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IMMUNOLOGICAL STUDIES IN PATIENTS WITH CHRONIC ACTIVE HEPATITIS

CYTOTOXIC ACTIVITY OF LYMPHOCYTES TO AUTOCHTHONOUS LIVER CELLS GROWN IN TISSUE CULTURE

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SUMMARY

The cytotoxic activity of lymphocytes against autochthonous liver cells was studied in patients with chronic liver diseases and in controls. Cytotoxicity of lymphocytes was observed in eight of ten patients with chronic active hepatitis, two patients with chronic persistent hepatitis, one patient with primary biliary cirrhosis, one patient with alcoholic hepatitis and carcinoma of the pancreas, and in three of five patients with acute viral hepatitis, but not in seven patients without liver alteration or with miscellaneous liver diseases. Serum was not cytotoxic, but in three patients it decreased the cytotoxicity of lymphocytes. Cytotoxicity was seen in both HBAg-positive and HBAg-negative patients, appears to be influenced by therapy, and does not correlate with autoantibodies.

These data support the hypothesis of an aggressive activity of lymphocytes in certain liver diseases.

INTRODUCTION

Several investigations in the last few years have indicated that certain patients with liver disease, especially those with chronic active hepatitis (CAH), exhibit humoral antibodies or cell-mediated immunity (Paronetto, 1973) toward a variety of antigens, some liver-specific and some unrelated to liver. It is not known, however, whether these immunological reactions have any relevance to the initiation or progression of the disease. Recent investigations in our laboratory have indicated that sera of patients with CAH and primary biliary cirrhosis (PBC) do not exhibit cytotoxic activity against cultures of liver cells (Paronetto, Gerber & Vernace, 1973). In an attempt to investigate whether a cell-mediated immune process plays a role in the pathogenesis of some liver diseases, we investigated the cytotoxic activity of lymphocytes against the patient's own liver cells grown in tissue culture.

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MATERIALS AND METHODS

Culture of liver cells

Liver cells were cultured from liver biopsy specimens following a previously described method (Demoise, Galambos & Falek, 1971). Early subcultures were used for the cytotoxic test.

Cytotoxicity

Cytotoxicity was measured by the method of Takasugi & Klein (1971). This test is based on the detachment or destruction of target cells by lymphocytes, and it is evaluated by counting the remaining target cells.

Approximately 200 liver cells were plated in microwells of a micro-test tissue culture plate (Falcon Plastics, Oxnard, California) with 10 μl RPMI 1640 (Gibco Grand Island, New York) medium containing 5% inactivated foetal calf serum and antibiotics. After 24 hr the medium was removed and replaced with 5 μl of medium and 10 μl of a suspension of patient lymphocytes. The 100:1 ratio of lymphocytes to target cells was adopted for all experiments.

At least two rows of microwells contained the lymphocytes from the patients. Other rows of microwells contained the following reactants: (a) 5 μl patient serum diluted 1:1 with 10 μl suspension of patient lymphocytes in a 100:1 ratio of lymphocytes to target cells; (c) 5 μl of phytohemagglutinin diluted 1:30 (PHA) (Difco, Detroit, Michigan) with 10 μl of medium; (d) 5 μl PHA with 10 μl lymphocytes, with a lymphocyte to target cell ratio of 100:1; and (e) 15 μl medium only. Dilution of sera and PHA was performed with RPMI 1640 medium. Plates were incubated at 37°C with air and 5% CO_2. After 3 days the fluid was removed by inversion and gentle shaking. Plates were then washed three times in phosphate-buffered saline and stained with Giemsa and the number of cells recorded.

Cytotoxicity was expressed according to the percentage reduction of the number of cells in relation to the control. Student's *t*-tests were performed to estimate the statistical significance of differences between cell counts in the rows of experimental and control wells. A difference was considered significant at a $P<0.05$ level.

In selected experiments cytotoxicity was also measured by the Hellström *et al.* (1971) modification of the Takasugi & Klein method. Falcon microtest II plates were used with the same reagents as in the previous method. The total volume of reagents in these experiments was 100 μl. The results of these experiments closely paralleled those obtained with the microcytotoxicity test of Takasugi & Klein.

Lymphocytes

When a monolayer or a continuous cell line was established (usually within 1 or 2 months) 30–50 ml of blood was obtained from the patient in 50 ml tubes containing 100 units of preservative-free heparin (Panheparin, Abbott, North Chicago, Illinois, 10,000 units). Lymphocytes were purified through a nylon column (Leukopak Fenwal Co., Morton Grove, Illinois) (Rubin, 1970).

