Clin. exp. Immunol. (1981) **45**, 143–151.

Histological and immunological investigation of liver-specific protein (LSP) immunized rabbits compared with patients with liver disease

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(Accepted for publication 16 January 1981)

SUMMARY

In this study 45 patients with a variety of liver diseases did not demonstrate T lymphocyte sensitivity to liver-specific protein (LSP) as assessed by lymphocyte transformation whereas LSP-immunized rabbits developed both cellular and humoral immunity to this antigen. Although these LSP-immunized rabbits demonstrated portal tract inflammation with some hepatocyte necrosis, rabbits immunized with antigens known to contaminate LSP developed similar lesions. These findings contrast with those of other investigators who have reported immune responsiveness to LSP in liver patients and chronic active hepatitis in LSP-immunized rabbits. In determining the significance of these studies it must be emphasized that all preparations of LSP contain an heterogeneous group of antigens and that the specific sensitizing antigen has yet to be indentified.

INTRODUCTION

Meyer zum Büschenfelde & Miescher (1972) described the isolation from human liver cytosol of two liver-specific antigens—liver-specific protein (LSP) located on hepatocyte plasma membrane and a cytoplasmic antigen known as LP2. Since immunological mechanisms involved in the initiation of hepatocyte damage may be directed against cell membrane components, the *in vitro* function of LSP in a variety of liver diseases has been repeatedly investigated. Miller *et al.* (1972) reported that leucocyte migration was inhibited in the presence of LSP in patients with chronic active hepatitis (CAH) and primary biliary cirrhosis (PBC). Sensitization to LSP was also found in lymphocyte transformation experiments (Thestrup-Pedersen, Ladefoged & Andersen, 1976; Ortona *et al.*, 1979). Lymphocyte cytotoxicity of cultured rabbit liver cells was described in patients with CAH, viral hepatitis (VH) and alcoholic hepatitis (Cochrane *et al.*, 1976a, 1976b, 1977) and it was implied that LSP was the principal target antigen. Employing a different assay, other investigators have also produced evidence in favour of LSP-directed cytotoxicity in CAH (Vogten *et al.*, 1978). The findings of antibody to LSP in the sera of liver patients (Jensen *et al.*, 1978; Kakumu *et al.*, 1979) supported the contention that hepatocyte cytotoxicity was mediated by antibody-dependent cell-mediated mechanisms (Cochrane *et al.*, 1976a). The significance of these reports of immunological reactity to LSP in man is supported by the finding that rabbits repeatedly immunized with liver fractions containing LSP developed a liver lesion with features similar to CAH (Meyer zum Büschenfelde & Hopf, 1974).

Thus reports to date suggest that LSP may act as a target antigen in liver disease. In evaluating

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0099-9104/81/0700-0143$02.00

these reports it is important to recognize that LSP preparations are biochemically heterogeneous and contain organ-non-specific as well as organ-specific determinants (Chisari, 1980; McFarlane, Wojcicka & Williams, 1980). It is not known which of the multiple antigenic determinants present in LSP preparations are responsible for the immunological phenomena which have been described.

In this study, using the lymphocyte transformation test, cell-mediated immunity to LSP was investigated in patients with liver disease and the results contrasted with those of similar experiments in rabbits immunized with LSP. In addition, the liver histopathology of LSP-immunized rabbits was compared with that in rabbits immunized with antigens known to contaminate LSP preparations. These contaminants detected by specific reaction with antisera in immunodiffusion studies include serum protein and organ-non-specific (kidney) antigens (Meyer zum Büschenfelde & Miescher, 1972; McFarlane *et al.*, 1977).

MATERIALS AND METHODS

Patients and controls. The 45 patients studied consisted of 16 with chronic active hepatitis, 12 with acute viral hepatitis, 13 with alcoholic liver disease and four with primary biliary cirrhosis. The diagnoses were based on established clinical and histological criteria (Sherlock, 1975).

The patients with chronic active hepatitis consisted of 11 females and five males, mean age 42 years (range 5–65 years). Nine were taking prednisolone at the time of study. Three were seropositive for hepatitis B surface antigen as measured by reverse passive haemagglutination (Wellcome Reagents).

