Clin. exp. Immunol. (1972) **10**, 99–108.

EXPERIMENTAL CHRONIC ACTIVE HEPATITIS IN RABBITS FOLLOWING IMMUNIZATION WITH HUMAN LIVER PROTEINS

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(*Received* 3 *November* 1971)

SUMMARY

Two liver-specific antigens are known: a water soluble protein (LP-2) and a water insoluble macromolecular low density lipoprotein (LP-1).

In this paper the relative role of the two antigens in the development of experimental immune hepatitis has been investigated. Immunization of rabbits with a human preparation containing both antigens, led in all animals to lesions characteristic of an immune hepatitis. Immunization of the animals with a purified water soluble liver protein proved less efficient: only two out of six animals developed characteristic lesions which were less severe than those in the first group. It was deduced that although not a prerequisite, the liver-specific lipoprotein plays an important supportive role in the development of immune hepatitis.

INTRODUCTION

In past years a water soluble liver-specific protein has been characterized immunologically (Licht, 1966; Meyer zum Büschenfelde, 1966, 1968; Meyer zum Büschenfelde & Miescher, 1971). Furthermore, using a partially purified water soluble liver fraction, it was possible to produce an experimental autoimmune hepatitis (Kössling & Meyer zum Büschenfelde, 1968, 1970; Meyer zum Büschenfelde & Kössling, 1971). Only recently were we able to detect a second liver-specific antigen which is water insoluble and which proved to be a macromolecular low density lipoprotein (Meyer zum Büschenfelde & Miescher, 1971).

MATERIALS AND METHODS

Animals

Twenty-one New Zealand White rabbits of both sexes, weighing $2{\cdot}5 \pm 0{\cdot}15$ kg were used, in single cages in a climatized animal room. The animals were fed with standard rabbit food.

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They were divided into three groups of eight, eight and five animals. The immunizing procedure is summarized in Table 1. One animal of group 1 and two animals of group 2 died before completion of the experiment.

TABLE 1. Immunization of three groups of rabbits

Date	Group 1 (eight animals) Sephadex G-100 fraction 1 (LP-1 + LP-2) (mg)	CFA* (ml)	Group 2 (eight animals) Sephadex G-200 fraction 2 (LP-2) (mg)	CFA* (ml)	Group 3 (five animals) CFA* (ml)	Bleedings	Animals deceased
1.10.69	10	2	5	2	2		
8.10.69	10	2	5	2	2		
15.10.69	10	2	5	2	2		
23.10.69	10	2	5	2	2		
29.10.69	10	2	5	2	2		
5.11.69	10	2	5	2	2		
12.11.69	10	2	5	2	2		
18.11.69	10	2	5	2	2		
26.11.69	10	2	5	2	2		
3.12.69	10	2	5	2	2		
18.12.69	—	—	—	—	—	+	Rabbit No. 4 of group 2
18.12.69	50	2	25	2	2		
8. 1.70		—	—	—	—	+	
21. 1.70	50	2	25	2	2		
28. 1.70	—	—	—	—	—	+	
5. 2.70	50	2	25	2	2		
11. 2.70	—	—	—	—	—	+	Rabbit No. 7 of group 1 Rabbit No. 2 of group 2
20. 2.70	50	2	25	2	2		
27. 2.70	—	—	—	—	—	+	
4. 3.70	50	2	25	2	2		
16. 4.70	—	—	—	—	—	+	
10. 6.70	—	—	—	—	—	+	
3. 7.70	—	—	—	—	—	+	All animals killed

* Freund's complete adjuvant.

The antigen used for immunization was obtained from apparently normal human liver as described elsewhere (Meyer zum Büschenfelde, 1968; Meyer zum Büschenfelde & Miescher, 1971). In summary, the crude liver homogenate was submitted to 150,000 *g*. The supernatant was fractioned by chromatography through Sephadex G-100. The first peak of this procedure was further fractioned by Sephadex G-200 chromatography, into a first peak containing the liver-specific lipoprotein (LP-1), and a second peak containing the liver-specific protein (LP-2). The Sephadex G-100 fraction 1 contained the two liver-specific antigens which made up at least 60% of this fraction. In addition, a few plasma proteins were also

present, in particular albumin and haptoglobulin. The proportion of the two liver-specific antigens was roughly 4:3 in favour of the liver-specific protein LP-2.

