Clin. exp. Immunol. (1980) **42,** 436–440.

Immune response to rabbit liver-specific lipoprotein in acute viral hepatitis

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

(Accepted for publication 6 June 1980)

SUMMARY

A serial prospective study of cell-mediated immunity to rabbit liver-specific lipoprotein (RLSP) has been done in 26 patients with acute viral hepatitis (AH) (18 $HBsAg^+$ and eight $HBsAg^-$) using a lymphocyte transformation test. An increased stimulation index was recorded in 56% of $HBsAg^+$ cases and in 63% of the $HBsAg^-$ group at the first determination within 2 weeks of presentation. A progressive return to normal values was observed during the course of the disease. In one patient, however, the stimulation index remained high at 6 months after presentation and liver biopsy showed the appearance of chronic active hepatitis. Results within the normal range of values were observed when a macromolecular kidney protein fraction was used as antigen: further evidence of an organ-specific component in RLSP preparation to which the immune response seems to be directed. These findings demonstrate the existence of a common and time-limited sensitization to RLSP in acute viral hepatitis irrespective of HBsAg status. It is suggested that RLSP may be a useful alternative to human LSP in evaluating immune reactions in liver diseases.

INTRODUCTION

Immune reactions directed at liver cell membrane determinants may be of importance in the development of hepatocellular injury in acute and chronic hepatitis, although the antigen/antibody system involved has not been clearly defined. Much of the evidence indicates a pivotal role of a macromolecular fraction of normal liver which has been termed 'liver-specific lipoprotein' (LSP) and which has shown organ-specificity but incomplete species-specificity (Meyer Zum Büschenfelde & Miescher, 1972; Hopf, Meyer Zum Büschenfelde & Freudenberg, 1974; McFarlane *et al.*, 1977; Hutteroth & Meyer Zum Büschenfelde, 1978). However, the detailed structure of LSP has not yet been determined and it has been suggested that it may contain both organ-specific and organ-non-specific determinants (Behrens & Paronetto, 1979; Chisari, 1980). In addition, the organ-specific component is probably labile and rapidly inactivated unless standardized preparative procedures are used such as the conditions of organ procurement, composition of buffers and storage temperatures (Chisari, 1980).

Cell-mediated immune reactions to liver antigens (in general) and to LSP (in particular) have been found in a high percentage of cases of chronic hepatitis patients, utilizing different assays (Smith *et al.*, 1972; Miller *et al.*, 1972; Meyer Zum Büschenfelde, Knolle & Berger, 1974; Thomson *et al.*, 1974; Meyer Zum Büschenfelde *et al.*, 1975; Lee, Reed & Mitchell, 1975; Thestrup-Pedersen, Ladefoged & Andersen, 1976; Realdi *et al.*, 1976; Cochrane *et al.*, 1976; Vogten *et al.*, 1978; Ortona

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

0099-9104/80/1200-0436$02.00

et al., 1979; Mieli Vergani *et al.*, 1979). The existence of such a sensitization has also been documented in the course of acute hepatitis (AH), although this event seems transient and time-limited (Meyer Zum Büschenfelde *et al.*, 1975; Thestrup-Pedersen *et al.*, 1976; Realdi *et al.*, 1976; Cochrane *et al.*, 1976; Lee *et al.*, 1977; Bartholomaeus, Reed & Joske, 1978).

In a former study (Ortona *et al.*, 1979) we have demonstrated that sensitization to LSP could also be documented with an antigen prepared from rabbit liver and that this rabbit LSP could be a valid alternative to the human preparation in the evaluation of cellular autoimmunity in chronic active liver diseases (CALD).

In the present work, we searched for the existence of sensitization to LSP in 26 patients with acute hepatitis, utilizing a lymphocyte transformation test (LTT) with rabbit LSP (RLSP) as antigen. Serial samples were obtained in all patients and the changes in cellular immunity to RLSP were followed during the course of the illness and compared with the clinical outcome.

