# In Vitro Cell-Mediated Cytotoxicity in Primary Biliary Cirrhosis and Chronic Hepatitis

### DYSFUNCTION OF SPONTANEOUS CELL-MEDIATED CYTOTOXICITY IN PRIMARY BILIARY CIRRHOSIS

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ABSTRACT The in vitro cytotoxic function and target cell specificity of peripheral blood lymphocytes from selected patients with primary biliary cirrhosis and hepatitis B surface antigen-negative chronic hepatitis were investigated using <sup>51</sup>Cr-labeled human Chang and EL-4 mouse sarcoma cell targets in assays of spontaneous cell-mediated cytotoxicity (SCMC) and mitogen-induced cellular cytotoxicity (MICC). In addition, antibody-dependent cellular cytotoxicity (ADCC) against Chang cells was assessed. At an effector-totarget cell ratio of 100:1, the mean SCMC against Chang cells was much less in patients with primary biliary cirrhosis than that in either the controls (P < 0.001) or the patients with chronic hepatitis (P < 0.005) whereas the value for patients with chronic hepatitis did not differ significantly from that of the controls. The mean SCMC against EL-4 mouse sarcoma cells was also less in patients with primary biliary cirrhosis than in the controls (P < 0.005) whereas the value for chronic hepatitis was not significantly different from that of the controls or patients with primary biliary cirrhosis. In contrast, MICC against both targets and ADCC against Chang cells were similar for each group. Comparison of SCMC and MICC against both target cells, measured simultaneously, showed similar cytotoxic potential against both target cells for each group. Effector cells capable of mediating cytotoxicity in each assay were defined by testing the cytotoxic function of lymphocyte subpopulations isolated from two representative patients with each disease using techniques of immunoabsorbent affinity chromatography and Fc receptor binding to antigen-antibody complexes. In both primary biliary cirrhosis and chronic hepatitis SCMC and ADCC were mediated by a subpopulation of lymphocytes which lack surface immunoglobulin (sIg-) and bear Fc receptors (Fc+). In contrast, MICC was mediated by sIg- cells which lack Fc receptors. Lymphocytes bearing sIg were not cytotoxic in any assay. These results establish a difference in cytotoxic function in primary biliary cirrhosis and chronic hepatitis by defining the presence of a defect in spontaneous cytotoxic function of sIg-, Fc+ lymphocytes against Chang cells in primary biliary cirrhosis.

### INTRODUCTION

In both primary biliary cirrhosis and chronic hepatitis it is possible that lymphoid cells become sensitized to hepatobiliary antigens or antigens cross-reacting with hepatobiliary antigens and are consequently cytotoxic for hepatic tissue (1, 2). This concept is supported by the demonstration that peripheral blood lymphocytes from patients with both of these diseases produce lymphokines when exposed to antigens contained in human liver homogenates (1, 3) or to a hepatospecific lipoprotein (4, 5). In addition, direct lymphocyte cytotoxicity against isolated xenogeneic hepatocytes has been reported to be increased in a majority of patients with chronic hepatitis and a minority of patients with primary biliary cirrhosis, compared to normal controls (6, 7). A variable degree of increased spontaneous cell-mediated cytolysis of allogeneic tar-

This work was presented in part at the 33rd Annual Meeting of the American Federation for Clinical Research, Atlantic City, N. J., 2 May 1976.

Received for publication 9 December 1976 and in revised form 11 July 1977.

get cells has also been reported in patients with chronic hepatitis (8-11) and primary biliary cirrhosis (10, 11). However, the hepatospecificity of the observed in vitro cytotoxicity and the nature of the effector cells mediating target cytotoxicity remain poorly defined.

To evaluate further cytotoxic function in primary biliary cirrhosis and chronic hepatitis, we have investigated the cytotoxic potential of peripheral blood lymphocytes in carefully selected patients with primary biliary cirrhosis and hepatitis B surface antigen (HB<sub>s</sub>Ag)<sup>1</sup>-negative chronic hepatitis using three distinct in vitro assays of cytotoxicity. These assays measured: (a) spontaneous cell-mediated cytotoxicity (SCMC); (b) mitogen-induced cellular cytotoxicity (MICC); (c)antibody-dependent cellular cytotoxicity (ADCC). Specificity of the cytotoxicity was evaluated by concurrently using as target cells an allogeneic, hepaticderived cell line (Chang liver) and a xenogeneic, nonhepatic control cell (EL-4 mouse sarcoma). In addition, studies were performed to identify the lymphocyte subpopulations capable of mediating each type of cytotoxicity in both diseases.