Patients

A total of twenty-six patients with CAH, chronic persistent hepatitis (CPH), acute viral

TABLE 1. Cytotoxicity of lymphocytes of patients with liver diseases and of controls against autochthonous liver cells

Patient			Lymphocyte cytotoxicity*	HBAg in serum	Treatment†
E.W.		PBC	48	0	Azathioprine
R.O.		CAH	20†	+	Steroids
H.E.		CAH	92	0	0
A.N.		CAH	46	0	Steroids
	after 10 months	CPH	0	0	Steroids
B.R.		CAH	100	+	0
P.A.		CAH	33	+	0
V.E.		CAH	83	+	0
	after 50 days		22†	+	Steroids
	after 70 days		0	+	Steroids
	after 100 days		100	0	Steroids
M.O.		CAH	40	+	Steroids
B.O.		CAH	34	0	Steroids
W.H.		CAH	33	0	Azathioprine
S.E.		CAH	0	0	Steroids
B.L.		CPH	52	0	0
K.L.		CPH	70	+	0
	after 100 days		86	+	0
R.A.		AVH	25†	+	0
L.E.		AVH	22†	+	0
M.E.		AVH	100	+	0
R.O.		AVH	98	+	0
S.C.		AVH	68	+	0
S.O.	Carcinoma of pancreas		19†	0	0
S.T.	Carcinoma of pancreas		0	0	0
W.E.	Cholangitis, subsiding		0	0	0
L.A.	Recurrent, cholestasis		0	0	0
M.A.	No changes, healed AVH		0	0	0
L.A.	No changes, healed AVH		22†	0	0
D.O.	Alcoholic hepatitis, carcinoma of pancreas		100	0	0
B.U.	No changes		10†	0	0

* Cytotoxicity expressed as percentage reduction of monolayer cells incubated with lymphocytes.

† Reduction of cells statistically not significant.

hepatitis (AVH), miscellaneous liver diseases, and patients without histological evidence of liver diseases were investigated (Table 1). Of the ten patients with CAH, six were male. Diagnosis was confirmed in all patients by histological examination.

Serological investigations

Hepatitis B antigen (HBAg) was detected by a double antibody radioimmunoassay method (Ausria-125, Abbott Laboratories, North Chicago, Illinois) and by counter current immunoelectrophoresis. Antibodies to smooth muscle, nuclei, and mitochondria were determined according to previously described methods (Doniach *et al.*, 1966; Paronetto,

1969). All sera were tested at a dilution of 1 : 10 (except for the detection of nuclear antibodies when undiluted sera were also used) on unfixed rat stomach and human kidney sections. Fluorescein-conjugated antihuman IgG (Behring Diagnostics, Inc., Woodbury, New York) was used and the fluorescein to protein ratio of this antiserum was 2·4 (Holborow & Johnson, 1967).

RESULTS

One patient with PBC, eight of ten patients with CAH, and two patients with CPH displayed cytotoxic activity of the lymphocytes against the cells derived from the patient's own liver cells (Table 1, Fig. 1). In one patient (V.E.) the lymphocytes, initially cytotoxic, failed to

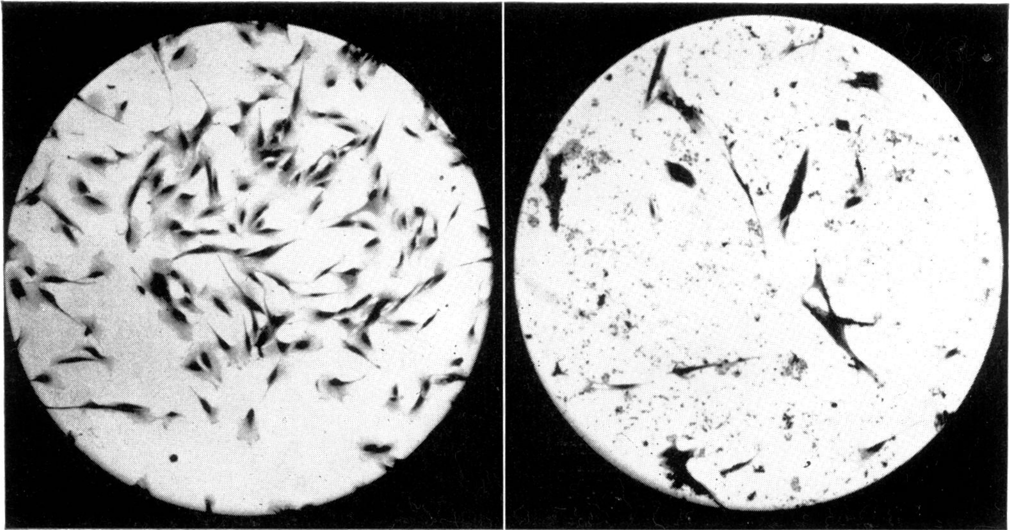

FIG. 1. Microwells containing liver cells from a patient (H.E.) with CAH incubated with medium (left) and patient's lymphocytes (right). Note in the right-hand photograph cellular debris and few markedly altered liver cells. (Both microwells stained with Giemsa; magnification ×25.)

