Clin. exp. Immunol. (1973) **15**, 9–16.

IMMUNOLOGICAL STUDIES OF AUSTRALIA ANTIGEN CARRIERS WITH AND WITHOUT LIVER DISEASES

J. O. NIELSEN, V. REINICKE, O. DIETRICHSON, V. ANDERSEN, M. THOMSEN AND E. ANDERSEN

University Clinic for Infectious Diseases, Blegdam Hospital, Second Medical Department, Kommunehospitalet, Copenhagen; Department of Medicine, Copenhagen County Hospital, St Elisabeth and Medical Department TA and Tissue Typing Laboratory of the Blood-Grouping Department, Rigshospitalet, Copenhagen, and Medical Department C, Gentofte Hospital, Gentofte, Denmark

(*Received* 20 *March* 1973)

SUMMARY

Examination of the cell-mediated and the humoral immune systems was carried out in two groups of persistent carriers of Australia antigen. Group A included eleven blood donors with an entirely normal liver histology and without previous history of liver disease. Group B included seven patients with an initial acute 'viral' hepatitis verified by biopsy. Subsequent biopsies revealed progression to chronic persistent hepatitis in three cases, to chronic aggressive hepatitis in three, and a suspicion of chronicity in one of the patients.

The patients in group B had significantly higher serum concentration of IgM and significantly lower serum concentration of complement C4 than the healthy carriers in group A. No differences were found between the two groups with respect to the presence of autoantibodies or the antibody response to vaccination with keyhole limpet haemocyanin. Delayed-type cutaneous reactions to 2,4-dinitrochlorobenzene, haemocyanin and PPD were equal in the two groups. The PHA-induced lymphocyte transformation was significantly lower in the group of patients with chronic hepatitis as compared to the healthy carriers, but none of the groups showed a statistically significant difference from a control group of eight laboratory technicians. It is concluded that a general immunodeficiency state is not a prerequisite for developing persistent Au-antigenaemia. The slightly impaired T-cell response to PHA found in patients with persistent Au-antigenaemia and chronic liver disease may be related to the liver disease rather than to the Au-antigen carrier state.

Correspondence: Dr J. O. Nielsen, Immunlaboratoriet, Blegdam Hospital, DK 2200 Copenhagen N, Denmark.

INTRODUCTION

Persistent Au-antigenaemia is correlated with chronic liver diseases in some cases (Nielsen *et al.*, 1971), though a carrier state without liver disease has also been clearly demonstrated (Reinicke *et al.*, 1972). It has been suggested that the great variation in hepatic involvement of Au-antigen carriers reflects individual variations in the immunological response (Dudley, Fox & Sherlock, 1972).

Furthermore, it has been suggested that an impaired immunological response of the host is a prerequisite for persistent Au-antigenaemia, since this state is found among patients with diseases associated with well-known immunological abnormalities (Miller, 1965, Wilson, Kirkpatrick & Talmadge, 1965). However, it is still uncertain whether the impaired immune response in these patients is a presupposition for the development of persistent Au-antigenaemia (Fouts, Byrne & Israel, 1970).

The aim of the present study was to elucidate whether there is any difference in the immunological response of Au-antigen carriers with and without liver diseases, and to investigate, whether the Au-antigen carrier state is associated with a non-specific immunodeficiency.

MATERIALS AND METHODS

The material comprised two groups of carriers of Au-antigen. Group A included eleven normal blood donors (The Blood Bank, Bispebjerg Hospital, Copenhagen) found to be persistent carriers of Au-antigen. The eleven carriers were selected from twenty-four consecutive blood donors with Au-antigenaemia described previously (Reinicke *et al.*, 1972). They represented all the carriers with a completely normal liver histology and none had a history of previous liver disease. Group B included seven patients with typical attacks of acute viral hepatitis. This diagnosis was in all cases verified by liver biopsy. These patients had persistent Au-antigenaemia associated with progression from acute to chronic liver disease as shown previously (Nielsen *et al.*, 1971). Repeated biopsies showed that chronic aggressive hepatitis developed in three patients and chronic persistent hepatitis in three, while in one patient the latest follow-up biopsy gave suspicion of progression to chronicity (de Groote *et al.*, 1968; Bianchi *et al.*, 1971). None of the patients received corticosteroids or other immunosuppressive drugs.

