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Lymphocyte transformation test with liver-specific protein and phytohaemagglutinin in patients with liver disease

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SUMMARY

Lymphocytes from thirty-four untreated patients with various liver diseases were stimulated in a lymphocyte transformation test with liver-specific protein (LSP). Eight of ten patients with chronic active or persistent hepatitis, two of five patients with non-alcoholic cirrhosis and six of nineteen patients with acute hepatitis showed a positive *in vitro* reactivity to LSP. In a control group of twelve persons without evidence of liver disease, eleven gave a negative response to LSP stimulation, whereas one person showed a positive response. Among fourteen patients with chronic hepatitis or non-alcoholic cirrhosis treated with prednisone at the time of the investigation, only one showed reactivity to LSP stimulation. Three patients in this group had previously had a positive reaction before prednisone was given. There was no statistically significant correlation between the reactivity to LSP stimulation and the presence or absence of hepatitis-associated antigen (HBAg) in serum, or with the biochemical liver parameters.

The response to *in vitro* stimulation with phytohaemagglutinin (PHA) was found to be significantly lower as compared with the control group in eleven patients with alcoholic liver disease and in the patients with acute hepatitis who had HBAg in serum. This decrease in reactivity could apparently not be ascribed to immunosuppressive factors in the patients' sera.

INTRODUCTION

It has been found in experimental studies in rabbits that immunization with a liver-specific protein induced a liver disease which histologically was similar to the changes seen in patients with chronic active hepatitis (Meyer zum Büschenfelde, Kössling & Miescher, 1972). This observation has initiated studies of immune reactions to liver-specific protein in patients with liver diseases, and migration-inhibition tests and cytotoxicity tests have provided evidence of the existence of cell-mediated immune responses against liver-specific protein and liver cells in patients with chronic hepatitis (Miller *et al.*, 1972; Thomson *et al.*, 1974; Paronetto & Vernace, 1975). We have used a lymphocyte transformation test to study the reactivity of peripheral blood lymphocytes from patients with various liver diseases after addition of liver-specific protein to the lymphocyte cultures.

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PATIENTS AND METHODS

Forty-five patients were investigated with liver-specific protein (LSP) in a lymphocyte transformation test. Five had chronic active hepatitis (CAH), ten chronic persistent hepatitis (CPH), eleven non-alcoholic cirrhosis (CH-NON-ALC) and nineteen had acute hepatitis (HA). Three patients, two with CAH and one with CH-NON-ALC, were studied both before and after steroid treatment, while eleven patients, two with CAH, three with CPH and six with CH-NON-ALC, were investigated only during steroid treatment. The remaining thirty-one patients did not receive any immunosuppressive treatment. The diagnoses were based on clinical and biochemial criteria and histological examination of liver-biopsy specimens. Twelve persons without liver disease were included as controls. Their biochemical liver parameters were normal, and tests for hepatitis-associated antigen (HBAg) were negative.

In forty-nine patients we studied the *in vitro* reactivity of lymphocytes to stimulation with phytohaemagglutinin (PHA); four had CAH, seven CPH, seven CH-NON-ALC; eleven patients had alcoholic liver disease (ALC), five with steatosis and six with cirrhosis, and twenty had acute hepatitis. Most of these patients, except for the eleven with ALC, were also investigated *in vitro* with LSP. The control group included eight persons from the control group stimulated with LSP, and seventy-three men from infertile couples investigated in the same period of time for *in vitro* reactivity to PHA stimulation with the same culture technique.

Liver-specific protein (LSP). LSP was prepared according to the method described by Meyer zum Büschenfelde *et al.* (1972). Normal human liver was obtained by autopsy, homogenized in 0·25 M sucrose at 4°C, and centrifuged at 150,000 *g* for 60 min. The supernatant was divided in aliquots of 8 ml and kept at −20°C. Prior to use, the supernatant was thawed and fractionated by sequential gel filtration on Sephadex G-100 and G-200 in a 0·1 M Tris-HCl, 0·2 M NaCl buffer (pH 8·0). The LSP appeared in the first peak eluted from the G-100 column and also from the G-200 column. The solution was concentrated in sucrose, dialysed against the starting buffer overnight, and the protein content was measured by Lowry's method. Before application to the lymphocyte cultures the antigen solution was adjusted to 250 μg protein/ml and sterile-filtered through a Millipore filter (0·22 μm). Four different livers were used during the investigation period and freshly prepared LSP was employed in all experiments. Two to five persons were investigated in each experiment. Gel filtration on Sepharose 6B as described by Miller *et al.* (1972) was not performed. Thus the antigen preparation probably contains other proteins than LSP, but the antigen preparation is referred to as LSP.