The LP-1 fraction was very unstable and proved unsuitable for immunization. The LP-2 fraction contained all of the liver-specific protein which made up more than 80% of the preparation. It was not possible to characterize the remaining 20%. For immunization, the Sephadex G-100 fraction 1 and the LP-2 fraction were used (see Table 1).

Serologic procedures

1. *Gel-diffusion* was carried out according to the technique of Ouchterlony.
2. *Immunoelectrophoresis.* The slide method of Scheidegger was used.
3. *Passive haemagglutination.* The microtitration technique described by Casey has been used as previously described (for 1, 2 and 3 see Meyer zum Büschenfelde & Miescher, 1971).
4. *Skin reactions.* Rabbits were skin tested with a 1% sterile solution of the first Sephadex G-100 fraction, as well as the second Sephadex G-200 fraction obtained from human as well as from rabbit liver homogenate. The solutions were injected into depilated skin areas on the back of the animals. The results were read after 24 and 48 hr.
5. *Absorption experiments.* Antisera were absorbed with various protein preparations in amounts of 25 mg/per ml immune serum. The sera were first incubated at 37°C for 30 min and subsequently for 24 hr at 4°C. They were examined after removing all precipitates by centrifugation.
6. *Fluoresceination procedure of human and rabbit LP-2.* The LP-2 fraction has been brought to a concentration of 10 mg/ml by diluting it in 0·85% sodium chloride solution containing 10% of a 0·5 M carbonate buffer, pH 9·5. Fluoresceine isothiocyanate (FITC) was added in a proportion of 5 mg of FITC per 100 mg of protein. The mixture was incubated for 14 hr at 4°C. After the first hour the pH was re-adjusted to 9·5. Following incubation the labelled protein was passed through Sephadex G-50. The main fluorescent fraction was collected and dialysed against 0·5 M tris-buffer for 3 days. The final solution was used at a concentration of 10 mg protein per ml. The absorption maximum of FITC labelled fraction LP-2 was found to be at 700 mμ.
7. *Immunofluorescence.* Thin (6–8 μm) cryostat sections were obtained from fresh organs (liver, kidney, spleen) of the rabbits. After air drying the sections were fixed in cold acetone for 10 seconds, washed three times for 5 min with PBS before being exposed to the following fluoresceinated agents: human LP-2, rabbit LP-2 and goat anti-rabbit γ-globulin. The anti-rabbit γ-globulin serum, obtained from Behring Werke, Marburg, was used in dilutions 1:4, 1:8 and 1:16. The preparations were incubated for 1 hr at 37°C in a humid chamber. They were then carefully washed in PBS and mounted in glycerine gelatine. Controls were performed by blocking experiments using the unlabelled reagents.
8. *Histology.* Fragments of liver, spleen, kidney, lymphnodes, lung, thyroid, heart and skeletal muscle were fixed in 10% buffered neutral formalin. Sections were prepared on paraffin embedded fragments. For selected purposes liver fragments were embedded in methacrylate for semi-thin sections (Kössling, 1970).

RESULTS

Serological studies

The immune response of the rabbits was assessed at regular intervals with passive haemag-

glutination of erythrocytes coated with human and rabbit LP-2 (Table 2). All animals of group 1 and group 2 developed antibodies reacting with human LP-2. Reactivity against rabbit LP-2 appeared only after a 5-month immunization period, and at weaker titres. At this time all animals of group 1 reacted with rabbit LP-2, but only two out of six animals of group 2 gave positive reactions. The animals in group 3 remained unreactive. The titres of positive reactions varied considerably: 1:2000 to over 1:1,000,000 against human LP-2 and 1:8–1:512 against rabbit LP-2.

TABLE 2. Results of passive haemagglutination tests using human (H) and rabbit (R) LP-2 as antigen. A titre of 1 : 4 or more is considered as positive

	Results expressed as number of animals with positive result/number of animals tested					
	Group 1		Group 2		Group 3	
Date of bleeding	HLP-2	RLP-2	HLP-2	RLP-2	HLP-1	RLP-2
18.12.69	8/8	0/8	8/8	0/8	0/5	0/5
28. 1.70	8/8	0/8	8/8	0/8	0/5	0/5
11. 2.70	8/8	0/8	8/8	0/8	0/5	0/5
27. 2.70	8/8	4/8	8/8	3/8	0/5	0/5
16. 4.70	7/7	5/7	6/6	3/6	0/5	0/5
10. 6.70	7/7	7/7	6/6	2/6	0/5	0/5

Absorption studies indicated that sera treated with human LP-2 lost their reactivity against human LP-2 while reacting more weakly with rabbit LP-2. Conversely, sera treated with rabbit LP-2 lost their reactivity against this liver protein while reacting practically to the same extent with human liver protein (Table 3).