The results obtained show that primary biliary cirrhosis is associated with markedly diminished SCMC against Chang cells compared to either normal controls or HB<sub>s</sub>Ag-negative chronic hepatitis, whereas MICC and ADCC in both patient groups are normal. The diminished spontaneous cytotoxic potential in primary biliary cirrhosis appears to be due to a selective defect in the functional capacity of a lymphocyte subpopulation which bears Fc receptors but not surface immunoglobulins (sIg) and which was shown to mediate this cytotoxicity. We conclude that primary biliary cirrhosis is associated with a disorder of cytotoxic function of the peripheral blood lymphocytes and in this respect differs from HB<sub>s</sub>Ag-negative chronic hepatitis.

#### **METHODS**

#### Individuals studied

All patients were referred to the Section on Diseases of the Liver, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health. 10 patients with primary biliary cirrhosis and nine with chronic hepatitis were selected for study using strict criteria which included: (a) Clinical features, serum biochemical and serologic tests, and hepatic histology consistent with the respective diagnosis; (b) A negative test for HB<sub>s</sub>Ag; (c) No history of the use of drugs known to cause the histologic lesion of chronic hepatitis (12); (d) No history of past or current alcohol abuse; (e) No clinical features of hepatic decompensation.

Patient characteristics are summarized in Table I. The primary biliary cirrhosis group included individuals with all histologic stages of this disease as described by Scheuer (13). The chronic hepatitis group included seven with chronic active hepatitis and two with chronic persistent hepatitis as currently defined by Boyer (14). Liver biopsies in each of the patients with chronic active hepatitis showed striking piecemeal necrosis of hepatocytes. One patient with chronic active hepatitis and one with primary biliary cirrhosis were receiving corticosteroid therapy for their hepatic disease while an additional patient with chronic active hepatitis (no. 12) was receiving prednisone 5 mg/day as physiologic replacement for the adrenal suppression that had resulted from previous use of pharmacologic doses of corticosteroid. None of the remaining 16 patients were receiving any form of immunosuppressive therapy. Each patient receiving corticosteroid was studied before a dose was given to minimize the acute effects of corticosteroid on circulating lymphocytes (15).

11 healthy normal volunteers of both sexes served as controls. None had a history of illness suggestive of acute hepatitis and none were receiving any medication.

## Serologic tests for hepatitis B virus (HBV) infection

A negative test for HB<sub>s</sub>Ag in the serum was required for selection of patients with both primary biliary cirrhosis and chronic hepatitis. HB<sub>s</sub>Ag was assessed by radioimmunoassay (Ausria II, Abbott Laboratories, Chemical Div., North Chicago, Ill.). In addition, sera were coded and simultaneously tested for antibody to hepatitis B surface antigen (anti-HB<sub>s</sub>) by radioimmunoassay (Ausab, Abbott Laboratories) and for antibody to hepatitis B core antigen (anti-HB<sub>c</sub>) using both complement fixation and counter immunoelectrophoresis techniques by Dr. Robert J. Gerety, Bureau of Biologics, Bethesda, Md., who had no knowledge of the diagnoses. Results shown in Table I indicate past HBV infection or seroconversion in patients nos. 6 and 18 and convalescence from HBV infection in patient no. 15 (16). By these criteria, no patient had evidence of HBV replication at the time of study.

#### Media and reagents

L-Glutamine-free RPMI (1640) medium and phosphatebuffered saline, pH 7.2, (PBS) were obtained from the Media Section, NIH. Eagle's minimum essential medium, nonessential amino acids, sodium pyruvate, gentamicin, and fetal calf serum (FCS) were purchased from Microbiological Associates, Bethesda, Md. FCS was incubated at 56°C for 30 min to inactivate complement before use. Penicillin-streptomycin solution, trypsin 0.25%, L-glutamine, and RPMI (1640) containing 25 mM Hepes buffer were acquired from Grand Island Biological Co., Grand Island, N.Y. Chang cell spinner culture medium was prepared from Eagle's minimum essential medium and contained 0.1 mM nonessential amino acids, 100 U/ml of penicillin, 100  $\mu$ g/ml streptomycin, 50  $\mu$ g/ml gentamicin, 1 mM sodium pyruvate, 4 mM L-glutamine, and 6% (vol/vol) FCS. RPMI (1640) medium was supplemented before use to contain L-glutamine 4 mM, penicillin 100 U/ml, strepto-