Qualitative determination of Au-antigen was performed by both immunoelectroosmophoresis and the Ouchterlony technique (Elling, Nielsen & Dietrichson, 1970; Prince & Burke, 1970), and identity reactions were obtained with several reference sera in all cases. Quantitative determination of Au-antigen in arbitrary units was performed by electrophoresis in antibody-containing gel (Elling, Nielsen & Dietrichson, 1973). Serum concentrations of IgG, IgM and IgA were determined as described by Weeke (1968) and complement C3 and C4 as described by Laurell (1966). The serum glutamic oxalacetic transaminase activity (SGOT) was determined by the routine method used at the Department of Clinical Chemistry.

Antinuclear antibodies (ANA) and mitochondrial antibodies (MA) were determined by the indirect fluorescent antibody technique, using FITC-labelled immunoglobulins against IgG and IgM on human thyroid, liver and kidney tissue from a O Rhesus-negative person. Smooth muscle antibodies (SMA) were demonstrated in serum specimens diluted 1:20 on

human uterine cells with the above mentioned labelled antisera (Christoffersen *et al.*, 1972).

Immunization with keyhole limpet haemocyanin (KLH) was performed by intracutaneous administration of 100 μg haemocyanin (Mann Research Laboratories) on day 1, 20 μg haemocyanin on day 14, and 100 μg haemocyanin on day 28. The antibody response was determined by the passive haemagglutination technique 2, 4, 6 and 8 weeks after the initial immunization.

For sensitization to 2,4-dinitrochlorobenzene (DNCB), a dose of 1 mg in 1·0 ml acetone was applied within a circular area of 1·5 cm in diameter (= 1·7 cm^2) on the volar aspect of the left forearm. No challenge dose was employed, and the area was examined for flare-up reaction on day 13 and 15. An erythema with induration was registered as a positive reaction. The Mantoux reaction was performed by intracutaneous administration of 0·1 ml tuberculin (Statens Seruminstitut, Copenhagen, 1 iu/ml). The delayed cutaneous reaction was registered 72 hr later.

For determination of the lymphocyte blast transformation response, the lymphocytes were isolated on a Ficoll–Isopaque gradient. 10^5 lymphocytes were cultured in 0·5 ml TC 199 with 20% pooled AB-serum. All cultures were set up in triplicate. The proliferative response was assayed by addition of ^{14}C-labelled thymidine for the last 24 hr of cultivation, followed by liquid scintillation counting (Dupont, Nielsen & Svejgaard, 1971). The results were expressed as counts per minute (cpm) minus background per 10^5 lymphocytes of the initial inoculum.

Phytohaemagglutinin P Difco (PHA) was employed at a wide range of concentrations (2–1600 μg/culture). Since abnormal dose-response curves have been demonstrated with lymphocytes from patients with immune deficiencies (Hosking, Fitzgerald & Simons, 1971). For antigenic stimulation, PPD without preservative (Statens Seruminstitut, Copenhagen) and an extract of *Candida albicans* (kindly provided by Dr N. Axelsen) were used. PPD was employed in concentrations of 0·05 and 0·02 μg/vial, and the Candida extract in final dilutions of 1:100 and 1:1000. The PHA-stimulated cultures were terminated after 96 hr and the antigen-stimulated cultures after 120 hr. Significance of stimulation was defined as cpm exceeding twice the cpm of parallel unstimulated cultures and being above 400 cpm.

The statistical analysis of the results was performed with the Student's *t*-test for the quantitative variables and with the χ^2 test for the qualitative variables. The rank correlation test was used in the lymphocyte transformation studies.

RESULTS

As shown in Table 1, the patients in group B had significantly higher serum concentrations of IgM and lower complement C4 concentrations as compared with the normal carriers in group A, however, the mean values in both groups were in the normal range. The SGOT values of group B were significantly higher than those of group A as might be expected. No significant differences were found between the remaining variables. It can be seen from Table 2 that no significant differences were found in any of the cutaneous reactions.