A similar result was obtained on skin testing. All animals of group 1 gave a positive reaction with human and rabbit liver protein while only two out of six animals of group 2 exhibited a cross reactivity with rabbit liver protein (Table 4).

The results of immunofluorescence studies on liver sections are summarized in Table 5, and illustrated in Fig. 1.

The histological findings are summarized in Table 6. All animals of group 1 exhibited the characteristic lesions of chronic active hepatitis (infiltration with lymphocytes and plasma cells within the portal space penetrating at different sites into the liver parenchyma, with piecemeal necrosis). Only two out of the six animals of group 2 developed a similar picture which may be qualified as chronic active hepatitis. Round cell infiltrations were found within the kidney and in the lungs of all the animals. The other organs (thyroid, lymphnodes, heart, skeletal muscle) appeared unaffected.

Fig. 2 shows the lesions in animal three of group 1, exhibiting quite extensive inflammatory changes within the portal space and penetrating into the liver parenchyma.

Fig. 3 shows the changes of piecemeal necrosis using a semi-thin section.

TABLE 3. Results of absorption studies (serum from blood obtained on June 10, 1970)

No. of animals	Test-antigen	Results of passive haemagglutination expressed in reciprocal titre Antigen used for absorption of serum: 0	HLP-2	RLP-2
Group 1				
1	HLP-2	256	0	256
2	HLP-2	256	0	256
3	HLP-2	512	0	128
4	HLP-2	256	0	512
5	HLP-2	256	0	512
6	HLP-2	512	0	1024
8	HLP-2	256	0	128
1	RLP-2	64	8	0
2	RLP-2	16	16	0
3	RLP-2	512	64	0
4	RLP-2	64	0	0
5	RLP-2	64	32	0
6	RLP-2	512	128	0
8	RLP-2	32	0	0
Group 2				
1	HLP-2	256	0	512
3	HLP-2	512	0	1024
5	HLP-2	512	0	512
6	HLP-2	512	0	1024
7	HLP-2	256	0	256
8	HLP-2	128	0	128
1	RLP-2	16	32	0
3	RLP-2	32	0	0
5	RLP-2	512	16	0
6	RLP-2	0	0	0
7	RLP-2	0	0	0
8	RLP-2	0	0	0

TABLE 4. Result of skin testing with human and rabbit liver fraction G 100-1 (containing LP-1 and LP-2), and with human LP-2

	Number of animals with positive skin test/number of animals tested			
	HLP G100-1	RLP G100-1	HLP-2	Saline
Group 1	7/7	7/7	7/7	0/7
Group 2	6/6	2/6	6/6	0/6
Group 3	0/5	0/5	0/5	0/5

