Harris, J. A., Diethelm, A. G., Mason, K. N., and Cobbs, C. G. (1971). *Alabama Journal of Medical Sciences*, 8, 105.
 Hill, R. B., Dahrling, B. E., Starzl, T. E., and Rifkind, D. (1967). *American Journal of Medicine*, 42, 327.
 Levine, T. H., Merrill, D. A., Kohler, C. F., and Clamon, H. N. (1970). *Transplantation*, 10, 141.
 Mackay, I. (1968). *Quarterly Journal of Medicine*, 37, 379.
 Mancini, G., Carbonara, A. O., and Hermans, J. F. (1965). *Immunochimistry*, 2, 235.
 Moorhead, J. F., et al. (1970). *British Medical Journal*, 4, 83.

Rodriguez-Iturbe, B., Serrano, H., Garcia, R., and Gallegos, B. (1971). *Transplantation*, 12, 405.
 Sahar, K., and Schwartz, R. S. (1965). *Journal of Immunology*, 95, 345.
 Swanson, M. A., and Schwartz, R. S. (1967). *New England Journal of Medicine*, 4, 163.
 Wells, J. V., and Jeremy, D. (1971). *Clinical Science*, 40, 393.
 Woodruffe, M. F. A., Nolan, B., Robson, J. S., and MacDonald, M. K. (1969). *British Medical Journal*, 1, 6.
 Zuhke, V., Deodhar, S. D., Nakamoto, S., and Kolff, W. J. (1967). *Transplantation*, 5, 135.

Specific Lymphocyte Stimulation by Purified, Heat-inactivated Hepatitis-B Antigen

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Summary

The ability of purified, heat-inactivated hepatitis-B antigen (HBAG) to stimulate sensitized lymphocytes in vitro was investigated with the lymphocyte stimulation test on lymphocytes from three groups of individuals. Stimulation was minimal in the lymphocytes of two out of 15 normal controls, whereas lymphocytes from nine out of 12 patients who had recovered from hepatitis B showed stimulation, as did lymphocytes from five out of 12 laboratory technicians who had been regularly exposed to HBAG but who had no history of hepatitis or signs of it in the previous two years. No differences were observed in the responses to phytohaemagglutinin of lymphocytes from persons in the three groups. HBAG and HBAG inactivated by heat were shown in immunodiffusion to be immunologically identical. Inactivated HBAG stimulated antibody production in guinea-pigs. These findings suggest that not only humoral but also cell-mediated immunity might be induced by vaccination with purified, heat-inactivated HBAG.

Introduction

The role of humoral and cell-mediated immunity against hepatitis-B antigen (HBAG), formerly called Australia antigen or hepatitis-associated antigen (H.A.A.), in hepatitis B (serum hepatitis) is not at present clear. In many viral diseases elimination of the virus depends on intact cell-mediated immunity. This may also be important in the case of hepatitis B (Dudley et al., 1972 a). Cell-mediated immunity against HBAG in patients recovered from acute hepatitis B has been studied with crude preparations of HBAG (Yeung Laiwah, 1971; Dudley et al., 1972 b). In vitro lymphocyte stimulation and leucocyte migration inhibition in the presence of serum containing HBAG or of liver tissue homogenates, however, might be due to other antigens. A test for cell-mediated immunity which used purified HBAG would therefore be preferable.

Experiments have shown that HBAG-positive serum is inactivated by heat (Krugman et al., 1970), and that active immunization with heat-inactivated serum provides some protection against hepatitis B infection (Soulier et al., 1972; Krugman and Giles, 1973). But whether the protection is due to humoral or cell-mediated immunity and whether the cell-mediated immunity has been induced by the vaccination is uncertain. We report here our investigations of the antigenic properties in vitro of purified, heat inactivated HBAG in relation to humoral and to cell-mediated immunity.

