history of cell-mediated and humoral immune response to HBsAg tested by LMI test and hemagglutination test on serial specimens of patients with nonparenterally transmitted hepatitis B virus infection. Despite the discrepancies mentioned above, there is no conflict with our demonstration of the disappearance of circulating HBsAg after acute hepatitis coinciding with the appearance of LMI and not with humoral antibody. Consistent with the hypothesis and observations of Dudley et al. (6, 7) that the cellular immune response plays an important role in the clearance of the infective agent, none of the investigators find positive LMI in chronic carriers of HBsAg. Hence, persistence of HBsAg in asymptomatic carriers may be considered a state of immunological tolerance to HBsAg. Our finding of irregular and delayed occurrence of anti-HBs are also consistent with the observations of Krugman and Giles (14). The absence of detectable antibodies for several months after disappearance of HBsAg may indicate that removal of HBsAg may be mediated solely by cell-mediated immunity to HBsAg and that late occurrence of anti-HBs may be a result of a secondary response to an additional exposure to HBsAg.

ACKNOWLEDGMENTS

We thank R. Fine, R. Owen, R. Nelson, R. Davies, S. Murphy, and J. Haupt for their cooperation in providing the clinical material for this study, P. Sutherland and C. Zipkin

for their assistance in the clinical follow-up of some of the patients, and L. Haroun and P. Thomas for their excellent technical assistance.

This investigation was supported by the Blood Resource Branch of the National Heart and Lung Institute under Public Health Service contracts NIH-71-2355 and 72-2958-B.

LITERATURE CITED

1. Blumberg, B. S., H. J. Alter and S. J. Visnich. 1965. A "new" antigen in leukemia sera. J. Amer. Med. Ass. **191**:541-546.
2. Center for Disease Control. 1974. Nomenclature of antigens associated with viral hepatitis type B. p. 29. Morbidity and Mortality Weekly Report. vol. 23: Center for Disease Control, Atlanta, Georgia.
3. Cherubin, C. E., and W. Szmuness. 1973. The emerging epidemiology of hepatitis B antigen. Ann. Intern. Med. 79:745-746.
4. Clot, J., O. Mattiew, S. Elharrar, M. Robinet-Levy, and J. M. Lemaire. 1973. Inhibitory effect of Australia positive sera on in vitro-stimulated T lymphocytes. Lancet **2**:576-577.
5. Cossart, Y. E. 1972. Epidemiology of serum hepatitis. Brit. Med. Bull. **28**:156-162.
6. Dudley, F. J., R. A. Fox, and S. Sherlock. 1972. Cellular immunity and hepatitis-associated Australia antigen liver disease. Lancet **1**:723-726.
7. Dudley, F. J., V. Guistino, and S. Sherlock. 1972. Cell-mediated immunity in patients positive for hepatitis-associated antigen. Brit. Med. J. **4**:754-756.
8. Frei, P. C., P. H. Erard, and R. Zinkernagel, 1973. Cell-mediated immunity to hepatitis-associated antigen (HAA) demonstrated by leukocyte migration test during and after acute B hepatitis. Biomedicine **19**:379-381.
9. Giustino, V., F. J. Dudley, and S. Sherlock. 1972. Thymus dependent lymphocyte function in patients with hepatitis-associated antigens. Lancet **2**:850-853.
10. Gocke, D. J., and C. Howe. 1970. Rapid detection of Australia antigen by counterelectrophoresis. J. Immunol. **104**:1031-1032.
11. Greenman, R. L., P. M. Kaplan, and W. S. Robinson. 1974. DNA polymerase in the core of the Dane particle of hepatitis B virus candidate. J. Virol. **13**:1231-1236.
12. Ibrahim, A. B., G. N. Vyas, and A. M. Prince. 1974. Studies on delayed hypersensitivity to hepatitis B antigen in chimpanzees. Clin. Exp. Immunol. **17**:311-318.
13. Ito, K., J. Nakagawa, Y. Okimoto, and H. Nakano. 1972. Chronic hepatitis— migration inhibition of leukocytes in the presence of Australia antigen. N. Engl. J. Med. **286**:1005-1006.
14. Krugman, S., and J. P. Giles. 1973. Viral hepatitis, type B (MS-2-strain). Further observations on natural history and prevention. N. Engl. J. Med. **288**:755-760.
15. Ling, C. M., and L. R. Overby. 1972. Prevalence of hepatitis B virus antigen as revealed by direct radioimmunoassay with ^{125}I-antibody. J. Immunol. **109**:834-841.
16. Nielsen, J. O., V. Reinicke, O. Dietrichson, V. Andersen, M. Thomsen, and E. Andersen. 1973. Immunological studies of Australia antigen carriers with and without liver diseases. Clin. Exp. Immunol. **15**:9-16.
17. Prince, A. M. 1968. An antigen detected in the blood during the incubation period of serum hepatitis. Proc. Nat. Acad. Sci. U.S.A. **60**:814-821.
18. Reed, W. D., C. G. Mitchell, A. L. W. F. Eddelston, W. M. Lee, R. Williams, and A. J. Zuckerman. 1974. Exposure and immunity to hepatitis-B virus in a liver unit. Lancet **1**:581-585.
19. Rosenberg, S. A., and J. R. David. 1971. *In vitro* assay for inhibition of migration of human blood leukocytes, p. 297. *In* B. R. Bloom and P. R. Glade (ed.), *In vitro* methods in cell-mediated immunity. Academic Press Inc., New York.
20. Shulman, N. R. 1970. Hepatitis-associated antigen. Amer. J. Med. **49**:669-692.
21. Vyas, G. N., and N. R. Shulman. 1970. Hemagglutination assay for antigen associated with viral hepatitis. Science **170**:332-333.
22. Vyas, G. N., E. W. Williams, G. G. B. Klaus, and H. E. Bond. 1972. Hepatitis-associated Australia antigen. Protein, peptides and amino acid composition of purified antigen with its use in determining sensitivity of the hemagglutination test. J. Immunol. **108**:1114-1118.
23. Vyas, G. N., H. A. Perkins, and R. Schmidt. 1972. Hepatitis and blood transfusion. Grune and Stratton, New York.
24. Yeung Laiwah, A. A. C., A. K. R. Chaudhuri, and J. R. Anderson. 1973. Lymphocyte transformation and leukocyte migration inhibition by Australia antigen. Clin. Exp. Immunol. **15**:27-34.
25. Zuckerman, A. J. 1972. Hepatitis-associated antigen and viruses. North-Holland Pub. Co., Amsterdam.