Clin. exp. Immunol. (1979) 38, 231–234.

Lymphocyte transformation test with rabbit liver specific lipoprotein (RLSP) in chronic active hepatitis

L. ORTONA, V. LAGHI, R. CAUDA & P. NERVO *Clinic of Infectious Diseases, Catholic University, Rome*

(*Accepted for publication* 24 *April* 1979)

SUMMARY

Cellular sensitization to rabbit liver specific lipoprotein (RLSP) has been investigated using a lymphocyte transformation test in patients with chronic active hepatitis (CAH).

A stimulation index greater than 2 was recorded in twenty out of twenty-five cases (eight of ten HBsAg positive and twelve of fifteen HBsAg negative) while values were lower than 2 in all the normal subjects.

These results confirm the finding of sensitization to LSP in chronic active hepatitis irrespective of HBsAg status and show that rabbit LSP can be used as an alternative to the human antigen in the lymphocyte transformation test, and is further evidence that this liver membrane lipoprotein has antigenic determinants which have species-cross-reactivity.

INTRODUCTION

Several investigations in the last few years have indicated that cell-mediated autoimmunity may be of importance in the development of chronic active liver diseases (CALD). The target antigen involved in this autoimmune reaction seems to be present in a macromolecular fraction of normal liver which has been termed liver-specific lipoprotein (LSP) and which has organ-specificity but incomplete species-specificity (Meyer zum Buschenfelde & Miescher, 1972; Hopf, Meyer zum Buschenfelde & Freudenberg, 1974; McFarlane *et al.*, 1977).

The LSP contains large amounts of phosphates and triglycerides and has a complex structure probably composed of sub-units. Studies done by polyacrylamide gel electrophoresis (PAGE) have demonstrated that LSP from different species (human, rabbit, rat, guinea-pig) shows similar mobility (Hutteroth & Meyer zum Buschenfelde, 1978).

Recently the species-unspecificity of LSP previously demonstrated by immunofluorescence studies (Hopf *et al.*, 1974) has been confirmed by immunodiffusion (Hutteroth & Meyer zum Buschenfelde, 1978). In fact, when testing both human LSP (HLSP) and rabbit LSP (RLSP) on immunodiffusion against anti-human LSP (prepared in sheep) two lines of precipitation were obtained: one common to HLSP and RLSP demonstrating the species-non-specific component and the other only for HLSP, indicating the presence of an additional species-specific part.

The central role of LSP in the development of CALD has been demonstrated in a rabbit model. Long-term immunization with HLSP induced chronic active hepatitis (CAH) with the appearance of cellular and humoral immunity against this antigen (Meyer zum Buschenfelde, Kossling & Miescher, 1972).

Numerous investigators have demonstrated sensitization of peripheral lymphocytes of CALD patients to liver membrane proteins and in particular to LSP. The following systems have been used: (1) lymphocyte transformation (Thestrup-Pedersen, Ladefoged & Andersen, 1976), (2) leucocyte migration inhibition (Smith *et al.*, 1972; Miller *et al.*, 1972; Meyer zum Buschenfelde, Knolle & Berger, 1974; Meyer zum Buschenfelde *et al.*, 1975; Lee, Reed & Mitchell, 1975; Realdi *et al.*, 1976) and (3)

Correspondence: Prof. L. Ortona, Istituto di Clinica delle Malattie Infettive, Università Cattolica del Sacro Cuore Largo A. Gemelli, 8, 00168 Roma, Italy.

0099-9104/79/1100-0231$02.00

cytotoxicity assays, namely against chicken red blood cells (RBC) coated by LSP (Vogten *et al.*, 1978), cultured rabbit liver cells (Thomson *et al.*, 1974; Cochrane *et al.*, 1976; Facchini *et al.*, 1978), liver cells of various origin in culture, autologous cells (Paronetto & Vernace, 1975; Wands & Isselbacher, 1975; Geubel *et al.*, 1976), Chang liver (Wands *et al.*, 1975; Vierling *et al.*, 1977; Kawanishi, 1977), and against rat liver (Fernandez-Cruz *et al.*, 1978).

In this study we have used a lymphocyte transformation test with rabbit LSP (RLSP) as antigen to study the sensitization of peripheral blood lymphocytes from patients with CALD to the species non-specific determinants of LSP. The antigen is rapidly denatured unless it is isolated within a few hours of death and it is not always possible to obtain LSP from man. Rabbit LSP has proved to be a suitable alternative antigen preparation.

MATERIALS AND METHODS

Twenty-five patients with CAH histologically diagnosed according to the criteria of De Groote *et al.* (1968) were studied. Fifteen (seven males and eight females) were HBsAg negative while ten (seven males and three females) were HBsAg positive.