Ten of the patients with viral hepatitis were studied within 5 weeks of the onset of their symptoms. All but one of the 11 patients with alcoholic liver disease had acute alcoholic hepatitis at the time of study. One patient with viral hepatitis and one with alcoholic liver disease were seropositive for hepatitis B surface antigen. The patients with primary biliary cirrhosis were all middle-aged females (mean age 58 years) with positive mitochondrial antibodies.

Twelve normal healthy control subjects and seven patients with other clinical disorders were also studied.

Antigen preparation. LSP was isolated in Tris–EDTA buffer from fresh human autopsy liver in a manner identical to that described by McFarlane *et al.* (1977). The presence of the liver-specific antigen in the preparation was confirmed by reaction against an antiserum kindly provided by Dr I. McFarlane. A less pure form of LSP was prepared by Sephadex G-100 gel filtration of liver cytosol and this fraction is reputed to contain the additional liver antigen LP2 (Meyer zum Büschenfelde & Miescher, 1972). A third liver 'microsomal' fraction was also prepared from the supernatant of a 16,300 *g* centrifugation of liver homogenate. This was recentrifuged at 105,000 *g* for 1 hr and the resultant pellet was resuspended in Tris/EDTA buffer and stored at −70°C.

A human kidney fraction was prepared by homogenization of fresh autopsy kidney (in Tris/EDTA buffer, pH 8·00) using a Potter homogenizer with Teflon pestle. The material was then centrifuged at 16,300 *g* for 15 min and the supernatant was stored at −70°C.

Heterologous serum was obtained from deer (*Cervus elaphus* and *Cervus nippon*) as part of a study of deer species identification.

The protein concentration of these fractions was measured according to Lowry *et al.* (1951) and, before use in lymphocyte cultures, fractions were sterile-filtered through a Millipore filter (0·22 μm). Following filtration of LSP, both protein concentration and immunoreactivity were reduced by approximately 50%. In immunodiffusion studies with the reference anti-LSP antiserum, filtered LSP demonstrated identity with unfiltered LSP except that the filtered material gave a less dense line of precipitation. This decrease in the precipitation band could be restored by reconcentration of the filtrate confirming that filtration did not cause a selective decrease in immunoreactive LSP.

Animals. Eighteen New Zealand white rabbits of both sexes were used. They were kept in single cages in an air-conditioned room and were fed with standard rabbit food. The animals were divided into four groups (Table 1) and rabbits were immunized intradermally each month for 1 year. The antigen fractions were sonicated with Freund's complete adjuvant at 4°C to form a water-in-oil emulsion. Groups A and C were immunized with LSP receiving 0·1 and 1·0 mg respectively per

Table 1. Scheme for immunization of rabbits. All animals were injected intradermally (i.d.) with 0·1–1·0 mg of antigen

	Rabbit groups			
	A	B	C	D
Number	6	6	3	3
Antigen	LSP	Serum	LSP	Kidney
mg i.d.	0·1	0·1	1·0	1·0

month. Group B rabbits were given 0·1 mg of heterologous (deer) serum and group D 1·0 mg of kidney antigen.

Lymphocyte cultures. Methods similar to those previously described were used (Feighery *et al.*, 1978). Mononuclear cells were obtained from heparinized blood by plasma gel (Laboratoire Roget Bellon) sedimentation and density-gradient centrifugation using Ficoll–Hypaque (Böyum, 1968). Cells were then washed twice with RPMI 1640 (GIBCO, Biocult) and resuspended in RPMI supplemented with 10% fetal calf serum and 100 units/ml of penicillin–streptomycin solution (GIBCO, Biocult). Different batches of fetal calf serum were used during these experiments. Each batch was selected as suitable by prior testing to ensure that cell growth was adequately supported and that neither intrinsic cytotoxic nor stimulatory factors were present.

One hundred thousand cells in 0·2 ml of culture medium were then added to the wells of a microculture plate (Sterilin microtitre F) and incubated at 37°C in a humidified 5% CO_2/air atmosphere. All cultures were performed in triplicate and cells were incubated with liver antigens and in control cultures with Tris/EDTA buffer. The liver antigens used were: LSP with a concentration range 0·2–100 μg/ml; LSP-LP2 with a concentration range 0·01–300 μg/ml; and a microsomal liver fraction using a similar concentration range. The capacity of cells to respond to mitogen was tested in all experiments and pokeweed mitogen (PWM, GIBCO) was used with a concentration range of 0·1–1,000 μg/ml. In some experiments sensitivity to purified protein derivative of tuberculin (PPD, Evan's Medical Co.) was tested at concentrations of 1, 10 and 20 μg/ml.