Materials and Methods

In vitro lymphocyte stimulation tests were done on lymphocytes from three groups of persons seronegative for HBAG. The first group consisted of 15 persons with no history or signs of hepatitis (normal controls); the second consisted of 12 patients who had recovered from acute hepatitis B (the interval between the disappearance of HBAG from their serum and the stimulation test varied from two to 72 weeks); and the third consisted of 12 laboratory technicians who had been regularly exposed to HBAG but who had shown no signs of hepatitis during a two-year period of monthly screening for serum alanine transaminase, serum aspartate transaminase, and HBAG and who had no history of hepatitis.

HBAG (subtypes ad and ay) was isolated from pooled HBAG-positive sera by a three-stage method (Houwen et al., 1973). Sera diluted 1:3 with phosphate-buffered saline were ultracentrifuged at 350,000 g for six hours and the pellets resuspended and chromatographed on Sepharose 4B. HBAG-positive fractions were pooled and concentrated. Human serum proteins were removed from the solution by immunoadsorption, and shown by immunodiffusion to have been removed. Moreover, this purified HBAG preparation raised a monospecific antiserum against HBAG in guinea-pigs.

The purified HBAG was heated in a waterbath at 100°C for 3 min. The HBAG titre had decreased by two dilution steps after the heating, probably owing to some precipitation and aggregation. HBAG was measured by radioimmunoassay (Ausria-125, Abbot Lab.). Hepatitis B antibodies (HBAb) were identified by counterelectrophoresis and reactions of identity by immunodiffusion.

For the lymphocyte stimulation tests suspensions of lymphocyte-monocytes were used which had been isolated from defibrinated blood by Ficoll-Isopaque gradient centrifugation according to the technique of Böyum (1968). Three hundred thousand lymphocytes were cultured in 12-75 mm Falcon tubes in 1 ml MEM-Eagle with 20% autologous serum supplemented with penicillin 100 IU/ml and streptomycin 100 µg/ml. Cultures

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with phytohaemagglutinin (PHA, Burroughs Wellcome) 5 µl/ml were incubated for three days at 37°C with 5% CO₂ in ambient air. Cultures with purified, heat-inactivated HB_{Ag} and control cultures without antigen were incubated under the same conditions for six days. HB_{Ag} was used in concentrations of 2, 10, and 50 µg protein/ml. Twenty-four hours before harvesting 0.075 µCi ¹⁴C-thymidine (Radiochemical Centre, Amersham, specific activity 56 mCi/mmol) was added.

The cells were harvested by filtration through a glass-fibre filter and washed twice in distilled water. Radioactivity in the dried filter was measured in a Packard liquid scintillation counter using 5 ml scintillation fluid and the results were expressed as disintegrations per minute (d.p.m.) per culture.

Cultures were done in quadruplicate, three for thymidine incorporation and one for morphological control. The arithmetical mean and standard deviation were calculated.

Results

Both active and heat-inactivated HB_{Ag} raised monospecific antisera in inoculated guinea-pigs, and both antigens were shown to be immunologically identical when immunodiffused with these monospecific antisera.

The results of the stimulation tests on lymphocytes from the three groups of persons are shown (fig. 1) as differences in d.p.m. between control cultures and cultures with HB_{Ag}. In most cases maximum stimulation by HB_{Ag} was found with 10 µg protein/ml, but in some cases it was with 50 µg protein/ml. Only the value of the maximum stimulation was recorded. The mean of the control cultures was 268 ± 175 d.p.m. A difference greater than 500 d.p.m. between the control cultures and cultures with HB_{Ag} was arbitrarily considered as evidence of stimulation by HB_{Ag}. This correlated roughly with morphological blast transformation.

FIG. 1—Results of in vitro stimulation by HB_{Ag} of lymphocytes from three groups of persons shown as differences in disintegrations per minute (d.p.m.) between control cultures and HB_{Ag} cultures.