PATIENTS AND METHODS

Twenty-six patients with acute viral hepatitis (AH) were investigated. The diagnosis was done on the basis of clinical, epidemiological and laboratory findings. None of the patients had a history of alcohol abuse and/or assumption of hepatotoxic drugs. In particular 18 (13 males and five females, median age 27 ± 13 years) were $HBsAg^+$, eight (five males and three females, median age 28 ± 11 years) were $HBsAg^-$ and also negative for anti-hepatitis A virus (HAV), anti-cytomegalovirus (CMV) and anti-Epstein–Barr virus (EBV).

A complete clinical and laboratory evaluation was done for each patient. In addition, the serum levels of AST, ALT (n.v. 7–50 iu), bilirubin and immunoglobulins were determined on the same day as the lymphocyte transformation test was done. For each patient three different determinations were performed: at 0–2, 2–4 and 4–6 weeks from the onset of symptoms.

All the patients were discharged having clinically recovered and with normal biohumoral values apart from one $HBsAg^-$ case in whom transaminases and serum immunoglobulins remained elevated.

Twenty normal subjects (14 males and six females, median age 27 ± 10 years) without liver disease were tested as controls. In this population all the biochemical liver parameters were normal and the HBsAg was negative.

The HBsAg and HAAb were determined by radioimmunoassay (AUSRIA II, HAV Ab, Abbott). Complement fixation and immunofluorescence were used for anti-CMV and anti-EBV.

Preparation of the LSP (RLSP) was done by double gel-filtration (Sephadex G-100 and Sepharose 6B) of a 105,000 ***g*** supernatant of rabbit liver homogenate, according to the method of McFarlane *et al.* (1977).

The lymphocyte transformation test (LTT) was performed as previously described (Ortona *et al.*, 1979). Briefly, RLSP was added to culture tubes at concentrations of 1·25 and 2·50 μg/ml; nothing was added to control tubes. All the tests were done in triplicate and the total lifespan for the cultures was 5 days. The results were expressed as stimulation index: SI = c.p.m. of RLSP-stimulated culture/c.p.m. of control culture. This was considered positive if greater than 2 (mean SI for normal subjects +2 s.d.). This result of 2 has been recorded using both RLSP concentrations. In each case tested the highest stimulation index obtained with two concentrations of RLSP was recorded.

In addition, in order to evaluate the organ-specificity of cellular autoimmunity to RLSP, a LTT with a macromolecular kidney protein fraction prepared in the same manner as RLSP was done in a selected number of AH patients and controls.

The statistical evaluation of the results was done using Student's *t*-test and factorial Fischer's χ^2 test (Laben 701 computer).

RESULTS

At 0–2 weeks an increased stimulation index was recorded in 10 (56%) of the 18 cases with acute $HBsAg^+$ hepatitis and in five (63%) of the eight $HBsAg^-$ cases (Fig. 1). At 2–4 weeks the SI was high

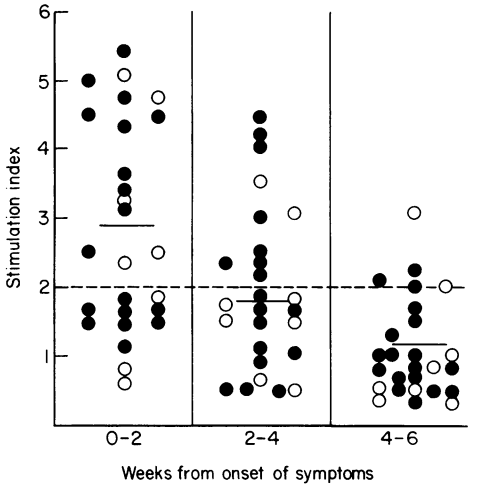

Fig. 1. Stimulation indices in patients with acute viral hepatitis according to the time of onset of symptoms. In each case tested, the highest stimulation index was recorded. The horizontal dotted line indicates the upper limit of normal. (•) $HBsAg^+$, (○) $HBsAg^-$.

in eight (44%) of the $HBsAg^+$ and two (25%) of the $HBsAg^-$ cases, but at 4–6 weeks the index remained abnormal in only three (12%) of the 26 cases (two $HBsAg^+$ and one $HBsAg^-$).