Four hours before the end of the incubation period (120 hr), 0·3 μCi of ^{3}H-thymidine (sp. act. 2·0 mCi/mmol; Radiochemicals) was added to each culture well. The contents of each well were then harvested onto glass-fibre discs (Whatman), washed in 10% trichloroacetic acid and dehydrated with methanol. The incorporation of radioactive thymidine in the dried discs was measured by liquid scintillation counting (Tricarb, Packard). Results are expressed as counts per minute (c.p.m.) of ^{3}H-thymidine uptake. In order to compare these results with those of other reports, a stimulation index was calculated in similar manner to Thestrup-Pedersen *et al.* (1976).

Histology. After completing the immunization schedule, rabbit liver specimens were obtained, fixed in formalin and processed by routine methods. Sections stained with haematoxylin & eosin were examined and the presence or absence of the following were noted: portal inflammation, periportal inflammation, lobular inflammation, piecemeal necrosis, lobular necrosis. These were defined as follows:

Portal inflammation: Inflammatory infiltrate confined to the portal tracts.
Periportal inflammation: 'Spill-over' of inflammation from the portal tract into the periportal area but without loss of hepatocytes.
Lobular inflammation: Inflammatory cells in the lobules with or without hepatocyte necrosis.
Piecemeal necrosis: Periportal inflammation with loss of hepatocytes.
Lobular necrosis: Loss of individual hepatocytes or groups of hepatocytes in the lobules.

Rabbits in groups C and D were biopsied before and after immunization. Preimmunization liver biopsies were obtained using a Tru-cut biopsy needle (Travenol Laboratories Inc.), the animals

receiving the neuroleptanalgesic *etorphine hydrochloride* in combination with methotrimeprazine (Immobilon, Reckett & Colman Pharmaceuticals).

RESULTS

In LSP-pulsed cultures (Fig. 1), seven antigen concentrations were used. None of the 45 liver patients (16 with chronic active hepatitis, 12 with viral hepatitis, 13 with alcoholic liver disease and four with primary biliary cirrhosis) demonstrated increased ^{3}H-thymidine incorporation when their lymphocytes were cultured with LSP. Eleven patients who were restudied and 21 control studies showed a similar lack of *in vitro* stimulation by LSP. In contrast, mononuclear cells from rabbits which had been primed to LSP *in vivo* demonstrated clear sensitization to the antigen when challenged *in vitro* and two examples of stimulated cultures are shown. Stimulation was found in the three immunized rabbits who were tested and in none of six non-immunized rabbits. In Fig. 2 the results from 45 liver patients following culture with LSP (10 μg/ml) are shown. Similar ^{3}H-thymidine incorporation was found at the other antigen concentrations tested. No significant increase in isotope incorporation was observed in any patient and in no instance was a stimulation index of greater than 2 found.

The liver antigen preparation which includes LSP and LP2 was used in further cultures (Fig. 3). Mononuclear cells from rabbits immunized with LSP demonstrated a clear blastogenic response to these antigens. High antigen concentrations (protein greater than 50 μg/ml) had an inhibitory effect on ^{3}H-thymidine incorporation. Lymphocytes from seven liver patients (four with chronic active hepatitis) were cultured with LSP-LP2 at a concentration range of 1–50 μg/ml, the sensitive range in immunized rabbits, but no significant increase in ^{3}H-thymidine uptake occurred. In only one instance was a stimulation index of greater than 2 found. The patient was a male with HBsAg-positive viral hepatitis who had a stimulation index of 2·1 at one antigen concentration (LSP-LP2, 1 μg/ml).

In other experiments using liver microsomes as antigen, similar results were obtained. The cells of two immunized rabbits were stimulated by this antigen preparation *in vitro* whereas those of three non-immunized rabbits and 12 liver patients were unresponsive. The different responses demonstrated by rabbit and human mononuclear cells could not be explained by a difference in cell viability since both cell types showed equal viability values following culture with liver antigens.