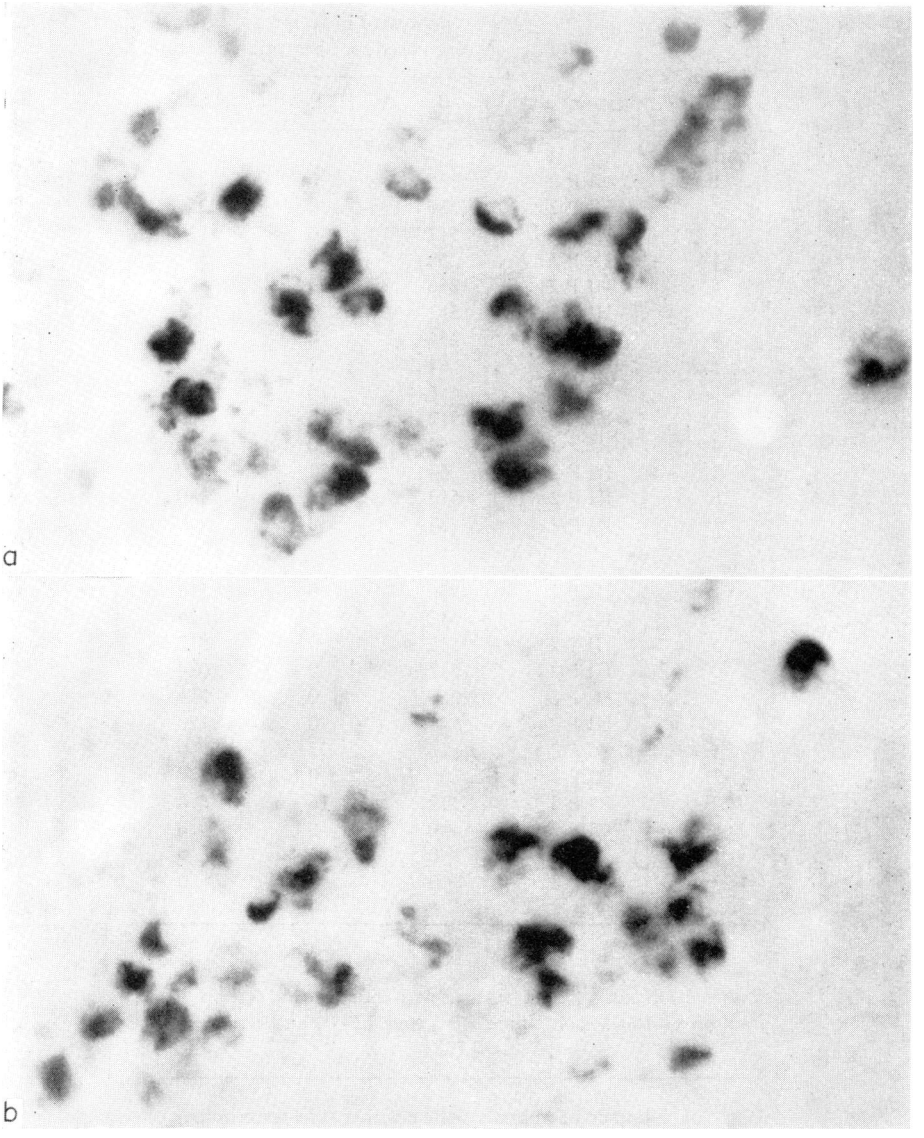

FIG. 1. Staining of mononuclear cells in the periportal space of rabbit No. 3, group 1 with (a) FITC labelled goat anti-rabbit γ-globulin, and with (b) FITC labelled RLP-2.

TABLE 5. Result of immunofluorescence studies on cryostatsections of all animals at the end of the experiment. Retention of mononuclear cells in the periportal space staining with FITC labelled anti-rabbit γ-globulin, with labelled HLP-2 as well as with labelled RLP-2

	Mononuclear cells in the periportal space staining with the following FITC labelled reagents:		
	Anti-rabbit Ig	HLP-2	RLP-2
Group 1	7/7	7/7	7/7
Group 2	4/6	4/6	2/6
Group 3	0/5	0/5	0/5

TABLE 6. Result of histologic examination

Group	Liver	Kidney
I/1	CAH-I	No lesion
I/2	CAH-I	No lesion
I/3	CAH-II	Infiltrates
I/4	CAH-I	No lesion
I/5	CAH-I	No lesion
I/6	CAH-I	No lesion
I/8	CAH-II	Infiltrates
II/1	CAH-I	Infiltrates
II/3	PI	No lesion
II/5	CAH-II	Infiltrates
II/6	PI	No lesion
II/7	PI	No lesion
II/8	PI	No lesion

Three degrees of liver damage can be distinguished:
Few periportal infiltrates: PI. Diffuse periportal infiltrates with impairment of periportal-parenchymal Barrier and scanty piecemeal necrosis (mild chronic active hepatitis): CAH-I. Diffuse periportal infiltrates with tendency to invade the liver parenchyma and piece-meal necrosis seen in every section: CAH-II. The spleen shows follicular hyperplasia throughout and a reactive red pulp. The kidneys of some animals exhibited a few round cell-infiltrates in the interstitium. The lungs exhibited round cell septal infiltrates in all animals.