<sup>&</sup>lt;sup>1</sup>Abbreviations used in this paper: ADCC, antibodydependent cellular cytotoxicity; anti-HB<sub>c</sub>, antibody to hepatitis B core antigen; anti-HB<sub>s</sub>, antibody to hepatitis B surface antigen; Con A, concanavalin A; E:T, effector to target cell ratio; FCS, fetal calf serum; HB<sub>s</sub>Ag, hepatitis B surface antigen; HBV, hepatitis B virus; MICC, mitogen-induced cellular cytotoxicity; PBS, phosphate-buffered saline pH 7.2; PHA, phytohemagglutinin-W; PWM, pokeweek mitogen; SCMC, spontaneous cell-mediated cytotoxicity; sIg, surface immunoglobulin; SRBC, sheep erythrocytes.

					Serum biochemical tests		Samla rivel tests					
	Patient	Age	Sex	Diagnosis	Total bilirubin*	Alkaline phos- phatase‡	SGPT§	AMA	ASMA¶	anti- HB,**	anti- HB <sub>c</sub> ‡‡	Pertinent medication
		yr										
1	A. C.	53	F	PBC, stage IV	5.7	444	81	+	+	0	0	
2	S. D.	44	F	PBC, stage IV	5.1	400	95	+	0	0	0	
3	P. M.	49	F	PBC, stage I–II	0.5	220	152	0	0	0	0	
4	L. H.	31	F	PBC, stage II–III	13.5	870	210	+	+	0	0	Prednisone, 10 mg by mouth per day
5	<b>H</b> . D.	61	F	PBC, stage I–II	1.0	215	165	+	0	0	0	
6	J. T.	55	F	PBC, stage III	5.4	400	85	+	+	+	0	
7	E. K.	39	F	PBC, stage I	0.8	590	270	+	0	0	0	
8	S. S.	45	F	PBC, stage I-II	0.4	177	82	+	0	0	0	
9	<b>W</b> . <b>W</b> .	38	F	PBC, stage III	1.1	192	101	0	+	0	0	
10	J. W.	45	F	PBC, stage II	2.3	390	138	+	0	0	0	
11	E. S.	51	М	САН	1.0	230	290	0	+	0	0	
12	J. M.	20	F	CAH, cirrhosis	0.8	56	50	0	+	0	0	Prednisone, 5 mg by mouth per day
13	P. A.§§	21	F	CAH, cirrhosis	0.3	36	14	0	0	0	0	Prednisone, 5 mg by mouth
14	G. B.	55	Μ	CAH	0.8	41	142	0	0	0	0	every other day
15	L. S.¶¶	27	F	CAH	0.6	34	185	0	+	+	+	
16	<b>F</b> . <b>C</b> .	48	F	CAH	0.8	118	290	0	0	0	0	
17	S. L.	20	F	CAH, cirrhosis	1.9	390	96	0	+	0	0	
18	S. W.	54	F	СРН	0.3	60	58	0	0	+	0	
19	J. H.	43	Μ	СРН	0.6	39	1000	0	0	0	0	

TABLE IPatient Population Characteristics

Abbreviations used: PBC, primary biliary cirrhosis; OAH, chronic active hepatitis; CPH, chronic persistent hepatitis. \* mg/100 ml serum, normal range 0.1-1.2.

‡ IU/liter, normal range 10-78.

§ IU/liter, normal range 2-45.

Antimitochondrial antibody.

¶ Anti-smooth muscle antibody.

\*\* Anti-HB surface antigen.

**‡**‡ Anti-HB core antigen.

§§ Spontaneous elevation of SGPT occurred within 2 wk of testing.