To demonstrate their capacity to proliferate in culture, all cell preparations were cultured in the

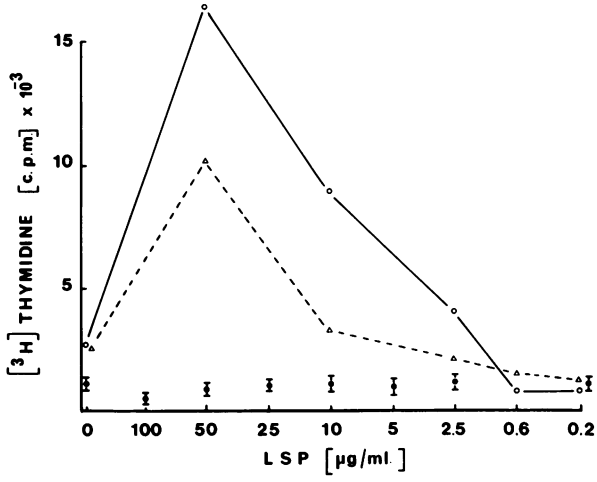


Fig. 1. Result of LSP-pulsed lymphocyte cultures in immunized rabbits (○, △) and liver patients (●). Points represent means and bars show standard errors. Evidence of stimulation is given by ^{3}H-thymidine incorporation.

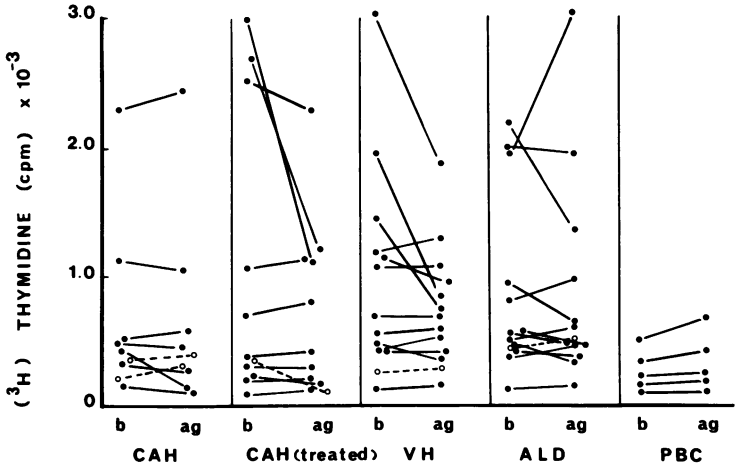


Fig. 2. Details of results in 45 liver patients after *in vitro* culture of their lymphocytes with LSP (10 μg/ml). HBsAg-negative patients (•), HBsAg-positive patients (o). Patients are grouped as follows: CAH = chronic active hepatitis, not on immunosuppressive treatment ($n = 7$); CAH (treated) = chronic active hepatitis, receiving immunosuppressive treatment ($n = 9$); VH = viral hepatitis ($n = 12$); ALD = alcoholic liver disease ($n = 13$); PBC = primary biliary cirrhosis ($n = 4$). Repeated studies in six patients are also shown. The results are expressed as c.p.m. of ^{3}H-thymidine uptake after culture with Tris-HCl buffer (b) or LSP (ag).

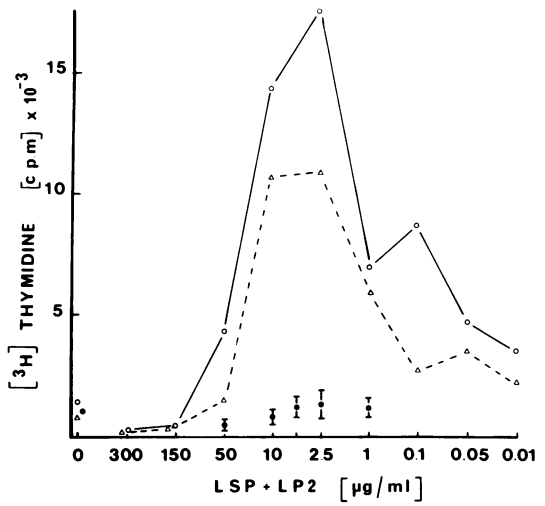


Fig. 3. Result of LSP-LP2-pulsed lymphocyte cultures in immunized rabbits (o, △) and liver patients (•). Points represent means and bars show standard errors. Evidence of stimulation is given by ^{3}H-thymidine incorporation.

presence of PWM and patients responded normally to this mitogen. In addition, some patients' cells were cultured with PPD and approximately half of them demonstrated sensitivity to this antigen.