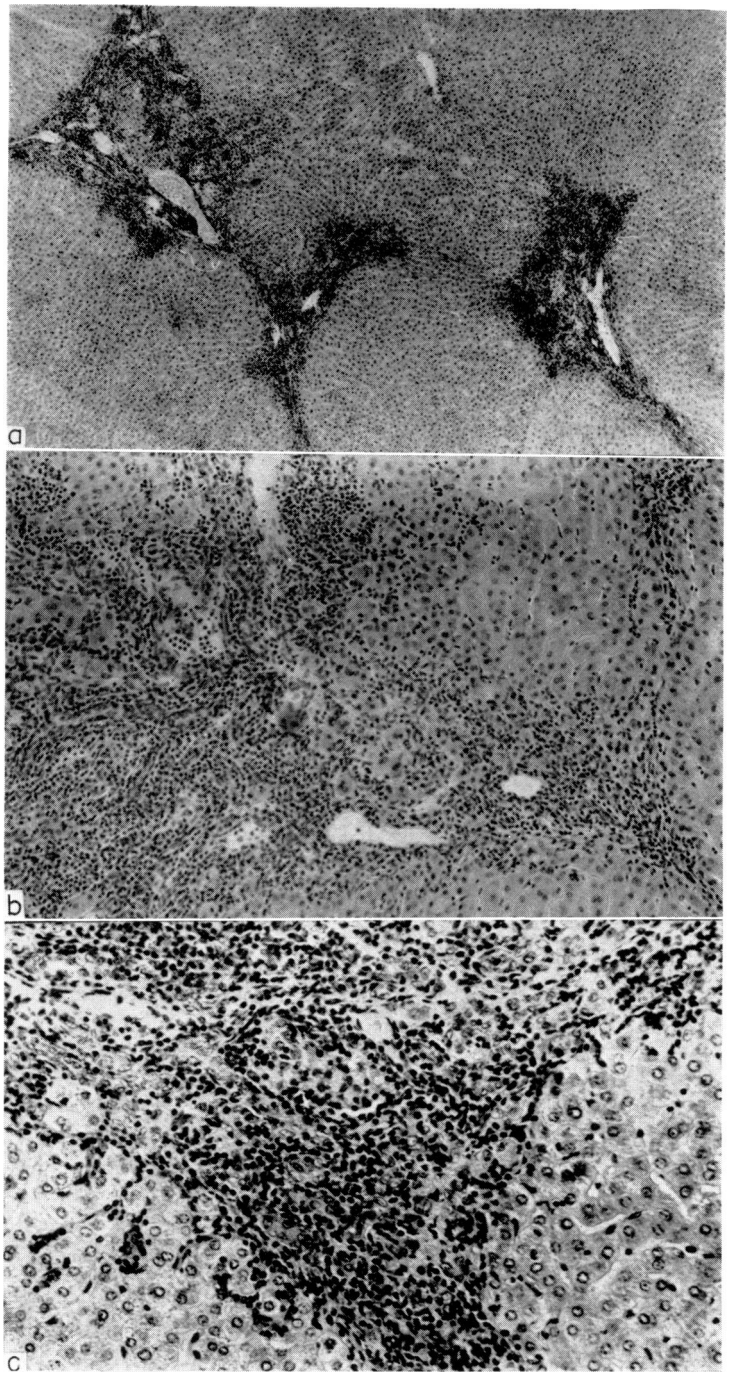

FIG. 2. Experimental autoimmune hepatitis after a 6-month course of immunization with human LP, Sephadex G-100 fraction 1. Diffuse portal and periportal infiltration with lymphoid cells, and invasion of liver parenchyma with formation of piecemeal necrosis (animal 3 of group 1). (a) Magnification $\times 32$; (b) magnification $\times 80$; (c) magnification $\times 200$.