Lymphocyte transformation test (LTT). Approximately 20 ml of venous blood was drawn into a test tube containing phenol-free heparin in order to produce a final concentration of 20 i.u./ml. A serum sample was also secured. The mononuclear cells were separated using Isopaque–Ficoll. They were washed twice in TC-199 with 5% inactivated pooled A serum and heparin 20 i.u./ml. The cell suspension was diluted in TC-199 with 15% pooled, inactivated A serum to give a final concentration of $0{\cdot}5 \times 10^6$ lymphocytes per ml. Portions of 2·0 ml of this suspension were placed in culture tubes, 15 × 150 mm. The LSP antigen was added to give concentrations of 0·5 μg/ml, 2·5 μg/ml and 12·5 μg/ml.

In some tubes, phytohaemagglutinin (PHA-P, Wellcome MR68) was added to give a concentration of 1 μg/ml. Nothing was added to the control cultures. Cultures were made in triplicate and incubated at 37°C. The duration of culture was 5 days for LSP antigen and 3 days for PHA-P-stimulated cultures. Twenty-four hours before harvesting, 0·2 μCi of [2-^{14}C]thymidine, specific activity > 50 μCi/mM, was added. The cells were harvested on Whatman glass-fibre filters (GF/82), washed in saline and alcohol, and the incorporated amount of labelled thymidine was measured in a Tri-Carb scintillation counter. The results are expressed either in disintegrations per minute (d/min) or as stimulation indices,

$$\text{SI} = \frac{\text{ct/min of antigen-stimulated culture}}{\text{ct/min of control culture}}.$$

A positive response was defined as an SI ⩾ 2·0. An SI between 2·0 and 3·0 was considered a weakly positive reaction, ⩾ 3·0 strongly positive.

Testing of serum for immunosuppressive factors. The serum samples were inactivated at 56°C for 30 min and kept at −20°C until use. The investigation for the presence of immunosuppressive factors was done using a LTT. The lymphocytes from the same three normal donors were used in all experiments. The lymphocytes were prepared as described above. A total of 2×10^5 lymphocytes were cultured in 0·2 ml TC-199 with 5% pooled A serum in microculture plates (Linbro Chemical Company, I.S.-MRC-96). The serum pool was added to inhibit the non-specific influence on the results from using different serum samples. Twenty microlitres of test serum was added to give a final total concentration of 15% serum in the culture. The lymphocytes were stimulated with PHA, 2·5 μg/ml, in the culture concanavalin A (Con A) 25 μg/ml of pokeweed mitogen (PWM), 25 μl/ml, which concentration was found to give optimal responses of lymphocytes in the culture system used. Three different mitogens were used because they may stimulate different

subpopulations of lymphocytes. Lymphocytes were also grown in 15% pooled A serum. We have expressed the results as percentages of the responses obtained when the same lymphocytes were cultured in 15% A serum alone, and then the mean percentage from the three donors was calculated.

RESULTS

Preliminary use of LSP in concentrations ranging from 0·5 μg to 50 μg/ml showed a distinct suppression of *in vitro* reactivity of lymphocytes at 50 μg LSP/ml. The concentrations giving the highest SI was found to vary from 0·5 μg to 12·5 μg LSP/ml, and concentrations of 0·5 μg, 2·5 μg and 12·5 μg LSP/ml were used in the experiments.

Fig. 1 shows the results from the thirty-four untreated patients after stimulation with LSP in the LTT. The highest SI obtained at one of three concentrations of LSP is recorded. In the thirty-four untreated patients the optimal LSP concentration was 0·5 μg/ml in ten cases, 2·5 μg/ml in nineteen cases and 12·5 μg/ml in six cases. It was not possible to correlate the variations in optimal concentrations of LSP to differences in different livers or antigen preparations. Generally, the reactivity of the lymphocytes to LSP was low, the highest SI observed being 4·5 in a patient with CPH. Three patients with CAH did show an SI $\geqslant$ 2·0; five out of seven patients with CPH, two of five with CH-NON-ALC and six of nineteen patients with HA reacted positively. All normal persons were negative, except one who showed a weakly positive response. It was a 61-yr-old man who was admitted to hospital for treatment of a minor cerebrovascular attack, and who had no clinical history of liver disease.

