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Lymphocyte Cytotoxicity in Human Liver Disease Using Rat Hepatocyte Monolayer Cultures

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Isolated rat hepatocytes were used to determine the cytotoxicity of peripheral blood mononuclear cells (PBM) from patients with alcoholic liver disease (ALD) and chronic active hepatitis (CAH). The specificity of the cytotoxic effect on liver cells was monitored using rat kidney cells. PBM from patients with CAH and ALD showed increased spontaneous cell-mediated cytolysis (SCMC) for hepatocytes. The SCMC against kidney cells was comparable for the patient groups and controls. Incubation of liver cells with antibody to human liver-specific protein significantly increased the cytotoxic activity of control PBM but did not block cytotoxicity by PBM of patients with CAH. Incubation of PBM from untreated CAH patients with thymosin fraction 5, a polypeptide extract of the thymus gland, significantly lowered cytotoxicity. Our findings suggest that rat liver cells provide a model for studies of cell-mediated immunity in human liver disease and that thymosin fraction 5 decreases the cytotoxic activity of sensitized lymphocytes in CAH.

INTRODUCTION

The cell-mediated immune system has been implicated as a possible mechanism for the hepatocellular destruction observed in a number of acute and chronic liver diseases (1–5). A variety of experimental models have been utilized to demonstrate cell-mediated cytotoxicity in patients with alcoholic liver disease, acute viral hepatitis, and chronic active hepatitis. The target cells for these studies have included Chang cells (6–10), rabbit and baboon hepatocytes (11–18), autologous human liver cells (8, 11, 12, 19, 20) and avian erythrocytes coated with purified liver cell membrane lipoprotein (21). The use of Chang liver cells has been questioned, prompted by concerns that these cells may no longer possess important antigenic determinants found on human, rabbit, and rat liver cells (22). Autologous human liver cells are difficult to obtain in sufficient number and are often unavailable in selected patients. Additionally, few of the studies to date have attempted to determine the specificity of cell-mediated cytotoxicity for the target liver cell.

We used monolayer cultures of isolated rat hepatocytes to study the immunologic mechanisms associated with the destruction of hepatocytes in alcoholic liver disease and chronic liver disease. Rat hepatocytes can be obtained in sufficient numbers and when grown in primary monolayer culture do not proliferate for several days. In addition, these cells demonstrate specific functions of hepatocytes seen *in vivo* (23). Close immunological cross reactivity has been demonstrated between the hepatocyte membrane liver-specific protein of rats and hu-

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mans (22, 24). Rat kidney cells, also grown in monolayer cultures, were used to assess the specificity of the peripheral blood mononuclear cell (PBM) interaction for liver parenchymal cells. The role of antibodies directed against liver-specific protein (LSP) in cell-mediated cytotoxicity was also examined.

Finally, PBM pretreated with thymosin fraction 5 were added to the hepatocyte monolayers to assess the possible effect of this immunomodulator on cell-mediated cytotoxicity. Thymosin, a polypeptide extract of the thymus gland, has been shown to induce the maturation of undifferentiated lymphocytes predetermined to follow the T-cell limb of the immune system. In addition, thymosin can influence the number and function of selective subsets of T cells including the immunoregulatory suppressor or helper cells (25).

PATIENTS AND METHODS

Patient and control subjects. A total of 50 patients were evaluated in the present study. Seven male patients, ages 32–68, had compensated alcoholic cirrhosis (CAC) on the basis of biopsy evidence of micronodular cirrhosis without superimposed alcoholic hepatitis (26), previous heavy alcohol intake, and demonstrated clinical and laboratory findings compatible with quiescent disease.

Ten patients consisting of nine males and one female, ages 35–58, had alcoholic hepatitis (AH). Although biopsies were sought, frequent coagulation deficiencies and poor clinical status precluded this procedure in 4 of the 10 patients. In them, the diagnosis of AH was based on clinical and laboratory findings similar to that used in a previous study of adrenocorticosteroid therapy in AH (27). Biopsies were accomplished in six patients and the diagnosis of AH (26) was histologically confirmed in each case.

Eight patients had the characteristic histologic features of chronic persistent hepatitis (CPH) (26) and three patients had unresolved hepatitis (26) (Table 1). Twenty patients had chronic active hepatitis (CAH) by clinical, biochemical, and histological criteria (26). Two additional patients with previous CAH were in remission and had the histologic finding of CPH following cessation of prednisone therapy at least 2 months earlier (Table 1).