¶ Titers of anti-HB<sub>c</sub> and anti-HB<sub>s</sub> unchanged for >9 mo.

mycin 100  $\mu$ g/ml, and the appropriate amount of FCS. The column medium used for Sephadex anti-human-(Fab')2 chromatography contained 2.5 mM sodium ethylenediaminetetraacetic acid, 0.3% (wt/vol) sodium bicarbonate, and 5% FCS in RPMI (1640) with 25 mM Hepes buffer. Human gamma globulin (Cohn fraction II) was obtained from Miles Laboratories, Inc., Kankakee, Ill. A solution of human gamma globulin, 10 mg/ml in RPMI (1640), was freshly prepared and centrifuged at 100,000 g for 1 h before use. Phytohemagglutinin-W (PHA) was obtained from Burroughs Wellcome Co., Triangle Park, N. C.; concanavalin A (Con A) from Miles Laboratories; and pokeweed mitogen (PWM) from Grand Island Biological Co. Each lectin was diluted to the appropriate concentration in plain RPMI (1640) and stored at -20°C until used. Lymphocyte Separator Reagent (iron carbonyl coated with poly-L-lysine) was obtained from Technicon Instruments Corp., Tarrytown, N. Y. Sterile sodium <sup>51</sup>Cr chromate (sp act 300 mCi/mg) was acquired from Amersham/Searle Corp., Arlington Heights, Ill. Rabbit antitrinitrophenyl-keyhole limpet hemocyanin serum used in the preparation of antigen-antibody coated plates was kindly provided by Dr. Pierre A. Henkart, National Cancer Institute.

### Target cell cultures

Chang cells for both spinner and monolayer cultures were obtained from Microbiological Associates. Spinner culture cells were maintained in spinner culture medium within magnetically-stirred glass spinner culture flasks. Monolayer cell cultures were cultivated with spinner culture medium in plastic flasks (Falcon 3024, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). Both cultures were incubated in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C and subcultured twice weekly. Monolayer cultures were divided into two lots. Lot I was harvested for subculturing by incubation with 0.25% trypsin for 5 min at 37°C. Lot II was not trypsinized but instead harvested mechanically by scraping with a sterile spatula. Cells were propagated for 12 wk in this

manner before use. EL-4 mouse sarcoma cells were propagated intraperitoneally in C57/B6 mice by innoculation of tumor ascites.

# Preparation of effector cells for cytotoxicity assays

#### MONONUCLEAR CELL SEPARATION

Mononuclear cells from fresh, heparinized venous blood were prepared using aseptic technique by diluting the specimen 1:1 with PBS and centrifuging 8–10 ml of the mixture over 3 ml of Ficoll-Hypaque (Litton Bionetics, Div. of Litton Industries Inc., Kensington, Md.) (17) at 400 g for 40 min. Cells at the interface were collected using a Pasteur pipette and washed three times in RPMI (1640) 5% FCS before quantitation using a standard hemocytometer and Türk's solution. Cells were then diluted in RPMI (1640) 5% FCS to the final concentration required for the cytotoxicity assay. Viability was  $\geq$ 97% as assessed by trypan blue exclusion.

#### ISOLATION OF MONONUCLEAR CELL SUBPOPULATIONS

To identify which lymphoid cell types mediate cytotoxicity in vitro, specific effector cell subpopulations were sequentially isolated on the basis of cell surface markers and functional capacity from two representative patients with primary biliary cirrhosis (nos. 7, 10) and chronic active hepatitis (nos. 15, 16). The cytotoxic capacity of each subpopulation from these four patients was assessed in assays of SCMC, MICC, and ADCC. The assays for enumeration of cells bearing sIg, Fc receptors of forming rosettes with sheep erythrocytes (SRBC) and the methods of isolation of lymphocyte subpopulations have been described elsewhere (18). The following summarizes the isolation procedure.

Macrophage-depleted lymphocytes. Mononuclear cells were prepared as described except that centrifugation on Ficoll-Hypaque was performed at 2,250 g for 5 min. Cells at the interface were washed three times and resuspended in RPMI (1640) 10% FCS. Cells capable of endocytosing iron were removed from the cell suspension after incubation with Lymphocyte Separator Reagent as described (18). The resulting mononuclear cells obtained have been shown to be morphologically homogeneous small lymphocytes contaminated with  $\approx 1\%$  macrophages (18).

Surface immunoglobulin-positive (sIg+) and negative (sIg-) lymphocytes. Lymphocytes depleted of macrophages were fractionated on the basis of the presence or absence of surface immunoglobulin using Sephadex anti-Fab immunoabsorbent affinity chromatography as described previously (18). Anti-Fab immunoabsorbent was prepared by covalently binding purified rabbit anti-human-(Fab')2 to Sephadex G-200 according to the method of Chess et al. (19). The first 15 ml of effluent was collected and used for studies of sIg- lymphocytes. This fraction contained an average of 75% and 71% of the macrophage depleted lymphocyte suspension applied to the column for the chronic active hepatitis and primary biliary cirrhosis patients, respectively. Analysis of the sIgcells obtained by this technique has shown that 80-85% form rosettes with SRBC, 10-15% bear Fc receptors, and only 1-2% are contaminating sIg+ lymphocytes (18).