The results of lymphocyte stimulation *in vitro* are shown in Table 3. These are the results obtained at optimal concentration of stimulant for each individual The PHA-induced transformation (Fig. 1) was significantly lower in group B than in group A ($P<0·05$). However, none of the groups differed significantly from a control group of eight laboratory technicians. As shown in Table 3, the unstimulated control activity was lower in group

TABLE 1. Biochemical and serological variables for eleven healthy carriers of Au-antigen (group A) and for seven carriers with liver diseases (group B)

Factor and normal range	Group A (11) mean (range)	Group B (7) mean (range)	Difference
SGOT (<25 units)	24 (15–50)	50 (18–130)	$P<0.01$
IgG g/l (7·2–15·1)	15·4 (10·5–19·0)	20·8 (12·1–41·0)	n.s.
IgM g/l (0·23–1·33)	0·64 (0·42–1·01)	1·25 (0·64–2·78)	$P<0.05$
IgA g/l (0·74–3·06)	1·50 (0·77–2·28)	1·38 (0·48–2·12)	n.s.
Complement C3 g/l (0·37–0·79)	0·77 (0·52–0·92)	0·80 (0·54–1·16)	n.s.
Complement C4 (40–135 U/l)	92 (65–125)	68 (48–129)	$P<0.05$
Platelets $10^3/\mu l$	201 (110–260)	177 (138–220)	n.s.
White blood-cells/μl	6·000 (5·200–10·600)	6·300 (4·700–8·300)	n.s.
Lymphocytes/μl	2·000 (1·300–2·500)	2·000 (1·600–2·800)	n.s.
Au-antigen in arbitrary units	135 (80–268)	156 (8–428)	n.s.
ANA positive	1	0	n.s.
SMA positive	0	0	n.s.
MA positive	0	0	n.s.

n.s., Not significant, $P>0.05$. ANA, antinuclear antibodies. SMA, smooth muscle antibodies. MA mitochondrial antibodies.

TABLE 2. Delayed type cutaneous reactions for eleven healthy carriers of Au-antigen (group A) and for seven carriers with liver diseases (group B)

Delayed type cutaneous reactions	Group A (11)	Group B (7)	Difference
Mantoux positive	10	6	n.s.
Haemocyanin (KLH) positive	8	3	n.s.
Dinitrochlorobenzen (DNCB) positive	5	5	n.s.
KLH and DNCB negative	3	2	n.s.

n.s., Not significant, $P<0.05$.

B after both 72–96 hr and after 96–120 hr. No differences were found in the responsiveness of the antigen-stimulated lymphocytes.

Following immunization with haemocyanin, four out of seven patients in group B responded with significantly raised antibody titres as compared with nine out of eleven of the healthy carriers in group A. This difference is not statistically significant.

DISCUSSION

As shown previously (Nielsen *et al.*, 1971), an Au-antigen carrier state can be induced by acute viral hepatitis. In contrast, the trigger mechanism for the healthy carrier state without any history of previous liver disease is still rather obscure. Extensive studies by Blumberg *et al.* (1969) seem to support the hypothesis that susceptibility to be a carrier of Au-antigen is controlled by an autosomal recessive gene.

Subsequent studies by Sutnick *et al.* (1970) showed that lymphocytes from patients with

TABLE 3. Results of lymphocyte transformation *in vitro*

	72–96 hr			96–120 hr				
	No.	Unstimulated cpm	PHA cpm	Unstimulated cpm	PPD		*C. albicans*	
					No. positive No. tested	Positive cpm	No. positive No. tested	Positive cpm
Group A	11	168±43	18421±2536	120±30	11/11	1435±339	11/11	1393±277
Group B	7	43±12	11007±1871	42±29	5/7	1928±804	6/7	1214±253
Controls	8	99±30	14353±2333	78±38	6/8	1175±269	8/8	1011±209

Values given are average cpm±SEM.
Group A includes eleven healthy carriers of Au-antigen.
Group B includes seven patients with chronic liver diseases and persistent Au-antigenaemia.

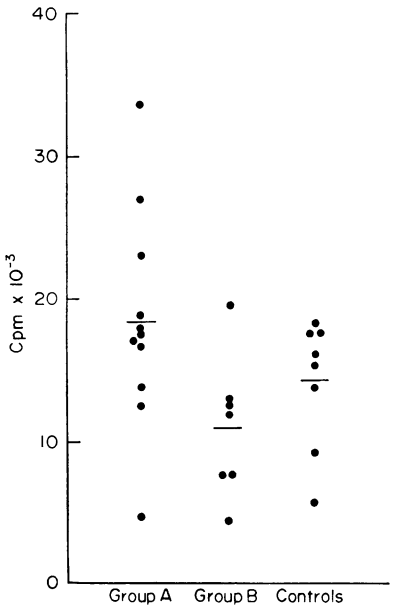

FIG. 1. PHA-induced lymphocyte transformation in eleven healthy carriers of Au-antigen (group A) and in seven antigen-carriers with chronic liver diseases (group B) compared to eight antigen-negative controls.