Among the six HA patients with a positive response *in vitro* to LSP, one had elevated SGOT and gamma-globulin, and three had increased levels of gamma-globulin more than 3 months after the acute attack of hepatitis. Of thirteen patients with a negative response to LSP stimulation during the acute attack of hepatitis, three continued to have elevated SGOT and gamma-globulin, and one had increased levels of gamma-globulin for more than

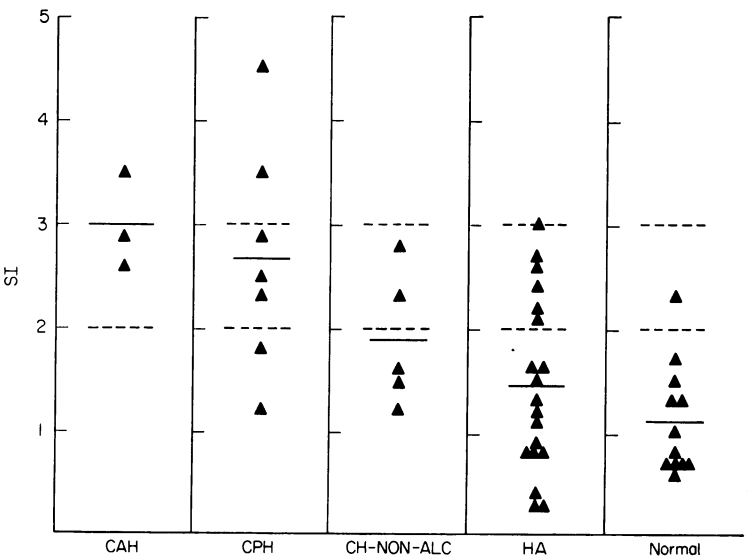

FIG. 1. Results obtained in patients with liver disease when their lymphocytes were stimulated with liver-specific protein (LSP) in a lymphocyte transformation test. The results are expressed by means of a stimulation index (SI). CAH, chronic active hepatitis (n = 3); CPH, chronic persistent hepatitis (n = 7); CH-NON-ALC, non-alcoholic cirrhosis (n = 5); HA, acute hepatitis (n = 19); normal, control group (n = 12). The dotted lines indicate SI = 2·0 and 3·0, respectively. The mean value in each group is indicated by a solid horizontal line in the column.

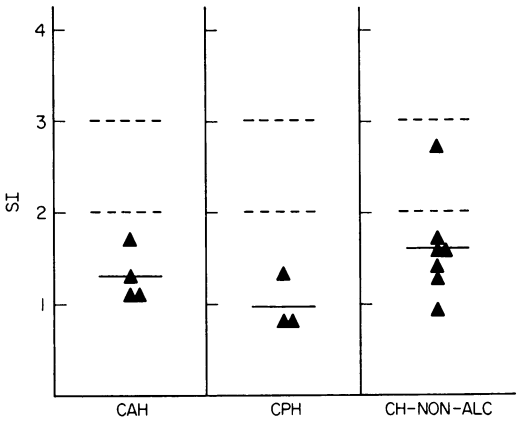

FIG. 2. Results from fourteen patients with various liver diseases after *in vitro* stimulation with liver-specific protein in a lymphocyte transformation test. All the patients were treated with prednisone at the time of investigations. For the explanation of the abbreviations, see Fig. 1.

3 months. The remaining nine patients had gone into complete remission. In the six patients with a positive *in vitro* reactivity to LSP stimulation, one had HBAg present in serum. In the LSP-negative group seven of thirteen showed the presence of HBAg. However, this difference was not significant ($P = 0{\cdot}15$, Fisher's exact test). The reactivity of the lymphocytes to LSP stimulation was not found to be correlated with the levels of liver enzymes in serum, or with the presence or absence of HBAg.