Liver biopsies of rabbits before immunization showed normal liver tissue. Table 2 shows the frequency of the various histopathological features in post-immunization rabbits.

There appeared to be no significant difference between the groups. Portal inflammation was present in virtually all liver biopsies (16 of 18) and there was no significant difference in the severity of the inflammation between the groups. Periportal and lobular inflammation were also of the same frequency and severity in those rabbits injected with LSP (groups A and C) as those injected with other antigens (groups B and D). Necrosis was slightly more frequent in LSP groups than in

Table 2. Histological features in post-immunization rabbits

Histological features	Rabbit groups A	B	C	D
Portal inflammation	4/6	6/6	3/3	3/3
Periportal inflammation	2/6	3/6	3/3	1/3
Lobular inflammation	2/6	2/6	2/3	3/3
Piecemeal necrosis	1/6	1/6	1/3	0/3
Lobular necrosis	1/6	0/6	2/3	1/3

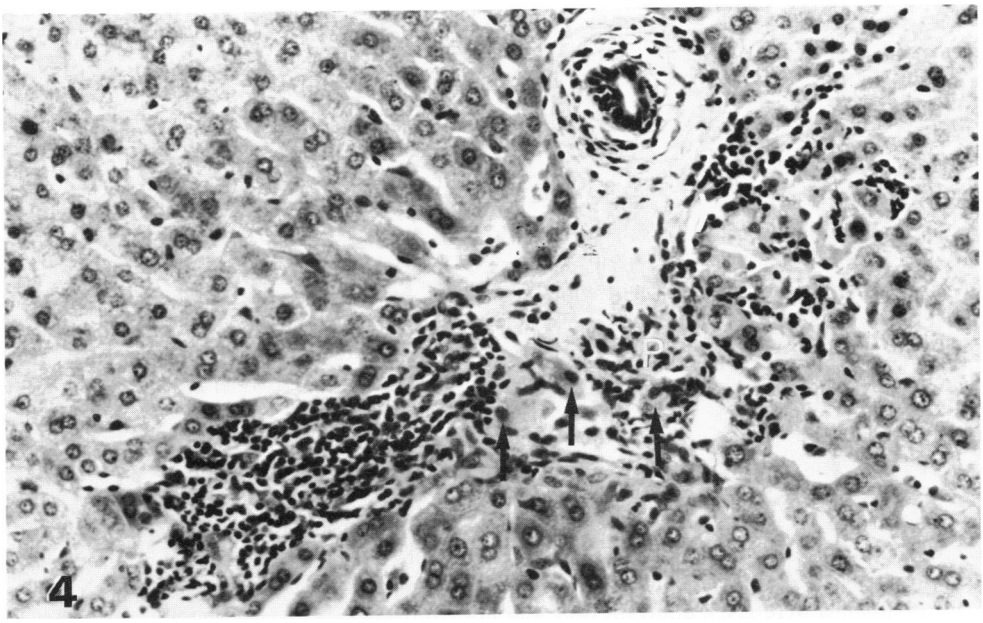
non-LSP groups—piecemeal necrosis two of nine and one of nine respectively and lobular necrosis three of nine and one of nine respectively. Piecemeal necrosis was mild in all cases and involved only part of the circumference of one or two portal tracts in the entire biopsy. The adjacent hepatocytes appeared morphologically normal, and there were no pale swollen hepatocytes such as occur in humans with chronic hepatitis. Typical liver histology post-LSP and post-deer serum immunization are shown in Figs 4 and 5 respectively.