show any activity when tested 50 and 70 days after the original biopsy; however, cytotoxicity reappeared 100 days after the original biopsy. In another patient with CAH (A.N.) a test repeated 18 months later showed lack of cytotoxicity of lymphocytes, while the liver biopsy exhibited chronic persistent (portal) hepatitis. In a patient with CPH (K.L.) cytotoxicity of lymphocytes was still present 100 days after the original biopsy. Serum of the patients was not cytotoxic; however, in three patients (B.R., V.E. and K.L.) the serum decreased the cytotoxic activity of lymphocytes from 100 to 36, 83 to 49, and 70 to 38, respectively.

Six of the twelve patients with CAH or CPH had HBAg in their serum. Lymphocytotoxicity was seen with equal incidence (five of six patients) in both the HBAg-positive and the HBAg-negative patients. Lack of cytotoxicity was associated with steroids or azathioprine treatment. Cytotoxicity of lymphocytes was also observed in three of five patients with HBAg-positive AVH. In these patients the serum was not cytotoxic and did not exhibit protective activity against autochthonous liver cells.

Seven of eight patients with miscellaneous liver diseases or without histological evidence of liver alteration did not exhibit reactivity of lymphocytes against autochthonous liver cells. The only exception was a patient with alcoholic hepatitis and concomitant carcinoma of the pancreas (D.O.). The lymphocytes of this patient exhibited complete destruction of the liver cell monolayer.

PHA-induced cytotoxicity of lymphocytes in all patients was investigated. Antibodies to smooth muscle were detected in only two patients with CAH (P.A. and R.O.), antibodies to nuclei were observed in a patient with AVH (M.E.) and antibodies to mitochondria were seen in the patient with PBC.

DISCUSSION

Using inhibition of migration of leucocytes as a correlate of cell-mediated immunity, previous investigators have demonstrated that patients with CAH and PBC have a cell-mediated immunity directed against liver extract or liver-specific antigen (Bacon, Berry & Bown, 1972; Miller *et al.*, 1972; Smith *et al.*, 1972). Inhibition of migration was, however, observed in only half of the patients with CAH when HBAg was used as antigen in this system (Dudley, Giustino & Sherlock, 1972). The present study indicates that when a different system is used patients with AVH and CAH exhibit lymphocytotoxicity against autochthonous liver cells.

Both HBAg-negative and HBAg-positive untreated patients with CAH exhibited cytotoxicity of lymphocytes. In four patients steroid therapy was associated with lack of reactivity, suggesting an inhibitory effect of steroid treatment on cytotoxicity. No correlation between cytotoxicity of lymphocytes and autoantibodies was detected. The significance of lymphocyte reactivity in patients with AVH is not clear. Two patients with AVH and cytotoxicity have recovered, while the third (S.C.) has elevated activity of transaminase 6 months after liver biopsy.

Previous work in our laboratory indicated that sera of patients with CAH and PBC are not endowed with cytotoxic activity against liver cells (Paronetto *et al.*, 1973). The present study, using an autochthonous system, confirms these data and furthermore suggests that sera might have a protective activity against cytotoxicity of lymphocytes. It is not known at present whether this protective activity is due to antibodies or to other serum factors.

The nature of the cells cultured from liver tissue is also unclear at present. The morphologic appearance of the cells (Guillouzo *et al.*, 1972) and their ability to synthesize a variety of serum proteins (e.g. albumin, fibrinogen, alpha-2 macroglobulin, beta lipoprotein, haptoglobin) (Le Guilly, Lenoir & Bourel, 1973), suggest the hepatocellular nature of subcultures of adult liver cells.

Since cytotoxicity with a heterologous line of liver cells may be due to histocompatibility differences, it is considered important that autochthonous liver cells be used. The activity of lymphocytes thus seems to be directed against autologous antigens; however, the organ specificity of the reaction is not as yet established. Further work is also necessary to assess whether the activity is directed toward neo-antigens appearing during cell replication in tissue culture. The uniformity of the results with various subcultures and the lack of reactivity of control patients militate against this possibility. The positive reaction obtained in a patient with alcoholic hepatitis may support the findings of altered cell-mediated

immunity to liver antigen in patients with alcoholic hepatitis (Sorrell & Leevy, 1972; Mihas & Bull, 1973).

These investigations thus indicate *in vitro* reactivity of lymphocytes of patients with some liver diseases and support the hypothesis that an *in vivo* aggressive activity of lymphocytes against liver cells plays a role in the pathogenesis of certain liver diseases.

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