The *in vitro* reactivity of lymphocytes to LSP stimulation from patients treated with prednisone appears from Fig. 2. Among fourteen patients treated with prednisone, thirteen

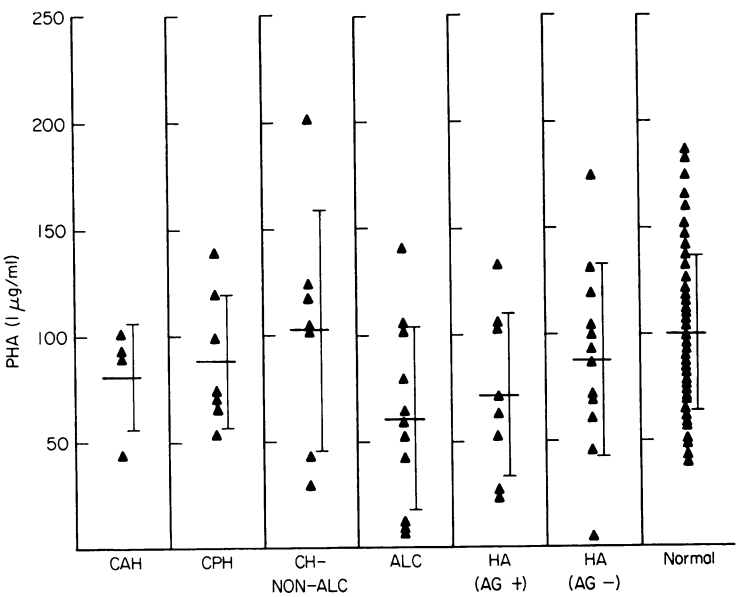

FIG. 3. Results in forty-nine patients with various liver diseases after *in vitro* stimulation of their lymphocytes with phytohaemagglutinin, 1 μg/ml, in a lymphocyte transformation test. The results are expressed in disintegrations per minute (d/min) $\times 10^{-3}$. The mean value $\pm$ 1 s.d. is indicated for each group. ALC, alcoholic liver disease ($n = 11$); HA (AG+), acute hepatitis with hepatitis-associated antigen (HBAg) ($n = 8$); HA (AG −), acute hepatitis without HBAg ($n = 12$); Normal, control group ($n = 81$).

showed no reactivity of their lymphocytes to LSP. Only one person with non-alcoholic cirrhosis gave a weakly positive reaction (SI = 2·7). Lymphocytes from three of the patients had also been tested with LSP before prednisone treatment was started, and all had a positive *in vitro* response at that time. This indicates that prednisone treatment has reduced the reactivity of the lymphocytes to stimulation with LSP *in vitro*.

The results after stimulation with PHA in concentrations of 1 μg/ml appear from Fig. 3. At the time of investigation, no prednisone treatment was given. The mean PHA response in eighty-one controls was 99·9±35·3 d/min, and the PHA responsiveness of lymphocytes from patients with chronic active or persistent hepatitis and non-alcoholic cirrhosis was not found to be significantly different from the range of responses in the control group. In eleven persons with alcoholic liver diseases, the response was significantly lower than that of the control group ($P<0·001$, Student's *t*-test). None of these patients had HBAg in serum. The mean PHA response in the six patients with alcoholic cirrhosis was 60·7±52·2 d/min (1 s.d.; $0·01<P<0·02$) and in the five patients with steatosis 59·3±36·1 d/min (1 s.d.; $0·01<P<0·02$). The mean PHA response in patients with acute hepatits was 80·8±42·0 d/min, and this was significantly lower than in the controls ($P<0·05$). The

TABLE 1. Mean response of lymphocytes from three normal persons when their lymphocytes were cultured in 5% A serum and 10% serum from patients with various liver diseases, expressed as a percentage of their response when cultured with 15% A serum alone

	PHA (2·5 μg/ml)	Con A (25 μg/ml)	PWM (25 μl/ml)	Number of sera
CAH	92·0±16·66	106·8±48·42	89·0±8·73	6
CPH	94·3±12·92	110·3±12·29	114·1±25·40	7
CH-NON-ALC	98·3±11·69	78·9±27·12	95·6±11·84	7
ALC	97·7±10·17	93·2±35·10	95·2±23·48	12
HA, HBAg+	100·4±13·73	95·8±15·91	103·9±23·44	7
HA, HBAg−	100·7± 7·71	104·8±23·11	90·5±10·46	11

PHA-induced lymphocyte transformation was also found significantly lower in eight HBAg-positive patients with HA than in eighty-one controls ($P<0·05$), while the difference of the mean response between twelve HBAg-negative HA patients and controls was not significant. The mean PHA response was lower in the group of HBAg-positive HA patients than in the group of HBAg-negative HA patients, but the difference was not significant.