A total of 34 normal control subjects (27 males, 7 females; ages 20–51) were studied. The control subjects were divided into two groups. Twenty-one controls (CT-1) were used to compute the cytotoxicity ratio (CR) (11) for each of 21 experiments. The number of patients evaluated in each experiment ranged from one to four. A second group of 13 control subjects (CT-2) were evaluated in random fashion throughout the study to monitor the CT-1 responses. In addition, the CR observed in the CT-2 was compared to the CR of the patient groups for the statistical analysis. All control subjects were healthy volunteers not known to have current or previous liver disease. Controls and patients with malignancy, with previous known immunodeficiency, with a history of recent viral upper respiratory illness, or recipients of blood transfusions within 4 weeks of the study were excluded.

Preparation of liver cells. Monolayer cultures of isolated rat liver parenchymal cells were prepared as previously described (28). Briefly, sodium nembutal (10 mg/100 g body wt) was injected intraperitoneally into male Sprague–Dawley rats

(200–250 g). The liver was perfused *in situ* with medium, consisting of calcium-free Hank's balanced salt solution (HBSS) containing 10 milliunits of insulin/ml, 0.5% bovine serum albumin (Calbiochem, La Jolla, Calif.), 2 units/ml of heparin, 1.0 m*M* of $MgSO_4$, 100 μg/ml of streptomycin, and 100 units/ml of penicillin. The medium was maintained at 37°C, gassed with 95% O_2/5% CO_2, and perfused through the liver with a polystatic pump for 15 min.

Enzyme perfusion medium was then infused for 20 min. This medium resembled the original perfusion medium, with the exception that heparin was absent and collagenase II, 0.05% (Worthington Chemicals, Freehold, N.J.) was added. The liver was transferred to a sterile disposable (100 × 10 mm) petri dish in an ice bath.

Glisson's capsule was cut and the cells gently dispersed. The cell suspension was filtered in sequence through a 253-μm mesh and then 64-μm mesh nylon gauze (Tobler, Ernst and Traber, Inc., Elmsford, N.Y.) into a sterile plastic centrifuge tube in an ice bath.

The cell suspension was centrifuged at 100*g* for 15 min at 4°C and the supernatant discarded. The pellet was then washed twice with ice-cold perfusion medium, centrifuged at 120*g* for 10 min, and resuspended in Ham's F-12 medium (Microbiological Associates, Bethesda, Md.) buffered with sodium bicarbonate at pH 7.4 and containing heat-inactivated, dialyzed fetal calf serum (FCS) (Reheis Chemical Co., Phoenix, Ariz.), 0.5 μg (11.9 milliunits) of insulin, 100 units of penicillin, and 100 μg of streptomycin/ml. The percentage of viable cells ranged between 96 and 98% by trypan blue exclusion. Antisera to rat membrane LSP (generously provided by Dr. K. H. Meyer zum Büschenfelde) was used as an organ marker and revealed that greater than 99% of the cells isolated were hepatocytes, using the indirect immunofluorescent technique as described previously (22).

Viable cells (1×10^5) were seeded into each well of a Falcon multiwell tissue culture plate (Falcon 3008) (at a density of 5×10^4 cells/cm^2) in a volume of 1 ml of Ham's F-12. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

After 6 hr incubation, the medium was changed. At 18 hr the Ham's F-12 was replaced by Waymouth HI-WO_5/BA_{2000} (International Scientific Industries, Gary, Ind.) culture medium. This allowed continuation of the culture in a serum-free milieu. This medium consisted of Waymouth's medium MB752/1 with 5 μg (11.9 milliunits) of insulin, 100 units of penicillin, and 100 μg of streptomycin/ml. The medium was changed daily. By the third day of incubation a monolayer was noted to cover the bottom of the well. Microscopic examination of each well was done to insure the presence of a uniform monolayer. Under these conditions, approximately 50% of the original inoculum remained attached to the culture plates, yielding 85–90% viable cells by trypan blue exclusion. Rabbit antisera to rat and human LSP revealed that greater than 95% of the cells in the monolayer still retained LSP by indirect immunofluorescent studies (22).

Preparation of kidney cells. The kidneys of the same animal were excised and placed in a sterile 100 × 10-mm petri dish containing 10 ml of Ca^{2+}-free HBSS buffered with sodium bicarbonate at pH 7.4 and supplemented with 100 μg/ml of streptomycin and 100 units/ml of penicillin. After removing the capsule, the kidneys were minced and washed repeatedly in HBSS.