The optimal concentration of RLSP was 1·25 μg/ml in 17 patients (nine at 0–2, six at 2–4 and two at 4–6 weeks from the onset of symptoms) while it was 2·50 μg/ml in 11 patients (six at 0–2, four at 2–4 and one at 4–6 weeks from the onset of symptoms).

At no time was there any statistically significant difference between the SI values in HBsAg-positive and -negative cases and a significant correlation could not be demonstrated between the SI values and AST, ALT, bilirubin and serum immunoglobulins at any stage of the disease.

The evolution of the three cases discharged from the hospital with a significantly elevated SI is summarized in Table 1. Two cases discharged with normal transaminases but slightly elevated SIs when retested after 6 months showed stimulation indices within the normal range and liver function tests remained normal. In contrast the third case discharged with elevated transaminases and more clearly elevated SI when retested after 6 months showed persistent sensitization to RLSP and elevated transaminases. The liver biopsy documented chronic active hepatitis (CAH). The remaining 23 cases retested after 6 months showed normal SIs and biohumoral indices.

The LTT with the macromolecular kidney protein fraction as antigen always showed a stimulation index within the normal range in five patients with AH at times when the SI with RLSP as antigen was significantly increased.

Table 1. Evolution of the cases discharged from the hospital with a significantly elevated stimulation index (SI) to RLSP

Patient	Age	Sex	HBsAg	AST at discharge (n.v. 7–50 iu)	SI to RLSP 0–2*	SI to RLSP 2–4*	SI to RLSP 4–6*	AST at month 6	SI to RLSP at month 6	Evolution
B.N.	35	F	+	49	5·6	4·3	2·3	35	0·8	Recovered
C.F.	19	M	+	40	4·7	4·3	2·1	41	1·6	Recovered
P.S.	18	M	−	293	4·8	3·2	3·4	280	4·2	CAH

* Weeks from onset of symptoms.

DISCUSSION

It now seems clear that tolerance to liver antigens is lost during the acute phase of viral hepatitis and it has been postulated that failure to restore tolerance is important in the development of CALD (Eddleston & Williams, 1974).

The existence of sensitization to LSP in the course of acute hepatitis has been widely demonstrated by studies utilizing different assays, namely leucocyte migration inhibition (Meyer Zum Büschenfelde *et al.*, 1975; Realdi *et al.*, 1976; Lee *et al.*, 1977), lymphocyte transformation (Thestrup-Pedersen *et al.*, 1976; Bartholomaeus *et al.*, 1978) and lymphocytotoxicity tests (Cochrane *et al.*, 1976).

In our experience it is not always easy to obtain suitable material from which to prepare human LSP and we have used rabbit LSP as a more readily available alternative in this and a previous study (Ortona *et al.*, 1979). The validity of this approach is based on extensive studies that demonstrate the organ-specificity but the incomplete species-specificity of this liver membrane macromolecular fraction (Meyer Zum Büschenfelde & Miescher, 1972; Hopf *et al.*, 1974; McFarlane *et al.*, 1977; Hutteroth & Meyer Zum Büschenfelde, 1978). Although it has been reported recently that LSP might contain some antigenic components which cross-react with kidney proteins (Behrens & Paronetto, 1979), the immunological importance of these cross-reacting determinants is, at present, unknown. In fact, in a microcytotoxicity test using target cells coated with liver antigen cross-inhibition with kidney proteins was not always recorded (Behrens, Vernace & Paronetto, 1979). Our preliminary results, using a macromolecular kidney protein fraction prepared in the same manner as RLSP in the LTT, seem to indicate that cross-reacting cell-mediated immunity between these two preparations is not evident in patients with acute viral hepatitis.