Immunosuppressive factors

The mean results of the study of fifty serum samples from patients with various liver diseases are listed in Table 1. It can be seen that with the addition of 10% test serum to the cultures, no evidence was found of the existence of immunosuppressive factor(s). The standard deviations are seen to be large, especially after stimulation with Con A. This is illustrated in Fig. 4, which shows the results after addition of test serum from twelve patients with alcoholic liver disease. The mean percentages after PHA stimulation show little variation, but when stimulated with Con A the test serum could induce a distinct suppression or a distinct increase in the responsiveness of lymphocytes from the three normal persons. The same phenomenon was also observed when the test serum came from patients with other liver diseases.

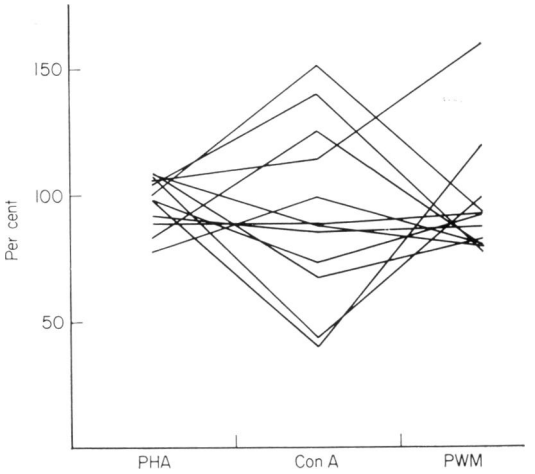

FIG. 4. Reactivity of normal lymphocytes from three persons stimulated with PHA, Con A and PWM. The results are expressed as the mean percentage from three persons when their lymphocytes were cultured in 5% A serum plus 10% serum from a patient, compared with the reactivity observed after addition of 15% A serum. The sera investigated are from patients with alcoholic liver disease (n = 12). For further details, see text.

DISCUSSION

It has been found that addition of autologous liver tissue from biopsy specimens can induce an increased transformation of lymphocytes from patients with chronic liver disease (Tobias, Safran & Schaffner, 1967; Sorrell & Leevy, 1972). Using the migration-inhibition test as a correlate of cell-mediated immunity, previous investigators have demonstrated that patients with chronic active hepatitis have a cell-mediated immune reaction to an extract of human foetal liver (Smith *et al.*, 1972). Miller *et al.* (1972) found that eleven of twelve patients with untreated chronic active hepatitis showed migration inhibition of their lymphocytes to LSP antigen. Using a cytotoxicity test, Paronetto & Vernace (1975) demonstrated that lymphocytes from ten of twelve patients with chronic active or persistent hepatitis reacted to a monolayer of autologous liver cells obtained from liver-biopsy specimens. Thomson *et al.* (1974) found a cytotoxic reactivity of lymphocytes in twenty out of twenty-two patients with chronic active hepatitis when culturing the lymphocytes on a monolayer of rabbit hepatocytes. Furthermore, they demonstrated that a rabbit antibody to LSP could block the cytotoxic reaction completely. On the basis of this observation they suggested that sensitization of lymphocytes to human LSP may be an important factor in the production of a lymphocyte-mediated hepatocyte injury.

Our LSP-antigen prepared on Sephadex G-100 and G-200 probably contains a certain amount of serum proteins. This could raise doubt as to the specificity of the sensitization in the patients. However, the maximum amount of allogeneic protein added to the cultures with the LSP-antigen is below the amount of allogeneic protein introduced by the use of 15% pooled A serum in the cultures. An increase of lymphocyte reactivity due to allogeneic serum in *in vitro* cultures does occur in some persons, but is eliminated when the results are expressed as SI. It seems less likely that the additional small amount of allogeneic serum constituents in the LSP preparation could induce the observed responses.