TABLE 1

CLINICAL, BIOCHEMICAL, AND SEROLOGICAL CHARACTERISTICS OF PATIENTS WITH CHRONIC HEPATITIS

Patient	Age (year)	Sex	Biopsy diagnosis	Total bilirubin[a]	Alkaline phosphatase[b]	SGOT[c]	SGPT[d]	Albumin[e]	HB_sAg (RIA)	Medication
1	60	F	CAH	0.6	125	419	452	4.2	–	–
2	30	F	CAH	2.6	513	804	392	3.4	–	–
3	63	M	CAH	1.1	212	57	24	2.9	+	–
4	64	F	CAH	1.1	368	226	124	3.5	–	–
5	72	F	CAH	2.3	101	610	464	3.5	–	–
6	24	M	CAH	0.5	40	32	5	3.8	+	–
7	30	M	CAH	1.1	114	540	662	3.4	–	–
8	64	F	CAH	0.7	150	137	93	4.1	–	–
9	52	F	CAH, cirr.	0.9	99	220	268	4.2	–	–
10	53	M	CAH	1.0	73	85	107	3.8	+	–
11	21	M	CAH, cirr.	0.4	54	34	67	3.8	–	Prednisone
12	23	M	CAH	0.9	78	140	270	3.5	+	Prednisone
13	22	M	CAH	0.2	167	122	223	3.6	+	Prednisone
14	33	F	CAH	0.4	109	192	382	3.6	–	Prednisone
15	42	M	CAH, cirr.	0.8	16	20	39	3.3	+	Prednisone
16	32	F	CAH	1.0	136	142	179	4.5	–	Prednisone
17	15	M	CAH	1.4	144	480	581	3.4	–	Prednisone
18	31	F	CAH	3.5	615	144	165	3.7	–	Prednisone
19	23	M	CAH	0.8	22	55	75	4.6		Prednisone
20	59	M	CAH	1.0	143	129	72	4.3	–	Prednisone

21	32	F	CAH, rem.	0.4	58	14	9	4.7	–	–
22	70	F	CAH, rem.	0.7	99	49	27	4.0	–	–
23	20	M	CPH	0.7	101	281	370	4.5	–	–
24	21	M	CPH	1.5	111	90	217	4.0	–	–
25	24	F	CPH	0.5	60	60	86	4.8	–	–
26	25	M	CPH	2.3	58	36	25	3.8	–	–
27	23	F	CPH	1.0	97	64	80	3.9	–	–
28	22	M	CPH	1.2	ND	115	173	4.6	–	–
29	37	M	CPH	0.5	411	77	16	3.6	–	–
30	27	F	CPH	0.3	16	57	70	4.2	–	–
31	20	M	UH	0.8	125	219	347	ND	+	–
32	22	F	UH	1.4	280	114	86	ND	–	–
33	23	M	UH	0.3	77	143	262	4.1	+	–

Note. Abbreviations used: CAH, chronic active hepatitis; CPH, chronic persistent hepatitis; UH, unresolved hepatitis; ND, not done; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase; cirr., cirrhosis; rem., remission; HB_sAg, hepatitis B-surface antigen.

[a] mg/100 ml serum, normal range 0.2–1.2.

[b] IU/liter, normal range 30–100.

[c] IU/liter, normal range 7–40.

[d] IU/liter, normal range 5–25.

[e] g/100 ml, normal range 2.4–5.4.

Trypsin medium (0.25%), consisting of Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline containing 100 μg/ml of streptomycin and 100 units/ml of penicillin was maintained at 37°C. The medium was used to trypsinize the washed kidney fragments on a magnet stirrer at 37°C for 20–30 min. The undigested fragments were allowed to settle and the supernatant decanted.

Trypsinization was repeated with fresh aliquots of trypsin every 15–20 min until most cells were released from the fragments, and the supernatants collected in a flask set in an ice bath. The cell suspension was centrifuged at 600*g* for 10 min and the supernatant discarded.

The growth medium which consisted of Eagle's minimum essential medium with Hank's salts containing 100 μg/ml of streptomycin, 100 units/ml of penicillin, and 10% heat-inactivated FCS, was maintained in an ice bath. The cells were resuspended in growth medium and washed three times at 600*g* for 5 min. Trypan blue exclusion testing was performed and the percentage of viable cells ranged between 90 and 95%.

The isolated epithelial cells, suspended in growth medium, were used for the cell cultures. Viable cells (1×10^5) were seeded into each well of a Falcon multiwell tissue culture plate (Falcon 3008) (5×10^4 cells/cm^2) in a volume of 1 ml of growth medium. The kidney cells, like the hepatocytes, were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The medium was changed daily. By the third day of incubation, 50% of the original inoculum remained attached to the culture plates and yielded 85–90% viable cells by the trypan blue exclusion test.

Isolation of PBM. Venous blood from patients or controls was collected in preservative-free heparin (1000 units/ml), diluted 1:1 with sterile 0.9% saline, and layered over Ficoll–Hypaque. Following centrifugation at 400*g* for 40 min at room temperature, the PBM contained in the interface were washed with saline and centrifuged at 400*g* for 15 min. The cells were washed twice more with saline and centrifuged at 400*g* for 10 min. Viability exceeded 95% by trypan blue exclusion and lymphocytes constituted 90–95% of the cell population with the remaining cells consisting of monocytes. The PBM were resuspended in saline, counted, and the final concentration adjusted to 1×10^7 cells/ml. Sera from patients and controls were heat inactivated at 56°C for 45 min.