The next 75 ml of effluent were collected and discarded in order to remove all nonabsorbed cells. Lymphocytes remaining on the column were then eluted after the addition of Cohn fraction II as described previously (18). The first 30 ml of eluate was collected for use in studies of sIg+ lymphocytes. Analysis of the cell fraction prepared in this fashion has shown that 92–96% of the cells are sIg+ by immunofluorescence and only 1–2% form rosettes with SRBC (18).

Fc receptor positive (Fc+), sIg-lymphocytes. A portion of the sIg-lymphocyte eluate was fractionated further by absorbing Fc+ cells onto the surface of plastic culture flasks (Falcon 3012) which had been coated with insoluble antigen-antibody complexes (18) prepared by the method of Alexander and Henkart (20). Analysis of the nonadherent cells has shown that 92-94% form SRBC rosettes and that <1% are contaminating Fc+ or sIg+ lymphocytes (18).

Lymphocytes bound to the antigen-antibody complex were dissociated by adding 10 ml of a solution of 0.01-M EDTA in PBS and rocking for 18 h at 4°C. Cells were then decanted and washed six times with RPMI (1640) before resuspension in RPMI (1640) 5% FCS.

#### Antisera preparation

Rabbit antisera against Chang cells were prepared as described elsewhere (18). Antiserum from a single animal was used in studies of ADCC and was not cytotoxic for Chang cells in the absence of effector cells.

#### Target cell labeling

Freshly obtained Chang cells from either spinner or monolayer cultures and EL-4 mouse sarcoma cells were suspended in 10 ml of RPMI (1640) 5% FCS and microliter portions counted in 0.25% trypan blue using a standard hemocytometer. Target cells were resuspended at a concentration of  $5 \times 10^6$  trypan blue-excluding cells/ml in RPMI (1640) 10% FCS. 1 ml was radiolabeled by incubating with 0.1 ml sodium <sup>51</sup>Cr chromate for 40 min at 37°C in an atmosphere of 95% air and 5% CO<sub>2</sub>. Extracellular <sup>51</sup>Cr was removed by washing the cells three times in RPMI (1640) 5% FCS and resuspending them at the appropriate concentration.

#### Cytotoxicity assays

Cytotoxicity assays in tissue culture tubes were performed in duplicate using a modification (21) of the method of Holm and Perlmann (22). 1-ml aliquots of effector cell suspension containing  $1 \times 10^6$  cells were first added to plastic tissue culture tubes (Falcon 3033). For assays of SCMC, 0.5 ml of RPMI (1640) 5% FCS was added to this suspension. For assays of MICC, 0.5 ml of RPMI (1640) 5% FCS containing PHA (4 µg/ml), Con A (20 µg/ml), or PWM (1:12.5 dilution) was added. Finally, 0.5 ml of <sup>51</sup>Cr-labeled target cells containing  $1 \times 10^4$  cells were added to each tube and the contents gently mixed. Tubes were then centrifuged at 550 g for 10 min and incubated in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> for 18 h. For experiments with effector to target cell ratios other than 100:1, the ratio was altered by varying the total number of effector cells while maintaining a constant target cell number.

After incubation, the cell pellet was gently resuspended in the supernate and recentrifuged at 550 g for 10 min. A 1.0ml aliquot of the supernate was placed in a separate tissue culture tube and both tubes were counted in a well type gamma scintillation counter (model 1085, Nuclear Chicago Corp., Div. Searle, G. D. & Co., Des Plaines, Ill.). The duration of counting for each sample was such that the standard deviation of counting for each sample was <3%. The percentage of <sup>51</sup>Cr released from the labeled target cells was calculated using the following formula: Percentage <sup>51</sup>Cr released

$$= \frac{2 \times \text{cpm in supernatant tube}}{\text{cpm in supernatant tube} + \text{cpm in pellet tube}} \times 100.$$

Cytotoxicity was expressed as the mean percentage of <sup>51</sup>Cr released in duplicate assays.