DISCUSSION

In this study 45 patients with a variety of liver diseases were found not to be sensitized to LSP as assessed by the lymphocyte transformation test (LTT). The study group included patients with CAH, PBC, VH and alcoholic liver disease and similar patients have been reported to demonstrate immune responsiveness to LSP using leucocyte migration inhibition and cytotoxicity assays (Miller *et al.*, 1972; Cochrane *et al.*, 1976a, 1976b, 1977; Vogten *et al.*, 1978; Kawanishi & MacDermott, 1979). Prednisolone has been reported to depress evidence of sensitization to LSP (Miller *et al.*, 1972) and nine of the 16 CAH patients were taking this medication at the time of study. The remaining eight patients were not on therapy but nonetheless their cells were not stimulated by LSP *in vitro*.

Although originally described as being very labile (Meyer zum Büschenfelde & Miescher, 1972), LSP in the Tris/EDTA buffer is stable for up to 18 months (McFarlane *et al.*, 1977). In case the potential lability of LSP could have been a factor in the negative results obtained, a more stable form of the antigen, LSP-LP2, was also used in some experiments. In addition, LSP was freshly prepared for many experiments. However, neither of these two manoeuvres resulted in LSP-induced transformation. Control experiments ensured that in all cultures, cells were capable of proliferating to a mitogen (PWM) and in many instances to a specific antigen (PPD).

In view of the biochemical heterogeneity of LSP, it is useful to distinguish between LSP the molecule and LSP the preparation (Chisari, 1980). In employing this terminology LSP the molecule is the organ-specific hepatocyte surface membrane antigen which, together with multiple other antigens included in this liver fraction, comprises LSP the preparation. It was shown in other studies that rabbits immunized with LSP developed a humoral response to many of the antigens contained in this preparation (Feighery *et al.*, 1981). The LTTs in these immunized rabbits demonstrated the presence of T lymphocytes sensitized to antigenic determinants present in LSP preparations but did not prove that the LSP molecule acted as an antigenic stimulus. There was no evidence that the Tris/EDTA buffer interfered with antigen-induced blastogenesis. The stimulation by liver microsomes suggests that antigens of this fraction cross-react with antigens contained in the LSP preparations. This suggestion is supported by the finding that rabbit anti-LSP reacted with microsomal liver in immunodiffusion studies (Feighery *et al.*, 1980). Microsomal stimulation could also be explained by the representation of endoplasmic reticulum antigens in the plasma membrane and it has been suggested that these two membrane systems may be in continuity (Emmelot *et al.*, 1964).



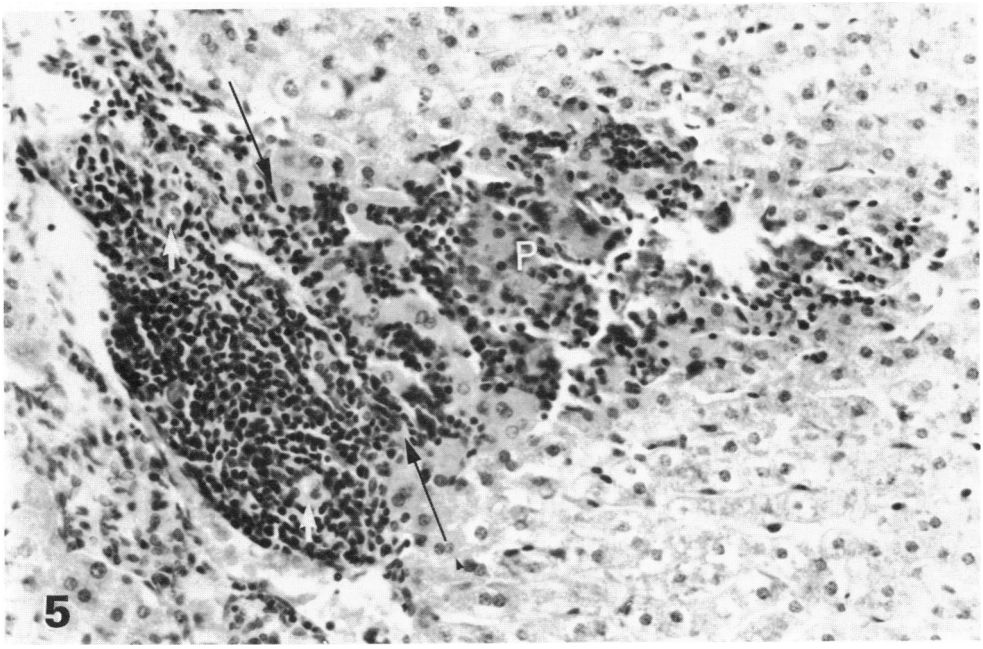


Fig. 4. Liver of rabbit (group C) injected with liver-specific protein. Lymphocytic infiltrate is seen in a portal tract with 'spill-over' into the lobule on the right (periportal inflammation). There is a small area of piecemeal necrosis (P) with loss of liver cells and occasional isolated hepatocytes (*arrows*). (H & E, × 265.)