Stimulation was minimal in the lymphocytes of two out of the 15 normal controls. The lymphocytes of nine out of the 12 patients who had recovered from hepatitis B showed stimulation, and HB_{Ab} was present in five before the test and in none during the test (table I). The lymphocytes of five out of the 12 laboratory technicians showed stimulation, and HB_{Ab} was present in three before the test and in four during the test (table II).

No significant differences were found in the results of stimulation by PHA of lymphocytes from the three groups of persons (fig. 2).

TABLE I—Time Relations of Lymphocyte Stimulation Tests with HB_{Ag} Conversion and HB_{Ab} in 12 Patients Recovered from Acute Hepatitis B

Patient No.	Lymphocyte Stimulation Test with HB _{Ag} *	Interval between HB _{Ag} Conversion and Test (weeks)	HB _{Ab}	
			Before Test	During Test
1	++	72	—	—
2	++	48	—	—
3	++	28	+	—
4	++	24	—	—
5	++	8	+	—
6	++	8	+	—
7	++	8	+	—
8	++	4	+	—
9	+	2	—	—
10	—	56	—	—
11	—	3	—	—
12	—	2	—	—

*Differences between HB_{Ag}-stimulated and control cultures: ++ = > 1,000 d.p.m. + = 500—1,000 d.p.m. — = <500 d.p.m.

TABLE II—Time Relations of Lymphocyte Stimulation Tests with HB_{Ab} in 12 Laboratory Technicians

Laboratory Technician No.	Lymphocyte Stimulation Test with HB _{Ag} *	HB _{Ab}	
		Before Test	During Test
1	++	+	+
2	++	+	—
3	+	—	+
4	+	—	—
5	+	—	—
6	—	+	+
7	—	—	+
8	—	—	—
9	—	—	—
10	—	—	—
11	—	—	—
12	—	—	—

*Footnote as in table I.

FIG. 2—Results of in vitro PHA stimulation of lymphocytes from three groups of persons expressed in disintegrations per minute (d.p.m.).

Discussion

Cell-mediated immunity against a specific antigen generally correlates with antigen-induced lymphocyte stimulation. In lymphocyte cultures both B- and T-lymphocytes will be stimulated by antigens (Vischer, 1972), but mainly the T-lymphocytes (Roitt *et al.*, 1969). Moreover, lymphocyte stimulation shows

the same carrier specificity as delayed hypersensitivity (Oppenheim *et al.*, 1967; Senyk *et al.*, 1971).

In-vitro lymphocyte stimulation, which is thought to represent mainly cell-mediated immunity against HBAG, was shown up to 72 weeks after the last signs of hepatitis in nine of our group of 12 patients who had recovered from the disease. Only five showed transient antibodies, and in these five an immune response against HBAG could be shown with in-vitro lymphocyte stimulation after the HB antibodies had disappeared. Two of the three patients who showed no stimulation were tested within four weeks of the disappearance of HBAG from their serum, but in that interval a cell-mediated immune response may not be detectable with in-vitro lymphocyte stimulation.

The fact that lymphocyte sensitization as well as antibody formation were also found in the group of laboratory technicians shows that a complete immune response can develop after exposures to HBAG which do not result in an attack of hepatitis. In two cases antibodies were detected without in-vitro lymphocyte stimulation. Perhaps such differences in the immune response may be related to the dose of HBAG, the subtype, the mode of transmission, or to genetic factors.

Most proteins lose their ability to induce antibody formation after heat-denaturation but remain as effective as untreated proteins in inducing delayed hypersensitivity (Gell and Benacerraf, 1959). Purified HBAG denatured by reduction and alkylation was found to have lost its ability to induce a humoral reaction in guinea-pigs but its ability to induce delayed hypersensitivity was unaffected (Vyas *et al.*, 1972). In contrast, we found that heat-inactivated purified HBAG elicited an antibody response in guinea-pigs. Moreover, it was found to be immunologically identical to untreated HBAG in immunodiffusion using monospecific antisera. Krugman and Giles (1973) found

antibodies against HBAG in some people after immunization with heat-inactivated HBAG-rich serum, while Soulier *et al.* (1972) found antibodies in all of seven persons immunized with a higher dose. In our studies purified, heat-inactivated HBAG stimulated sensitized lymphocytes in vitro.