Spontaneous release of  ${}^{51}$ Cr was calculated in this manner after incubating labeled target cells with medium alone. The addition of each of the three mitogens to the medium did not affect the magnitude of spontaneous  ${}^{51}$ Cr release from target cells.

Cytotoxicity assays in microtiter plates (18) were used in studies of ADCC and for determination of the cytotoxic capacity of isolated lymphocyte subpopulations in primary biliary cirrhosis and chronic active hepatitis to permit more rapid processing of large numbers of samples. Chang target cells were prepared as described and suspended at a concentration of  $1 \times 10^5$  cells/ml in either RPMI (1640) 5% FCS alone or medium containing either rabbit anti-Chang antiserum or PHA at concentrations of 1:500 and 2 µg/ml, respectively.

Six 0.1-ml aliquots of each of the above target cell suspensions were placed in tissue culture tubes for determination of the total <sup>51</sup>Cr radioactivity added to microtiter plate wells. 0.1 ml of effector cells suspended in RPMI (1640) 5% FCS and 0.1 ml of target cell suspension were added to the Vbottom wells of plastic microtiter plates (Cooke Laboratory Products, Div. Dynatech Laboratories Inc., Alexandria, Va.) in triplicate. Plates were centrifuged at 55 g for 5 min and incubated in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C for 18 h. After incubation, plates were centrifuged at 550 g for 10 min and 0.1-ml aliquots of supernate were carefully removed from each well and placed in tissue culture tubes for scintillation counting. Spontaneous release of <sup>51</sup>Cr was determined from quadruplicate assays in which radiolabeled Chang cells had been incubated with medium alone, medium plus anti-Chang antiserum, and medium plus PHA.

Percentage release of <sup>51</sup>Cr in microtiter cytotoxicity experiments was calculated using the following formula:

Percentage <sup>51</sup>Cr release = 
$$\frac{2 \times \text{supernate cpm}}{\text{Total cpm/well}} \times 100.$$

Cytotoxicity was expressed as the mean percentage of  ${}^{51}Cr$  released in triplicate assays.

#### Statistical analysis

Group data were compared using Student's t test (23).

#### RESULTS

# Evaluation of cytotoxicity assays using unfractionated mononuclear cells

Optimal effector cell to target cell ratio (E:T) for SCMC. Results of SCMC assays against Chang target cells for controls and patients with primary biliary cirrhosis and chronic hepatitis are shown in Fig. 1. Cytotoxicity was detectable at an E:T as low as 5:1 and reached plateau values at an E:T  $\geq$  100:1 for each group. Use of an E:T of 100:1 in these studies permits meaningful comparison of the maximum cytotoxic potential in both control and patient groups by ensuring a situation of effector cell excess.



FIGURE 1 SCMC against Chang cells as a function of the quantity of effector cells employed for controls (N = 2), primary biliary cirrhosis (N = 3), and chronic active hepatitis (N = 3). Cytotoxicity expressed as the mean  $\pm 1$  SE of the percentage of <sup>51</sup>Cr released.

Spontaneous release of <sup>51</sup>Cr from radiolabeled target cells. Radiolabeled target cells were incubated without effector cells during each cytotoxicity study. Serial results of spontaneous release of <sup>51</sup>Cr showed little variation, averaging  $26.2\pm0.4\%$  (±SE) from Chang and  $20.9\pm0.2\%$  from EL-4 mouse sarcoma cells, respectively. The values for Chang cells compared favorably with the  $26.3\pm1.0\%$  release obtained with spinner culture Chang cells by Holm and Perlmann after 24 h incubation (22). The small variation of these values emphasizes the predictability of target cell behavior.

Reproducibility of SCMC and MICC. Over the course of these studies, SCMC and MICC were periodically retested in a single control individual at an E:T of 100:1. Results of seven determinations of SCMC against Chang targets averaged 55.8±1.2% and six determinations against EL-4 mouse sarcoma targets averaged 34.2±4.5%. MICC using PHA averaged 82.1±1.5% against Chang and 82.7±2.1% against EL-4 mouse sarcoma targets. A similar degree of reproducibility was evident using Con A or PWM. The small degree of variability in assays of SCMC and MICC suggest that the capacity to mediate each function is a stable property in normal humans and is in accord with similar observations by Holm and Perlmann using Chang targets (22). In addition, these results suggest that extended subculturing of target cells did not result in alterations of the surface membranes of target cells which might systematically alter susceptibility to lysis by effector cells.