Fig. 5. Liver of rabbit (group B) injected with deer serum. Dense lymphocytic infiltrate in a portal tract closely related to two small bile ducts (*short arrows*). There is piecemeal necrosis with loss of liver cells and extension of the inflammatory infiltrate into the lobule (P). Long arrows mark original limit of portal tract. (H & E, × 270.)

The results presented here are in conflict with two previous studies which reported *in vitro* reactivity to LSP using the LTT (Thestrup-Pedersen *et al.*, 1976; Ortona *et al.*, 1979), although the level of stimulation recorded by Thestrup-Pedersen *et al.* (1976) was generally low. In both earlier studies, a macroculture technique was used, Tris/EDTA buffer was not added to the control cultures and the results were expressed as a transformation index. These technical differences may in part explain the conflicting results obtained.

If the LSP molecule is a target antigen in liver disease, why did LSP fail to stimulate in these experiments? The possible explanations include: (1) The LSP preparation used may not allow the presentation of the LSP molecule in a manner suitable for lymphocyte stimulation. This might involve inhibition of blastogenesis by other factors present in the preparation and it was noted in this study and in other reports (Thestrup-Pedersen *et al.*, 1976; Ortona *et al.*, 1979) that high antigen protein concentrations inhibited ^{3}H-thymidine uptake. (2) Patients with these liver disorders may not possess T lymphocytes sensitized to LSP. The finding that T lymphocytes are not the effector cells in rabbit hepatocyte cytotoxicity experiments is in support of this explanation (Cochrane *et al.*, 1976a). Positive leucocyte migration inhibition in the presence of LSP (Miller *et al.*, 1972) might be considered to represent the effect of sensitized T lymphocytes, but it has also been reported that migration inhibition may involve other mechanisms including the interaction of antigen with cytophilic antibodies on the surface of polymorphonuclear leucocytes (Bloom, 1971; Rocklin, 1974).

Meyer zum Büschenfelde & Hopf (1974) reported that rabbits immunized intraperitoneally with LSP-LP2 developed CAH or liver cirrhosis. The rabbits in that study were immunized over a prolonged period (143 weeks) and at each inoculation given a large quantity of protein (25 mg). Control animals were given adjuvant only and not control antigens. In the study reported here, rabbits were immunized intradermally with Sepharose 6B-purified LSP. Although portal inflammation developed in the livers of these rabbits, neither CAH nor cirrhosis was found and this may be related to the shorter immunization schedule and smaller quantities of antigen administered. However, animals immunized with antigens known to contaminate LSP preparations, serum protein and organ-non-specific antigens, demonstrated similar liver lesions, and the outcome of their long-term immunization is open to speculation. This finding emphasizes the importance of identifying precisely the antigen(s) which induce(s) the liver lesions described by Meyer zum Büschenfelde & Hopf (1974).

It is likewise important to identify the antigen responsible for the *in vitro* reactivity to LSP described in patients. Further studies are required before it can be assumed that this reactivity is directed against a liver-specific plasma membrane target and that this is peculiar to patients with liver disease. In this regard it is known that liver mitochondrial antigens, not considered to be organ-specific, caused leucocyte migration inhibition not only in patients with liver disease but also in patients with other autoimmune disorders (Brostoff, Roitt & Doniach, 1969; Ohta, Nonomura & Nishimura, 1976).

Although LSP preparations appear to contain an organ-specific hepatocyte plasma membrane antigen, further evidence against the purity of this preparation is the finding that LSP does not demonstrate the typical biochemical properties of a plasma membrane fraction (Feighery & Weir, 1980). Improved methods of liver antigen purification are required to help determine the role of these antigens in liver disease.

Conleth Feighery was the recipient of a grant from the Medical Research Council of Ireland.

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