A complete clinical and laboratory evaluation was done at the time of the present study. Levels of serum aspartate transaminase (AST), bilirubin, alkaline phosphatase, albumin, total globulin and immunoglobulin were determined. None of the patients who participated in the present study had ever received immunosuppressive treatment.

Twelve normal subjects without liver disease were tested as a control. In this population all the above biochemical liver parameters were normal and also the HBsAg was negative.

The HBsAg determination was conducted using a radioimmunoassay (AusRIA II, Abbot).

Preparation of the antigen. The rabbit LSP was prepared by Sephadex G100 gel filtration (Tris-EDTA buffer pH 8·0) of the 105,000 *g* supernatant of rabbit liver homogenate and further purified over Sepharose 6 B according to the method of McFarlane *et al.* (1977). This permits to have highly purified preparation but 2% of albumin as only detectable contaminant.

The rabbit LSP obtained in this way was further chemically characterized as a lipoprotein in the various systems used: (1) lipid electrophoresis on Whatman II chromatography paper (2) starch-gel electrophoresis and (3) microzone electrophoresis.

The protein content was measured by the Lowry method and the concentration of LSP adjusted to 250 μg/ml and sterile filtered through a millipore filter 0·22 μm.

Prior to use the preparation of RLSP was dialysed overnight against 0·1 M Tris HCl pH 8·0 containing 0·2 M NaCl which was done in order to remove EDTA. The presence of EDTA could interfere in fact with the immunological reactivity of peripheral lymphocytes (Meyer zum Buschenfelde personal communication).

Lymphocyte transformation test (LTT). Twenty ml of venous blood was drawn into a test tube containing heparin (20 i.u./ml). The lymphocytes, separated by Isopaque-Ficoll were washed in RPMI 1640 Hepes Medium and then were diluted in the same medium supplemented by 20% pooled fresh human AB heat inactivated serum (Istituto Sieroterapico Milanese) to produce a final concentration of 5×10^5 cells/ml. Two and one half ml of this cellular suspension were cultured in sterile test tube at 37°C in an atmosphere of 95% O_2 and 5% CO_2. The RLSP was added to the single culture at the following concentrations: 1·25 μg/ml and 2·5 μg/ml. Nothing was added to the control tubes. All the tests were done in triplicate. The total lifespan for the culture with RLSP was five days. Twenty four hours before harvesting, the cultures were added to 0·2 μCi of ^{3}H-Thymidine. At the time, the cells, collected on Whatman GF/C glass fibre filter paper, were washed with water and alcohol and the incorporation of the isotope was measured in a gamma-counter. The results were expressed as stimulation index.

$$SI = \frac{\text{cpm of RLSP stimulated culture}}{\text{cpm of control culture}}$$

An index >2 (mean SI for normal subjects ± 2 s.d.) was considered as a positive result. In each case tested, the highest stimulation index obtained with two concentrations of RLSP was recorded.

RESULTS

Preliminary addition of RLSP in concentrations ranging from 0·5 μg/ml to 30 μg/ml demonstrated a suppression of *in vitro* reactivity of lymphocytes at 30 μg/ml. The higher stimulation indexes were obtained at lower concentrations of this antigen and in particular between 1·0 μg/ml and 3·0 μg/ml. Therefore, in the present study it was decided to use the concentrations of 1·25 μg/ml and 2·50 μg/ml.

A positive result was obtained in twenty out of twenty-five CAH patients, eight out of ten HBsAg positive and twelve out of fifteen HBsAg negative (Fig. 1). In twelve patients the optimal concentration of RLSP was 1·25 μg/ml while in eight patients it was 2·50 μg/ml. All the normal subjects gave negative results.

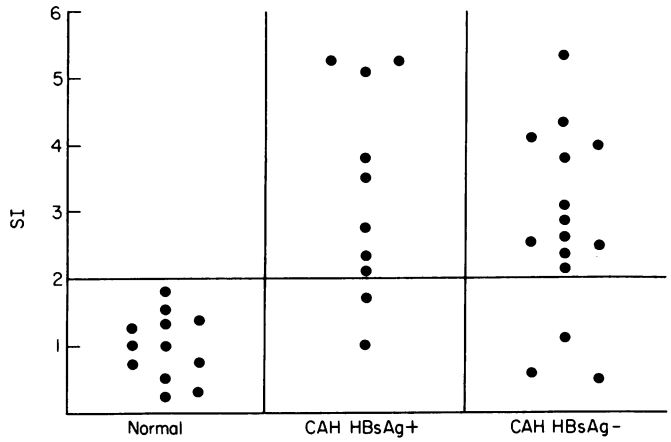

FIG. 1. Results obtained in chronic active hepatitis using a lymphocyte transformation test with rabbit liver specific lipoprotein (RLSP). In each case tested, the highest stimulation index obtained with two concentrations of RLSP was recorded. (The line at 2 indicates the mean SI for normal subjects ± 2s.d.)