Down's syndrome (patients with a high incidence of Au-antigenaemia) were hyporesponsive to PHA stimulation. However, no difference was found between patients without Au-antigen and patients with persistent Au-antigenaemia. Recently Giustino, Dudley & Sherlock (1972) found an impaired lymphocyte response to PHA stimulation in patients with chronic liver disease and persistent antigenaemia as compared with normal controls without Au-antigenaemia, and they concluded that persistence of Au-antigen is associated with impaired T-lymphocyte function. However, the present results show clearly that the PHA induced T-cell response in healthy carriers of Au-antigen (group A) is quite normal. Hence, it can be concluded that persistent Au-antigenaemia *per se* is not associated with a depression of the PHA induced lymphocyte response. In the patients with chronic liver diseases (group B) we did not find a statistically significant decrease. We are inclined to believe that the impaired T-cell response found by Giustino *et al.* (1972) in patients with chronic liver disease and persistent antigenaemia is related to the liver disease rather than to the carrier state, since immunological abnormalities have been reported previously in patients with chronic liver disease (Smith *et al.*, 1972) and since we found a significantly impaired T-cell response in antigen carriers with liver diseases as compared to carriers with no liver disease.

The absence of a T-cell immunodeficiency state in carriers of Au-antigen with or without liver disease shown in the present paper does not exclude the existence of a more specific 'defect' or 'tolerance' of the immune system to Au-antigen as suggested by Giustino *et al.* (1972), but this is still a hypothesis without experimental evidence at present.

While failure to demonstrate an immunodeficiency state in carriers of Au-antigen may reflect the inadequacy of the test system available at present, it also stresses the importance of

a more varied interpretation of Au-antigen and Au-antigenaemia. The immunochemical reactive Au-antigen can at present be separated into three morphological types of particles and into at least two antigenic subtypes. At present there is evidence of a correlation between the particle pattern and the liver disease (Nielsen, Nielsen & Reinicke, 1972; Nielsen, Nielsen & Elling, 1973), and evidence has been found recently that suggests a relationship between the antigenic subtypes and the clinical and histological severity of the liver diseases (Nielsen & Le Bouvier, 1973).

ACKNOWLEDGMENTS

The authors are indebted to Professor Hemming Poulsen and Dr Per Christoffersen, The Institute of Pathological Anatomy, Kommunehospitalet, Copenhagen, for performing histological examination of the liver biopsies, and to Dr Bo Dupont and Dr Preben Elling for advice and criticism during the planning of this study. The patients in group B are included in the Copenhagan Hepatitis Acuta Program.

This work was supported by grants from the P. Carl Petersen Foundation and the Danish State Medical Research Council No. 512–1132/71, 1631/72 and 1945/72, 1520/72 and by grants from The Medical Research Foundation for Copenhagen, Faroe Islands and Greenland No 44,71/72.

REFERENCES

BIANCHI, L., DE GROOTE, J., DESMET, V., GEDIGK, P., KORB, G., POPPER, H., POULSEN, H., SCHEUER, P.J., SCHMID, M., THALER, H. & WEPLER, W. (1971) Morphological criteria in viral hepatitis. *Lancet*, **i**, 333.

BLUMBERG, B.S., FRIEDLAENDER, J.S., WOODSIDE, A., SUTNICK, A.I. & LONDON, W.T. (1969) Hepatitis and Australia antigen. Autosomal recessive inheritance of susceptibility to infection in human. *Proc. nat. Acad. Sci.* (Wash.), **62**, 1108.

CHRISTOFFERSEN, P., DIETRICHSON, O., FABER, V. & POULSEN, H. (1972) The occurrence and significance of abnormal bile duct epithelium in chronic aggressive hepatitis. *Acta. path. microbiol. scand.* **80**, 294.