Cytotoxicity studies. On the third day of culture, 100 μCi of ^{51}Cr (sodium chromate, 1.0 μCi/ml, Amersham Corp., Arlington Heights, Ill.) was added to each of the wells containing liver and kidney monolayers. The hepatocytes were incubated for 90 min and the kidney cells for 3 hr both at 37°C in humidified air with 5% CO_2. After incubation, the cells were washed three times with culture medium to remove all excess unbound free ^{51}Cr. In preliminary studies, washed rat hepatocyte and kidney monolayers exhibited counts of 8290 ± 1574 and 9356 ± 2515 cpm (mean ± SD of five experiments), respectively following incubation with ^{51}Cr.

Additional experiments using PBM to target cell ratios of 10:1, 20:1, and 60:1 were accomplished on three patients with CAH. No cytotoxicity was seen at the 10:1 ratio. While cytotoxicity was elicited at the 20:1 and 60:1 ratios, the former yielded consistently higher values. PBM of patients and controls were added to 3-day-old liver and kidney monolayer cultures at a ratio of 20:1. Triplicate samples were prepared. PBM (1×10^6) in 0.1 ml of HI-WO_5/BA_{2000} was added to the

labeled liver cells. Similarly, 1×10^6 PBM in 0.1 ml of growth medium was added to the labeled kidney monolayers. In parallel wells, homologous or autologous serum, at a final concentration of 10% was added with the PBM. One set of wells contained only liver cells and culture medium, and another set, kidney cells and growth medium. PBM (1×10^6) were incubated with a previously determined optimal concentration of 40 μg/ml of pyrogen-free (clinical quality) calf thymosin fraction 5 (29) for 20 min in a water bath at 37°C. These were then added to a set of wells containing liver cells. A final set of wells containing liver cells was incubated for 30 min with rabbit antisera to human LSP, diluted 1:4 with $HI\text{-}WO_5/BA_{2000}$. The anti-LSP was prepared and tested for specific reactions toward LSP as described previously (30). In addition, the antisera was absorbed, in turn, with lyophilized rat plasma, rat spleen cells and kidney homogenates, and finally with human PBM at a 3:1-volume ratio of serum to cells (22). The anti-LSP was not cytotoxic for rat hepatocytes in the absence of effector cells as determined by trypan blue examination of hepatocytes incubated with the antisera at 37°C for 6 hr. PBM from patients with CAH or controls were then added to these wells.

The final volume of each well was brought to 1.5 ml by adding culture medium to all wells with liver cells and growth medium to the wells with kidney cells. The cultures were then incubated at 37°C for 6 hr in a rocking chamber in a humidified atmosphere containing 5% CO_2.

Following incubation, the entire reaction mixture in each well was transferred into a 12 × 75-mm plastic test tube (Falcon 2054) using a rubber policeman and a Pasteur pipette. The mixture was centrifuged at 300*g* for 10 min. One-half milliliter of the supernatant was collected, without disturbing the pellet, by using a pipetman and transferred to another 12 × 75-mm glass tube. The original tube with 1 ml of supernatant and the cell pellet was designated the pellet tube.

All samples were counted for their radioactivity in an AutoGamma spectrophotometer, Model 5320 (Packard Instruments Co., Downer's Grove, Ill.). Background counts per minute were subtracted from the supernatant and pellet tubes.

Calculations of cytotoxicity. The percentage of ^{51}Cr release from labeled liver or kidney cells was calculated according to the following equation:

$$\%\ \text{specific}\ ^{51}\text{Cr release} = \frac{3 \times \text{cpm in supernatant tube}}{\text{cpm in supernatant tube} + \text{cpm in pellet tube}} \times 100.$$

Cytotoxic activity was expressed as the mean percentage ± SD of ^{51}Cr released from triplicate determinations.

The cytotoxicity ratio (CR) (11) for each patient or individual CT-2 was calculated as follows:

$$\frac{\%\ \text{specific}\ ^{51}\text{Cr release of each patient or CT-2}}{\%\ \text{specific}\ ^{51}\text{Cr release of the corresponding CT-1}}$$

The CR of each patient and CT-2 was calculated using the single CT-1 subject in each experiment. Comparison of the CR between the patient groups and the CT-2 group was accomplished using Student's independent *t* test. Changes in the CR within study groups were analyzed by applying Student's dependent *t* test.