DISCUSSION

The mechanisms involved in the pathogenesis of CAH remain poorly understood although much of the evidence implicates cellular autoimmune reactions (Eddleston & Williams, 1974).

In order to evaluate the pathogenetical role of liver membrane proteins and in particular of LSP as a possible target antigen in CALD, various experimental studies have been done utilizing different immunological assays.

The results of these studies have demonstrated that a common sensitization of peripheral blood lymphocytes from CALD patients to HLSP exists, either using leucocyte migration inhibition (Smith *et al.*, 1972; Miller *et al.*, 1972; Meyer zum Buschenfelde *et al.*, 1974; Meyer zum Buschenfelde *et al.*, 1975; Lee *et al.*, 1975) or lymphocyte transformation (Thestrup-Pedersen *et al.*, 1976). An improvement of leucocyte migration inhibition was also documented in CAH patients who have undergone an immunosuppressive treatment.

As well as the data obtained with human LSP, Realdi *et al.* (1976), using a preparation of rabbit liver proteins as antigen, inhibition of leucocyte migration in 53% HBsAg positive CAH and 75% HBsAg negative CAH was found. An improvement was documented in the four cases treated. Thomson *et al.* (1974) and Cochrane *et al.* (1976) found the presence of lymphocyte cytotoxicity against rabbit liver cells in culture in 90% of patients with untreated CAH showed no difference between HBsAg positive and negative cases. The cytotoxicity could be blocked by adding LSP or aggregated IgG therefore suggesting that LSP was the target antigen primarily involved and that the cytotoxic reaction was of the antibody dependent cellular cytotoxicity type, mediated by Fc-receptor-bearing cells (K-cells).

The data we have obtained demonstrate that this sensitization to LSP can also be detected with an antigen not prepared from human liver and that RLSP can be a suitable alternative to the human liver antigen in the study of immune reactions in CALD. This is an indirect confirmation of the importance of sensitization to the species non-specific determinants of LSP. Our findings demonstrate that the sensitization to RLSP in CAH is irrespective of HBsAg status which is not in accord with the data of Realdi *et al.*, (1976), who found a non-statistically significant increase in leucocyte migration inhibition to rabbit liver proteins in CAH HBsAg negative.

A possible objection to our results might be that these positive stimulation indices were due not to the organ-specific part of LSP, but to the species-specific portion to which our patients could have been sensitized in the past. However, this seems highly improbable because the sensitization was present only in CAH patients and not in any of the normal subjects tested. This disease specificity of the immune response to LSP has been confirmed using a leucocyte migration test by Meyer zum Buschenfelde *et al.* (1975), who found no evidence of sensitization to LSP in any control patients with diseases not involving the liver.

We are grateful to Dr A. L. W. F. Eddleston and Dr I. G. McFarlane of the Liver Unit King's College Hospital, London, for their valuable advice.

REFERENCES

COCHRANE, A.M.G., MOUSSOUROS, A., THOMSON, A.D. EDDLESTON, A.L.W.F. & WILLIAMS, R. (1976) Antibody-dependent cell-mediated (K-cell) cytotoxicity against isolated hepatocytes in chronic active hepatitis *Lancet*, i, 441.

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

EDDLESTON, A.L.W.F. & WILLIAMS, R. (1974) Inadequate antibody response to HBsAg or suppressor T cell defect in development of active chronic hepatitis. *Lancet*, ii, 1543.

FACCHINI, A., STEFANINI, G.F., BERNARDI, M. MIGLIO, F., GASBARRINI, G. & LABO, G. (1978) Lymphocytotoxicity test against rabbit hepatocytes in chronic liver diseases. *Gut*, **19**, 189.

FERNANDEZ-CRUZ, E., ESCARTIN, P., BOOTELLO, A., KREISLER, M. & SEGOVIA, J.M. (1978) Hepatocyte damage induced by lymphocytes from patients with chronic liver diseases, as detected by LDH release. *Clin. exp. Immunol.* **31**, 436.

GEUBEL, A.P., KELLER, R.H., SUMMERSKILL, W.H.J., DICKSON, E.R., TOMASI, T.B. & SHORTER, R.G. (1976) Lymphocyte cytotoxicity and inhibition studied with autologous liver cells: observations in chronic active liver disease and the primary biliary cirrhosis syndrome. *Gastroenterology*, **71**, 450.