DUDLEY, F.J., FOX, R.A. & SHERLOCK, S. (1972) Cellular immunity and hepatitis-associated, Australia antigen liver disease. *Lancet*, **i**, 723.

DUPONT, B., NIELSEN, L.S. & SVEJGAARD, A. (1971) Relative importance of four and LA loci in determining mixed-lymphocyte reactions. *Lancet*, **ii**, 1336.

ELLING, P., NIELSEN, J.O. & DIETRICHSON, O. (1970) Australia antigen in patients with various liver diseases. *Acta. path. microbiol. scand.* B **78**, 657.

ELLING, P., NIELSEN, J.O. & DIETRICHSON, O. (1973) Quantitative studies of Australia antigen in acute and chronic hepatitis. *Vox Sang.* (In press).

FOUTS, D.W., BYRNE, E.B. & ISRAEL, H.L. (1970) Hepatitis-associated antigen and sarcoidosis. *Lancet*, **ii**, 1257.

GIUSTINO, V., DUDLEY, F.J. & SHERLOCK, S. (1972) Thymus dependent lymphocyte function in patients with hepatitis-associated antigen. *Lancet*, **ii**, 850.

DE GROOTE, J., DESMET, V.J., GEDIGH, P., KORB, G., POPPER, H., POULSEN, H., SCHEUER, P.J., SCHMID, M., THALER, H., UEHLINGER, E. & WEPLER, W. (1968) A classification of chronic hepatitis. *Lancet*, **ii**, 626.

HOSKING, C.S., FITZGERALD, M.G. & SIMONS, M.J. (1971) Quantified deficiency of lymphocyte response to phytohaemagglutinin in immune deficiency diseases. *Clin. exp. Immunol.* **9**, 467.

LAURELL, C.B. (1966) Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Ann. Biochem.* **15**, 45.

MILLER, D.G. (1965) Hodgkin's disease, lymphosarcoma and chronic lymphocytic leukemia. *Immunological Diseases* (Ed. by M. Samter), p. 373. Little & Co., Boston.

NIELSEN, J.O., DIETRICHSON, O., ELLING, P. & CHRISTOFFERSEN, P. (1971) Incidence and meaning of per-

sistence of Australia antigen in patients with acute viral hepatitis: Development of chronic hepatitis. *New Engl. J. Med.* **285,** 1157.

NIELSEN, J.O., NIELSEN, M.H. & REINICKE, V. (1972) Absence of Dane particles in Au-antigen carriers with a histologically normal liver. *Lancet*, **ii,** 934.

NIELSEN, J.O., NIELSEN, M.H. & ELLING, P. (1973) Differential distribution of Australia antigen-associated particles in patients with liver diseases and normal carriers. *New Engl. J. Med.* **288,** 484.

NIELSEN, J.O. & LE BOUVIER, G.L. (1973) Subtypes of Australia antigen among patients and healthy carriers in Copenhagen: A relationship between the subtypes and the degree of liver damage in acute viral hepatitis. *New Engl. J. Med.* (In press).

PRINCE, A.M. & BURKE, K. (1970) Serum hepatitis antigen (SH): Rapid detection by high voltage immuno-electroosmophoresis. *Science*, **169,** 593.

REINICKE, V., DYBKJAER, E., POULSEN, H., BANKE, O., LYLLOFF, K. & NORDENFELT, E. (1972) A study of Australia-antigen-positive blood donors and their recipients, with special reference to liver histology *New Engl. J. Med.* **286,** 867.

SMITH, M.G.M., GOLDING, P.L., EDDLESTON, A.L.W.F., MITCHELL, C.G., KEMP, A. & WILLIAMS, R. (1972) Cell-mediated immune response in chronic liver diseases. *Brit. med. J.* **1,** 527.

SUTNICK, A.I., RAUNIO, V.K., LONDON, W.T., MILLMAN, I. & BLUMBERG, B.S. (1970) New immunologic relationship of Australia antigen. *Vox Sang.* **19,** 296.

WEEKE, B. (1968) Carbamylated human immunoglobulins tested by electrophoresis in agarose and antibody containing agarose. *Scand. J. clin. Lab. Invest.* **21,** 351.

WILSON, W.E., KIRKPATRICK, C.G. & TALMADGE, D.W. (1965) Suppression of immunologic responsiveness in uremia. *Ann. Intern. Med.* **62,** 1.