### SCMC and MICC against Chang cells

Results of SCMC and MICC against Chang cells, for the controls and patients with primary biliary cirrhosis and HB<sub>s</sub>Ag-negative chronic hepatitis, are recorded in

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	Control	РВС	СН	Control vs. PBC	Control vs. CH	CH vs. PBC
SCMC	$52.7 \pm 2.2*$	$37.2 \pm 2.5$	49.7±3.0	<i>P</i> < 0.001‡	NS§	P < 0.005
MICC						
PHA	$77.5 \pm 2.6$	$69.5 \pm 2.6$	$74.0 \pm 3.3$	NS	NS	NS
Con A	$65.3 \pm 4.2$	$57.1 \pm 1.8$	$63.7 \pm 3.4$	NS	NS	NS
PWM	$76.2 {\pm} 2.8$	$72.3 \pm 3.5$	$75.4 \pm 3.6$	NS	NS	NS

TABLE II Cytotoxicity Against Chang Cells

Abbreviations used: PBC, primary biliary cirrhosis; CH, chronic hepatitis.

\* Mean±1 SE of the percent <sup>51</sup>Cr released from radiolabeled target cells at an E:T = 100:1. Spontaneous release from target cells incubated with medium alone =  $26.2\pm0.4\%$  and with medium plus mitogen =  $26.4\pm0.4\%$ .

‡ Student's t test.

§ Not significant.

Table II. The mean value of SCMC for chronic hepatitis patients did not differ significantly from that of control individuals. In contrast, the mean value of SCMC for primary biliary cirrhosis patients was significantly less than that of either control individuals  $(37.2 \pm 2.5\% \text{ vs. } 52.7 \pm 2.2\%, P < 0.001)$  or patients with HB<sub>s</sub>Ag-negative chronic hepatitis  $(37.2\pm2.5\% \text{ vs})$ . 49.7 $\pm$ 3%, *P* < 0.005). The low values of SCMC in the patients with primary biliary cirrhosis did not appear to be attributable to the degree of cholestasis. Within the primary biliary cirrhosis group the mean value for SCMC for the five patients with total serum bilirubin concentrations >2.0 mg/100 ml was 38.8%, whereas the corresponding mean for the other five patients was 35.6%. Furthermore, the correlation coefficients between values for SCMC and serum total and direct bilirubin concentrations and serum alkaline phosphatase activity were 0.12, 0.09, and 0.31 respectively. In addition, SCMC in a 55-yr-old female with severe cholestasis (total bilirubin 18 mg/

100 ml, direct bilirubin 10 mg/100 ml, and alkaline phosphatase 400 IU/liter) due to large duct biliary obstruction was 49.9% and in a simultaneously studied normal control subject was 48.7%. SCMC in the patients with primary biliary cirrhosis could not be related to the duration of symptoms attributable to the disease or the hepatic histologic stage of the disease as defined by Scheuer (13). MICC against Chang cells was similar for controls and patients with primary biliary cirrhosis and chronic hepatitis using each mitogen.

# SCMC and MICC against EL-4 mouse sarcoma cells

Cytotoxicity assays against EL-4 mouse sarcoma cells were performed concurrently with assays using Chang cells and the results are shown in Table III. As with cytotoxicity against Chang cells, the mean value of SCMC for patients with chronic hepatitis did not dif-

 TABLE III

 Cytotoxicity Against EL-4 Mouse Sarcoma Cells

	Control	РВС	СН	Control vs. PBC	Control vs. CH	CH vs. PBC
SCMC	$43.5 \pm 2.7*$	$31.0 \pm 2.6$	$34.1 \pm 5.4$	P < 0.005‡	NS§	NS
MICC PHA Con A PWM	$77.0\pm2.4$ $49.4\pm2.6$ $63.3\pm3.4$	$71.9 \pm 4.4$ $38.3 \pm 5.1$ $51.5 \pm 3.8$	$63.9 \pm 5.9$ $42.1 \pm 6.0$ $47.5 \pm 5.3$	NS NS P < 0.05	NS NS P < 0.025	NS NS NS

Abbreviations used: PBC, primary biliary cirrhosis; CH, chronic hepatitis.

\* Mean±1 SE of the percent <sup>51</sup>Cr released from radiolabeled target cells at an E:T = 100:1. Spontaneous release from target cells incubated with medium alone =  $20.9\pm0.2\%$  and with medium plus mitogen =  $20.4\pm0.3\%$ .