HOPF, U., MEYER ZUM BUSCHENFELDE, K.H. & FREUDENBERG J. (1974) Liver-specific antigens of different species. II. Localization of a membrane antigen at cell surface of isolated hepatocytes. *Clin. exp. Immunol.* **16**, 117.

HUTTEROTH T.H. & MEYER ZUM BUSCHENFELDE K.H. (1978) Clinical relevance of the liver-specific lipoprotein (LSP). *Acta Hepato Gastroenterol.* **25**, 243.

KAWANISHI, H. (1977) *In vitro* studies on IgG-mediated lymphocyte cytotoxicity in chronic active liver disease. *Gastroenterology*, **73**, 549.

LEE, W.M. REED, W.D. & MITCHELL, C.G., (1975) Cellular and humoral immunity to hepatitis B surface antigen in active chronic hepatitis. *Brit. Med. J.* **1**, 705.

MCFARLANE, I.G., WOJCICKA, B.M., ZUCKER, G.M., EDDLESTON, A.L.W.F. & WILLIAMS, R. (1977) Purification and characterization of human liver-specific membrane lipoprotein (LSP). *Clin. exp. Immunol.* **27**, 381.

MEYER ZUM BUSCHENFELDE, K.H. & MIESCHER, P.A. (1972) Liver specific antigens. Purification and characterization. *Clin. exp. Immunol.* **10**, 89.

MEYER ZUM BUSCHENFELDE, K.H., KOSSLING, F.K. & MIESCHER, P.A. (1972) Experimental chronic active hepatitis in rabbits following immunization with human liver proteins. *Clin. exp. Immunol.* **11**, 99.

MEYER ZUM BUSCHENFELDE, K.H., KNOLLE, J. & BERGER J. (1974) Cellulare Immunreaktionen gegenuber homologen leberspezifischen Antigenen (HPL) bei chronischen Leberentzundungen. *Klin. Wschr.* **52**, 246.

MEYER ZUM BUSCHENFELDE K.H., ALBERTI, A., ARNOLD, W. & FREUDENBERG, J. (1975) Organ-specificity and diagnostic value of cell-mediated immunity against a liver-specific membrane protein: studies in hepatic and non-hepatic diseases. *Klin. Wschr.* **53**, 1061.

MILLER, J., SMITH, M.G.M., MITCHELL, C.G., REED, W.D., EDDLESTON, A.L.W.F. & WILLIAMS, R. (1972) Cell-mediated immunity to a human liver-specific antigen in patients with active chronic hepatitis and primary biliary cirrhosis. *Lancet*, ii, 296.

PARONETTO, F. & VERNACE, S. (1975) Immunological studies in patients with chronic active hepatitis. Cytotoxic activity of lymphocytes to autochthonous liver cells grown in tissue culture. *Clin. exp. Immunol.* **19**, 99.

REALDI, G., ALBERTI A., BERTOLOTTI, F., TREMOLADA, F., DIODATI, G. & CADROBBI, P. (1976) Immunoreazioni umorali e cellulari verso HBsAg e verso proteine fegato-specifiche in corso di epatite acuta e cronica. *Min. Gastroenterol.* **22**, 208.

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

THESTRUP-PEDERSEN, K., LADEFOGED, K. & ANDERSEN, R. (1976) Lymphocyte transformation test with liver-specific protein and phytohaemagglutinin in patients with liver disease. *Clin. exp. Immunol.* **24**, 1.

THOMSON, A.D., COCHRANE, M.A.G. MCFARLANE, I.G., EDDLESTON, A.L.W.F. & WILLIAMS, R. (1974) Lymphocyte cytotoxicity to isolated hepatocytes in chronic active hepatitis. *Nature (London)*, **252**, 721.

VIERLING, J.M., NELSON, D.L., STROBER, W., BENDY, B.M. & JONES, A. (1977) *In vitro* cell mediated cytotoxicity in primary biliary cirrhosis and chronic hepatitis. *J. Clin. Invest.* **60**, 1116.

VOGTEN, A.J.M. HADZIC, N., SHORTER, R.G., SUMMERSKILL, W.H.J. & TAYLOR, W.F. (1978) Cell-mediated cytotoxicity in chronic liver disease: a new test system. *Gastroenterology*, **74**, 883.

WANDS, J.R., PERROTTO, L., ALPERT, E. & ISSELBACHER, K.J. (1975) Cell mediated immunity in acute and chronic hepatitis. *J. Clin. Invest.* **55**, 921.

WANDS, J.R. & ISSELBACHER, K.J. (1975) Lymphocyte cytotoxicity to autologous liver cells in chronic active hepatitis. *Proc. Nat. Acad. Sci. USA*, **72**, 1301.