Our results obtained by an LTT are in agreement with the observations in the studies mentioned above. Eight of ten untreated patients with chronic active or persistent hepatitis gave a positive response to LSP stimulation. Of nineteen patients with acute hepatitis, six showed a positive reaction to LSP stimulation and four of these had a protracted

disease. However, an SI $\geqslant 2{\cdot}0$ is not necessarily correlated with protracted course of hepatitis because, at the time of investigation, four of thirteen patients with no *in vitro* reactivity to LSP also showed signs of protracted hepatitis.

Prednisone treatment clearly changes the response of lymphocytes to LSP stimulation in the LTT. Miller *et al.* (1972) found no migration inhibition in four patients with chronic active hepatitis who were treated with prednisone or azathioprine. Paronetto & Vernace (1975) saw no cytotoxicity of lymphocytes in three of seven patients with CAH after treatment with steroids or azathioprine. However, Thomson *et al.* (1974) found that only in two of eighteen patients receiving various immunosuppressive drugs did the cytotoxic activity of their lymphocytes on a monolayer of rabbit hepatocytes fall within the normal range. Our observation in the LTT are in accordance with the clinical observation that prednisone is of value in the treatment of chronic active hepatitis (Murray-Lyon, Stern & Williams, 1973). Whether patients with acute hepatitis and a positive reaction to LSP stimulation would benefit from prednisone treatment is, however, uncertain.

The decreased responsiveness of lymphocytes to PHA stimulation found in patients with liver disease was seen both in patients with steatosis and cirrhosis. Our observation is not in agreement with those of Hsu & Leevy (1971), who found a normal reactivity to PHA stimulation *in vitro* in eleven patients with alcoholic cirrhosis. On the other hand, they observed an inhibitory plasma factor in the same patients. The decreased PHA response in patients with alcoholic cirrhosis may be supported by the observations of Bernstein *et al.* (1974), who found that patients with alcoholic cirrhosis have a significant reduction ($P<0{\cdot}01$) in the number of T cells in the peripheral blood. However, they did not see a similar change in the number of T cells in patients with steatosis.

The depressed PHA response in patients with acute hepatitis confirms the findings of other authors (Agarwal *et al.*, 1971; Clot *et al.*, 1973; Newble *et al.*, 1975). The patients with HBAg present in serum had a significantly depressed reactivity compared with the control group, whereas the PHA responses in patients without HBAg were not significantly reduced. This could be due to the increased amount of HBAg present in the former group, thereby inducing a suppression of *in vitro* reactivity of lymphocytes. It may also explain the observation that only one of the six patients with positive *in vitro* reactivity to LSP stimulation had HBAg in serum, whereas this antigen was present in the serum of seven of thirteen patients with negative *in vitro* reactivity to LSP. It is in accordance with the temporary suppression of *in vitro* reactivity of lymphocytes occurring after tuberculin skin testing in normal persons (Thestrup-Pedersen, 1974).

Several authors have reported the existence of immunosuppressive factors in serum (Clot *et al.*, 1973; Newberry *et al.*, 1973; Newble *et al.*, 1975). But in one study it was found that HBAg-positive plasma in a concentration of 20% significantly enhanced the response of normal lymphocytes after PHA stimulation (Giustino, Dudley & Sherlock, 1972). Most experiments have been performed with the use of 20 or 25% serum or plasma in the cultures, whereas we applied the test serum in a concentration of 10% plus 5% pooled A serum. The addition of A serum to all cultures was done because addition of a single plasma sample from different donors in a concentration of 15% can induce a ten-fold variation in the *in vitro* reactivity of normal lymphocytes to PHA stimulation, whereas pooling of three plasma samples reduces the variation to two-fold (Mangi, Dwyer & Kantor, 1974). The variability observed in the cultures stimulated with Con A also indicates an influence from the test serum on this mitogen, probably due to a binding of Con A to glycoproteins in serum, thereby changing the actual concentration of the mitogen in the culture. In our investigation, the depressed PHA response could not be ascribed to immunosuppressive factors in serum. Thus, the decrease in the PHA response seems to be confined, at least in part, to the cells in the culture (Clot *et al.*, 1973; Newble *et al.*, 1975).

We conclude that *in vitro* reactivity of peripheral blood lymphocytes to liver-specific

protein can be demonstrated by means of an LTT. The test may be used to assess the efficiency of an immunosuppressive therapy.

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