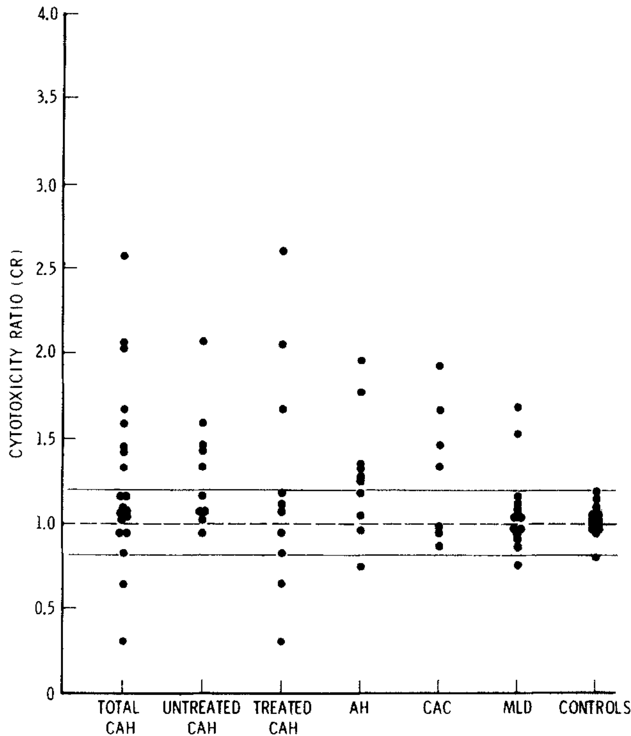

FIG. 1. Cytotoxicity ratios in chronic active hepatitis (CAH), alcoholic hepatitis (AH), compensated alcoholic cirrhosis (CAC), and miscellaneous liver diseases (MLD). The bracketed area represents the mean ± 2 SD of the CR for the 13 controls (CT-2).

RESULTS

Lymphocytes from 13 CT-2 subjects showed spontaneous cell-mediated cytotoxicity (SCMC) with a mean CR of 1.02 ± 0.09 for rat hepatocytes. Positive evidence of cytotoxicity was determined to be a CR of 1.20 which represents 2 SD above the mean CR obtained in these 13 CT-2 subjects (Fig. 1, Table 2). Lymphocytes from 20 patients with CAH demonstrated increased cytotoxicity with a CR of 1.27 ± 0.52. Although eight of these patients had CRs greater than 1.20, there was no significant difference in the CR between the 20 patients and the CT-2. When these patients were divided into treated and untreated groups of 10 patients each, the SCMC in the untreated group was significantly higher than the CT-2. The CR of patients with CAH on prednisone therapy did not differ significantly from the CT-2.

As shown in Table 2, lymphocytes from patients with AH and CAC were significantly more cytotoxic for rat hepatocytes than were allogeneic normal lymphocytes ($P < 0.02$ and $P < 0.05$, respectively). No significant difference in SCMC was noted between the CT-2 and the group of patients with miscellaneous liver disease, which included patients with CPH, unresolved hepatitis, and CAH in remission.

TABLE 2

CYTOTOXICITY RATIO (CR) IN CAH, ALCOHOLIC LIVER DISEASE, AND CONTROLS

		Incubation conditions			
Study groups	Number of subjects	Patient lymphocytes alone	Patient sera alone	Patient lymphocytes plus autologous sera	Normal allogeneic lymphocytes plus 10% test sera
CAH (total)	20	1.27 ± 0.52	1.08 ± 0.25	1.32 ± 0.58	1.27 ± 0.42[c]
CAH (treated)	10	1.23 ± 0.68	1.10 ± 0.28	1.39 ± 0.70	1.32 ± 0.39[b]
CAH (untreated)	10	1.32 ± 0.34[a]	1.05 ± 0.24	1.25 ± 0.47	1.22 ± 0.46
AH	10	1.28 ± 0.35[b]	1.10 ± 0.36	1.37 ± 0.38	1.26 ± 0.30[b]
CAC	7	1.31 ± 0.40[c]	1.03 ± 0.25	1.26 ± 0.27	1.60 ± 1.05
Miscellaneous liver disease	13	1.08 ± 0.25	0.95 ± 0.23	1.18 ± 0.27	1.38 ± 0.46[b]
Controls (CT-2)	13	1.02 ± 0.09	0.99 ± 0.11	0.97 ± 0.22	0.97 ± 0.22

Note. Results are expressed as means ± SD. P values are derived from Student's independent t test as compared to controls.

[a] $P < 0.01$.

[b] $P < 0.02$.

[c] $P < 0.05$.

The influence of serum factors on *in vitro* lymphocyte cytotoxicity was examined in the patient groups. To begin with, none of the heat-inactivated sera from the various patient groups and CT-2 were cytotoxic for rat hepatocytes (Table 2). When the CR was determined when lymphocytes were incubated in the presence of 10% heat-inactivated autologous serum, as seen in Table 2, the CR was not significantly altered in any of the patient groups or CT-2 from the CR obtained with lymphocytes alone.