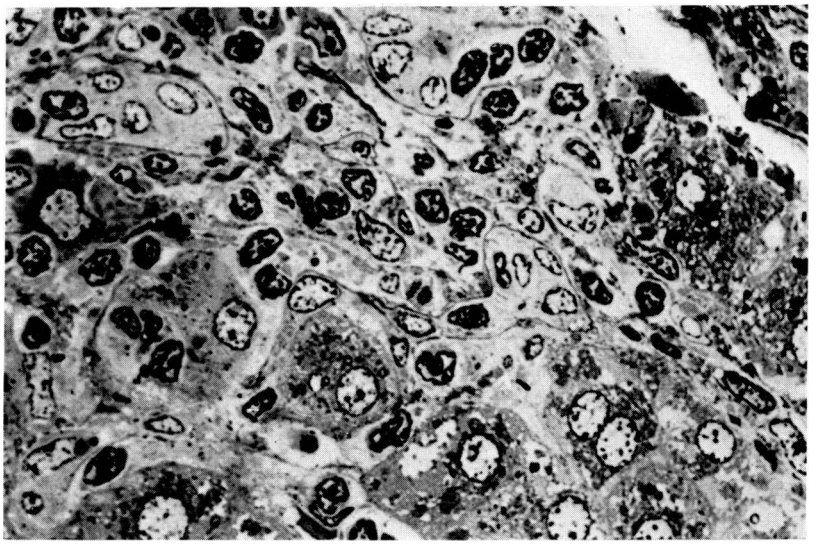

FIG. 3. Piecemeal necrosis. Semi-thin methacrylate section, Movat-staining. (animal 3 of group 1). Magnification: × 900.

DISCUSSION

Previous studies have indicated that experimental 'active chronic hepatitis' can be induced in rabbits by using heterologous liver protein for immunization. This experimental disease may exhibit progression, even 3–5 months after the last immunization injection. Similar lesions, although of much lower intensity, were obtained when chemically altered homologous protein was used for immunization (Meyer zum Büschenfelde, 1970).

Since then it has been possible to isolate two distinct liver-specific antigens. This raises the question as to which of the two antigens is responsible for the experimental autoimmune hepatitis. It would have been desirable to immunize one group with the liver-specific lipoprotein (LP-1), the second group with liver-specific protein (LP-2) and a third group with both antigens together. Unfortunately, LP-1 proved to be very unstable and we could not obtain a sufficient amount of this antigen for the purpose of immunization. For this reason we could only compare the effect of immunization with the Sephadex G-100 fraction 1, with the effect of immunization with fraction LP-2. The two liver-specific proteins constituted about 60% of the first antigen. In addition it contained 2–3 plasma proteins. The LP-2 fraction contained mainly the liver-specific protein which made up more than 80% of this fraction.

The group of rabbits immunized with the mixture of the two antigens developed lesions similar to those observed in human chronic active hepatitis, to the same extent as was observed in previous experiments using the supernatant of a crude liver homogenate submitted to 150,000 *g*. In the group of animals immunized with LP-2, only a small proportion of animals produced qualitatively similar lesions, but quantitatively much less severe. This difference cannot be due to the amount of antigen used since we intentionally immunized the animals with an excess of antigen. In prior experiments it was shown that the amount of

the antigen within a certain range did not influence the development of autoimmune hepatitis, which proved to be a function of the duration of immunization.

The dosage of the two preparations used for immunization was calculated to have approximately an equal amount of liver-specific protein in each. The difference in the effect, in terms of antibody formation to homologous liver protein as well as autoimmune hepatitis, can not therefore be due to differences in the amount of liver-specific protein. Rather, it must be ascribed to a substance present in the Sephadex G-100 fraction and absent in the LP-2 fraction. The difference in the two fractions resides mainly in the presence of the liver-specific lipoprotein in the former (about 20% of total weight) but not in the latter, and in the contamination of the former preparation with plasma proteins. Previous experiments showed that the immunization of rabbits with human plasma proteins in Freund's adjuvant does not produce autoimmune hepatitis. It appears thus more likely that the difference of the two groups is due to the fact that the liver lipoprotein is only present in the Sephadex G-100 fraction. Since no immune response to human LP-1 could be found in animals of group 2 immunized with LP-2, one may assume that LP-1 is not a prerequisite in the development of the hepatic lesion. However, it may play a helper role in producing a membrane damage allowing release of the intra-cellular liver-specific protein. Meanwhile, such a direct reaction has been demonstrated in liver perfusion experiments (Meyer zum Büschenfelde, Jeunet & Miescher, 1971). It should be mentioned that a delayed hypersensitivity reaction to this lipoprotein may be equally operative in producing membrane damage, thus making the intracellular hepatic antigen or antigens accessible to an immune reaction.

More experiments are necessary to better assess the relative role of the two liver-specific proteins in producing autoimmune hepatitis. In particular, we are attempting to isolate the liver-specific lipoprotein in sufficient amount to carry out a direct immunization experiment.

ACKNOWLEDGMENT

This work was supported by the Deutsche Forschungsgemeinschaft, and in part by the Swiss National Fund Project Nr. 5260 and by the Henry M. and Lillian Stratton Foundation.

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