The present results show the existence of a common sensitization to RLSP in the acute phase of hepatitis irrespective of HBsAg status. However, this autoimmune reaction seems to be transient having disappeared in almost all cases by weeks 4–6 from the onset of symptoms. This finding of a time-limited sensitization to LSP is substantially in accord with former studies done only in acute $HBsAg^+$ hepatitis, utilizing leucocyte migration inhibition either to human LSP (Meyer Zum Büschenfelde *et al.*, 1975; Lee *et al.*, 1977) or to rabbit liver proteins (Realdi *et al.*, 1976). The persistence of cellular sensitization to LSP after the acute phase of the disease could be another index to detect the presence of a continued autoimmunity which seems to be of such crucial importance in the development of chronic active liver disease.

We are indebted to Dr A. L. W. F. Eddleston of the Liver Unit, King's College Hospital, London for his valuable advice.

REFERENCES

BARTHOLOMAEUS, W.N., REED, W.D. & JOSKE, R.A. (1978) Autoimmune responses to liver antigens in man regulated by suppressor T lymphocytes. *Gastroenterology*, **75**, 954 (abstract).

BEHRENS, U.J. & PARONETTO, F. (1979) Studies on 'liver-specific' antigens. I. Evaluation of the liver specificity of 'LSP' and 'LP-2'. *Gastroenterology*, **77**, 1045.

BEHRENS, U.J., VERNACE, S. & PARONETTO, F. (1979) Studies on 'liver specific' antigens. II. Detection of serum antibodies to liver and kidney cell membrane antigens in patients with chronic liver disease. *Gastroenterology*, **77**, 1053.

CHISARI, F.V. (1980) Liver specific protein in perspective. *Gastroenterology*, **78**, 167.

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*, **1**, 441.

COCHRANE, A.M.G., MOUSSOUROS, A., SMITH, A., THOMSON, A.D., EDDLESTON, A.L.W.F. & WILLIAMS, R. (1976) Autoimmune reaction to a liver specific membrane antigen during acute viral hepatitis. *Gut*, **17**, 714.

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.

HOPF, U., MEYER ZUM BÜSCHENFELDE, 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 BÜSCHENFELDE, K.H. (1978) Clinical relevance of the liver-specific

lipoprotein (LSP). *Acta Hepatogastroenterol.* **25,** 243.

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

LEE, W.N., REED, W.D., OSMAN, C.G., VAHRMAN, J., ZUCKERMAN, A.J., EDDLESTON, A.L.W.F. & WILLIAMS, R. (1977) Immune responses to the hepatitis B surface antigen and liver-specific lipoprotein in acute type B hepatitis. *Gut,* **18,** 250.

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

MEYER ZUM BÜSCHENFELDE, K.H., KNOLLE, J. & BERGER, J. (1974) Cellulare Immunreaktionen gegenüber homologen leberspezifischen Antigenes (HPL) bei chronischen Leberentzundungen. *Klin. Wochenschr.* **52,** 246.

MEYER ZUM BÜSCHENFELDE, 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. Wochenschr.* **53,** 1061.

MIELI VERGANI, G., VERGANI, D., JENKINS, P.J., PORTMANN, B., NOWAT, A.P., EDDLESTON, A.L.W.F. & WILLIAMS, R. (1979) Lymphocyte cytotoxicity to autologous hepatocytes in HBsAg-negative chronic active hepatitis. *Clin. exp. Immunol.* **38,** 16.

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.

ORTONA, L., LAGHI, V., CAUDA, R. & NERVO, P. (1979) Lymphocyte transformation test with rabbit liver specific lipoprotein (RLSP) in chronic active hepatitis. *Clin. exp. Immunol.* **39,** 231.

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. *Minerva Gastroenterol.* **22,** 207.

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 disease. *Br. Med. J.* **i,** 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, A.M.G., MCFARLANE, I.G., EDDLESTON, A.L.W.F. & WILLIAMS, R. (1974) Lymphocyte cytotoxicity to isolated hepatocytes in chronic active hepatitis. *Nature,* **252,** 721.

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