When allogeneic normal lymphocytes were incubated in the presence of sera from the patient groups, the cytotoxic activity of the normal lymphocytes was significantly increased in sera from patients with MLD, CAH, and AH (Table 2). No such cytotoxic effect was noted when the CT-2 lymphocytes were incubated in homologous control sera.

Thymosin effect on lymphocyte cytotoxicity. Incubation of PBM for 20 min with thymosin fraction 5 prior to addition to hepatocyte cultures decreased cytotoxicity in untreated CAH, and in patients with AH and CAC. This lowered SCMC attained statistical significance in patients with untreated CAH only. No significant alteration in the CR was noted in the 18 CT-1 or 11 MLD subjects studied (Table 3).

Antibody-dependent cellular cytotoxicity (ADCC). To evaluate the candidacy of the LSP as a target antigen in these studies, antisera to human LSP was added to the monolayers of rat hepatocytes. This was followed by the addition of lymphocytes to the incubating wells. The CR was increased in seven CT-1 subjects studied but not significantly altered in the eight patients with CAH that were evaluated (Table 4). That the antisera to human LSP reacted with LSP on the cell surface of rat hepatocytes has been shown previously (22).

Target cell specificity. Lymphocytes from a total of 21 patients with CAH, AH, CAC, and CPH were incubated with monolayer cultures of rat kidney. There was no significant difference in SCMC between the patient and control groups. When 9 of the 21 patients who demonstrated SCMC against liver cells were looked at as a

TABLE 3
INFLUENCE OF THYMOSIN FRACTION 5 ON LYMPHOCYTE CYTOTOXICITY

Study group	Number of subjects	CR (mean ± SD)	
		Lymphocytes alone	Lymphocytes + thymosin
CAH (total)	20	1.27 ± 0.52	1.18 ± 0.57
CAH (treated)	10	1.23 ± 0.68	1.23 ± 0.75
CAH (untreated)	10	1.32 ± 0.34	1.14 ± 0.35[a]
AH	10	1.28 ± 0.35	1.13 ± 0.42
CAC	7	1.31 ± 0.40	1.16 ± 0.28
MLD	11	1.06 ± 0.23	1.13 ± 0.26
Controls (CT-1)	18	1.00 ± 0.00	1.13 ± 0.29[b]

[a] $P < 0.02$, Student's dependent *t* test.
[b] Not significant, one-sided *t* test.

group, none showed significant cytotoxic activity against kidney cells (CR 1.57 ± 0.31 for liver versus 1.04 ± 0.08 for kidney) (Fig. 2).

Association of cytotoxicity to duration of illness. Patients with CAH who showed *in vitro* SCMC had a mean duration of illness of 25.9 ± 12.0 months (mean ± SEM) as contrasted to 39.4 ± 15.0 months in CAH patients who did not have *in vitro* cytotoxic activity. This difference was not significant.

Correlation of cytotoxicity to biochemical tests of liver function. Analysis of biochemical parameters did not reveal any significant relationship between the serum total bilirubin, SGOT, SGPT, alkaline phosphatase, and albumin values, and *in vitro* cytotoxicity.

DISCUSSION

The results of these studies indicate that rat hepatocytes may be utilized to assess *in vitro* cell-mediated cytotoxicity in liver disease. In addition, the SCMC activity seen against rat hepatocytes in patients with CAH, AH, and CAC but not against rat kidney cells underscores the probable specificity of the lymphocyte target organ interaction. The isolation of target cells utilizing enzyme preparations of collagenase or trypsin has been shown not to result in appreciable changes in

TABLE 4
CR IN PRESENCE OF ANTIBODY TO HUMAN LSP

Study groups	Number of subjects	Incubation conditions	
		Lymphocytes alone	Lymphocytes + anti-LSP
CAH (total)	8	1.45 ± 0.52	1.50 ± 0.54
CAH (treated)	4	1.62 ± 0.69	1.81 ± 0.58
CAH (untreated)	4	1.29 ± 0.28	1.19 ± 0.26
Control (CT-1)	7	1.00 ± 0.00	1.38 ± 0.40[a]

Note. Mean ± SD.
[a] $P < 0.05$. Compared to lymphocytes alone, one-sided *t* test.