These findings suggest that vaccination with purified, heat-inactivated HBAG might induce not only humoral but also cell-mediated immunity against serum hepatitis. Further studies are needed to show whether cell-mediated rather than humoral immunity against HBAG offers protection against hepatitis B.

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References

- Böyum, A. (1968). *Scandinavian Journal of Clinical and Laboratory Investigation*, 21, 77.
 Dudley, F. J., Fox, R. A., and Sherlock, S. (1972 a). *Lancet*, 1, 723.
 Dudley, F. J., Giustino, V., and Sherlock, S. (1972 b). *British Medical Journal*, 4, 754.
 Gell, P. G. H., and Benacerraf, B. (1959). *Immunology*, 2, 64.
 Houwen, B., Marrink, J., Vos, J., Molenaar, I., and Nieweg, H. O. (1973). *Vox Sanguinis*, Suppl., 24, 114.
 Krugman, S., Giles, J. P., and Hammond, J. (1970). *Journal of Infectious Diseases*, 122, 432.
 Krugman, S., and Giles, J. P. (1973). *New England Journal of Medicine*, 288, 755.
 Oppenheim, J. J., Wolstencroft, R. A., and Gell, P. G. H. (1967). *Immunology*, 12, 89.
 Roitt, I. M., Graves, M. F., Torrigiani, G., Brostoff, J., and Playfair, J. H. L. (1969). *Lancet*, 2, 367.
 Senyk, G., Williams, E. B., Nitecki, D. E., and Goodman, J. W. (1971). *Journal of Experimental Medicine*, 133, 1294.
 Soulier, J. P., *et al.* (1972). *American Journal of Diseases of Children*, 123, 429.
 Vischer, T. L. (1972). *Clinical and Experimental Immunology*, 11, 523.
 Vyas, G. N., Rao, K. R., and Ibrahim, A. B. (1972). *Science*, 178, 1300.
 Yeung, Laiwah, A. A. C. (1971). *Lancet*, 2, 470.

Changes in Antigenic Nature of Lymphocytes Caused by Common Viruses

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Summary

Healthy human lymphocytes were incubated in the presence of influenza A2/Singapore/57, herpes simplex type 1, or adenovirus type 2. After two days the cultures were inactivated by irradiation. Fresh lymphocytes taken from the same donor were then found to react to the virus-treated cells in short-term cultures. We suggest that this reactivity is due to a change in the surface characteristics of the lymphocytes brought about by the presence of the virus. This may account for anomalous reactions in mixed lymphocyte cultures, and a similar effect in vivo might cause accelerated graft rejection.

Introduction

Recently we reported unexpected reactivity between the lymphocytes of two identical twins (Pegrum *et al.*, 1972). At the time it was noticed that the twin whose cells caused the reactivity was suffering from a heavy cold. When he recovered no reaction occurred between these siblings' lymphocytes. This observation suggested the possibility that the surface antigens on the stimulating lymphocytes had been altered by the viral infection. Such a change might account for the observation that lymphoblastoid cell lines obtained from patients with glandular fever cause reactivity of the subject's own lymphocytes after recovery (Steel and Hardy, 1970).

To test this hypothesis further lymphocytes were cultured in a nutrient medium with three viruses. After incubation cultures were irradiated and the cells washed. These were then placed in contact with fresh autologous lymphocytes and the reactivity was assessed.

Materials and Methods

PREPARATION OF VIRUSES

Influenza A2/Singapore/57 virus was grown at 37°C in the allantoic fluid of 10-day-old fertile hens' eggs. After three days

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