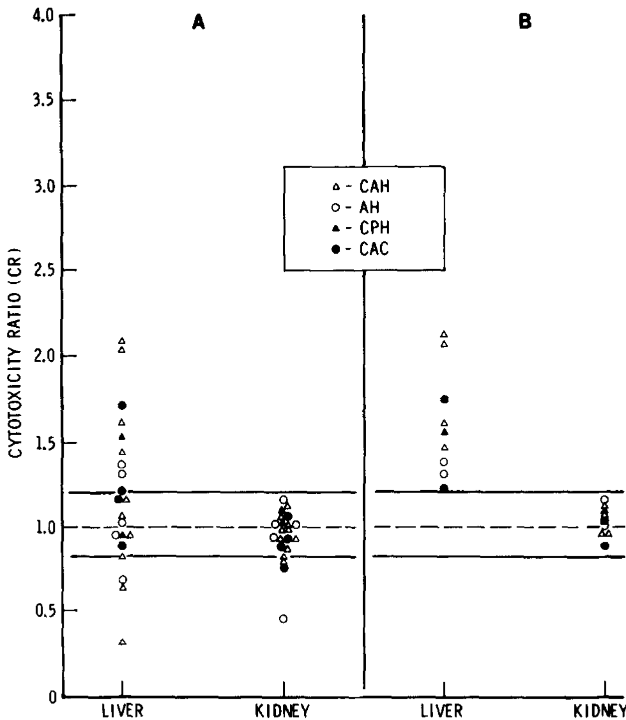

FIG. 2. Cytotoxicity ratios for liver versus kidney monolayer cultures. (A) In 21 patients examined; (B) in 9 patients with demonstrated cytotoxicity for liver cells.

organ specific antigens located on rat hepatocyte membranes (31). In other investigations of SCMC in CAH, lymphocytes from these patients demonstrated cytotoxicity against Chang cells isolated with trypsin (20, 32, 33). In one report, increased SCMC was observed when either Chang cells treated with trypsin or human hepatocytes isolated with collagenase were used as target cells (20). That the LSP and presumably other liver-specific antigens were still present on our target rat hepatocytes was confirmed using antisera to LSP both on the day of isolation and on the third day of culture. No attempt was made to insure that organ-specific antigens remained on the kidney cells after 3 days of culture. However, retention of organ-specific kidney antigens has been reported following trypsinization and for an interval of at least 80 hr (34).

Our data on cell-mediated cytotoxicity in CAH and AH support the conclusions of other investigations using a variety of target cells. Unlike several studies of lymphocyte cytotoxicity in CAC (8, 17), we were able to demonstrate that lymphocytes from patients with CAC still manifest *in vitro* cytotoxic capability. The decreased *in vitro* cytotoxicity shown by patients with CAH on steroid therapy supports the findings of others (6, 7, 16, 19, 20), suggesting further that corticosteroids inhibit the effector phase of lymphocyte-mediated cytotoxicity (35).

Several mechanisms have been suggested to account for *in vitro* cell-mediated cytotoxicity in CAH and AH. The first, SCMC involves a specific interaction between the sensitized T lymphocyte and target cell antigen(s) with subsequent

injury or death of the target cell. This system appears to be antibody independent (36).

It has been postulated that LSP may play a role in the pathogenesis of CAH (11, 21, 37–40) and perhaps in AH (17). We were not able to demonstrate a decrease in cytotoxicity in CAH using blocking antibody to human LSP. Conceivably, the anti-LSP may have blocked SCMC but promoted ADCC or alternatively, the cytotoxicity observed in CAH may have represented a summation of SCMC and ADCC effect. The ADCC of nonimmune lymphocytes against rat hepatocytes was increased, presumably through the action of Fc-receptor K cells. This suggests that mechanisms other than that directed against LSP may be in play in this system, including cytotoxic activity directed against other liver-specific antigens that have been described (41, 42), or against antigens yet to be identified. It is possible that a postulated surface immunoglobulin-negative (sIg−) Fc-receptor-bearing (Fc+) non-T lymphocyte (33) is the effector cell in rat liver SCMC. This effector cell does not require the presence of antibody and presumably would not be blocked by anti-LSP, if sensitized to another antigen.

ADCC is a second mechanism proposed to account for *in vitro* cell-mediated cytotoxicity and requires the presence of an antibody specific for the target cell antigen and an effector cell possessing an Fc receptor (36, 43). The quantity of antibody required is substantially less than that needed for complement-dependent cytotoxicity (36).

B-cell-enriched, but not T-cell-enriched fractions from patients with CAH have been shown to possess cytotoxic activity against isolated rabbit hepatocytes (12). The cytotoxicity was blocked by aggregated IgG suggesting that sIg−, Fc+ K cells present in the B-cell fraction were the effector cells. However, no specific antibody was identified in their sera-free system, thus leaving open the true identity of the effector cell. Kakumu *et al.* (32) also showed that PBM from patients with CAH were cytotoxic for Chang cells and that the effector cells were K but not B cells. Although the target Chang cells in their systems were not coated with antibody, if ADCC was the actual mechanism for the cytotoxic reaction, IgG antibodies against surface antigens on the Chang cells must have been present in their system. The IgG may have been produced by the lymphocytes or present in the FCS used. Vogten *et al.* (21), using ^{51}Cr-labeled avian erythrocytes coated with liver cell membrane lipoprotein as target cells and PBM as aggressors, showed increased cytotoxicity in patients with chronic active liver disease. Cytotoxicity was inhibited by free lipoprotein, antilipoprotein, and aggregated Ig.

A possible explanation for the ADCC-like mechanism of cytotoxicity in the absence of known antibody is offered by Vierling *et al.* (33). They have suggested that in normal humans and in the absence of antibody, SCMC against Chang cells is mediated by sIg−, Fc+ lymphocytes that are not T lymphocytes. It is not known if these lymphocytes are the same subpopulation as K cells.

In the present study, incubation of allogeneic nonimmune lymphocytes in the presence of patient sera in MLD, CAH, and AH resulted in increased cytotoxicity, presumably ADCC, against rat hepatocytes. This suggests that sera of patients with MLD, CAH, and AH contain a factor(s), possibly IgG, that allows normal Fc receptor lymphocytes to demonstrate cytotoxic activity against target cells. The

enhanced cytotoxic activity of normal lymphocytes in the presence of sera from patients with MLD may be explained by the presence of antibody to hepatic antigens (38–40).

We were not able to demonstrate significant inhibitory activity by sera from patients with CAH or AH on cytotoxicity by autologous lymphocytes in contrast to the findings of other investigators (6, 10, 19, 21). As mentioned previously, antibodies to hepatic antigens present in autologous sera (39) may have blocked SCMC and promoted ADCC.

It is not possible to determine in the present study which mechanism or effector cell is operative in *in vitro* cell-mediated cytotoxicity as unseparated peripheral mononuclear cells were used. The possible role of the monocyte cannot be addressed as these cells were not removed from the lymphocyte population. Our data would suggest, however, that there is increased SCMC with either a sensitized T lymphocyte or a sIg−, Fc+ non-T lymphocyte as effector cells. These cells presumably operate in an antibody-free environment. It is possible, however, that adsorbed cytophilic antihepatocyte antibodies may have induced an ADCC mechanism and contributed an additive effect to the observed SCMC. While addition of antisera to LSP appears to promote cytotoxicity by allogeneic nonimmune K lymphocytes, the presence of the antisera might not necessarily alter the increased cytotoxic effect of PBM from patients with CAH if both the K cell and the sIg−, Fc+ non-T lymphocyte are from the same population of null cells.

The observation that thymosin fraction 5 significantly lowered SCMC in untreated CAH raises the possibility that this thymic extract influenced a subpopulation(s) of lymphocytes involved directly, or perhaps indirectly, in cytotoxic activity. Thymosin did not effect lymphocyte viability and the SCMC of the CT-1 and MLD was minimally increased.

The endocrine thymus has an important regulatory role in the ontogenesis, function, and senescence of the T lymphoid system. The thymus produces thymosin which may act within the gland or at sites outside the thymus with induction of maturation and differentiation of T cells (25). Thymosin fraction 5 has been shown to increase T-cell counts and induce cellular immune competence *in vitro* and *in vivo* in a number of primary and secondary immunodeficiency syndromes (25, 44–46). On interaction with thymosin, null cells possessing T-lymphocyte antigens can form spontaneous rosettes with sheep red blood cells (SRBC), a functional capacity of mature T cells (47). Thymosin fraction 5 consists of at least 12 polypeptides with differing biologic activities which may act at discrete points in the differentiation of T cells already capable of forming SRBC rosettes (48). Thus, thymosin has been reported to influence the number and function of T-cell subsets which in turn can exert immunoregulatory control over B-cell immunoglobulin synthesis (49). In addition, a radiosensitive, thymosin-responsive suppressor cell has been described in humans (50).

Thomas *et al.* (51) have shown that T-cell numbers are reduced in patients with CAH and that incubation with thymosin increased T-cell counts. In a similar fashion, we have shown that decreased T-cell counts in AH can be increased with thymosin (52).

Diminished Con A-induced suppressor cell activity has been described in pa-

tients with systemic lupus erythematosus which was significantly reversed *in vitro*, when PBM were incubated with thymosin (53). Diminished suppressor cell activity has also been described in CAH accounting, perhaps, for increased cytotoxicity (4, 54). In addition, thymosin has been shown to enhance Con A-induced suppressor cell activity in patients with CAH (54). The decrease in cytotoxicity seen with thymosin in the present studies may reflect an induced suppressor cell effect or a direct alteration of the cytotoxic effector cell.

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