‡ Student's t test.

§ Not significant.

fer significantly from that of control individuals. Again, the mean value for SCMC in patients with primary biliary cirrhosis was significantly less than that of controls ( $31.0\pm2.6\%$  vs.  $43.5\pm2.7\%$ , P < 0.005). Although the mean value of SCMC in primary biliary cirrhosis was lower than that in chronic hepatitis, the difference was not statistically significant.

Mean values for MICC against EL-4 mouse sarcoma cells were similar for each group using either PHA or Con A. Results with PWM, however, showed that mean values were significantly less in patients with primary biliary cirrhosis compared to controls ( $51.5\pm3.8\%$  vs.  $63.3\pm3.4\%$ , P < 0.05) and in patients with chronic hepatitis compared to controls ( $47.5\pm5.3\%$  vs.  $63.3\pm3.4\%$ , P < 0.025).

### ADCC against Chang cells

ADCC against this target was evaluated in five controls, five patients with primary biliary cirrhosis, and three patients with chronic active hepatitis at an E:T of 1, 5, 10, 25, and 100 to 1. These results are shown in Fig. 2. Cytolysis of antibody coated target cells was similar for each group at each ratio.

# Evaluation of target cell specificity of SCMC and MICC

To evaluate whether peripheral blood lymphocytes from patients with primary biliary cirrhosis or chronic hepatitis were preferentially cytotoxic to Chang cells,



FIGURE 2 ADCC against Chang cells for controls (N = 5), primary biliary cirrhosis (N = 5), and chronic active hepatitis (N = 3). Cytotoxicity expressed as the mean  $\pm 1$  SE of the percentage of <sup>51</sup>Cr released.

simultaneously determined values for SCMC and MICC against both target cells were compared. For this comparison, each patient's results in these assays of cytotoxicity were expressed as a fraction of the corresponding results determined concurrently in control individuals. These values are summarized in Table IV. The relative capacity of lymphocytes to mediate SCMC against both target cells did not differ for patients with either primary biliary cirrhosis or chronic hepatitis indicating an absence of preferential cytotoxicity for Chang cells. The corresponding results for MICC were similar.

# Effect of trypsin treatment of Chang cells on SCMC

The Chang cells used in the above studies were propagated in spinner culture and not exposed to trypsin. In contrast, Chang cells, when propagated as monolayer cultures, are routinely harvested with trypsin. Studies were performed to assess the possibility that trypsin might expose or modify antigens on the Chang cell surface membrane which are important for the recognition of target cells by lymphocytes. Assays of SCMC were performed concurrently against: (a) Chang cells in spinner culture; (b)Chang cells in monolayer culture propagated by mechanical harvesting and not exposed to trypsin; (c) Chang cells as in b which were harvested for the SCMC study with a single exposure to trypsin; (d)Chang cells in monolayer culture which had been propagated with repetitive trypsin exposure. Results of SCMC, expressed relative to the value for SCMC against Chang cells from spinner culture, are shown in Fig. 3 for two controls, three patients with primary biliary cirrhosis, and three patients with chronic hepatitis. SCMC against Chang cells cultured as monolayers with or without trypsin exposure was comparable to SCMC against Chang cells from spinner cultures for controls and patients.

### Cytotoxic capacity of lymphocyte subpopulations in primary biliary cirrhosis and HB<sub>s</sub>Ag-negative chronic hepatitis against Chang cells

ADCC. Representative results of ADCC using lymphocyte subpopulations as effector cells are shown in Fig. 6. In each of the two patients with primary biliary cirrhosis as well as the two patients with chronic active hepatitis, ADCC was mediated exclusively by sIg-, Fc+ lymphocytes. This identification of the ADCC effector cell is in agreement with the results of others (24) and this cell has been termed a K lymphocyte (25).

SCMC. Representative results of SCMC assays us-

 TABLE IV

 Comparison of Cytotoxicity Against Chang and EL-4 Mouse Sarcoma Cells

		РВС				СН				
	Chang	EL-4	Р	C	hang	EL-4	Р			
SCMC	$0.72 \pm 0.04*$	$0.88 \pm 0.10$	>0.1‡	0.96	±0.07	$0.95 \pm 0.1$	2 >0.9			
MICC PHA Con A PWM	$0.90 \pm 0.07$ $0.89 \pm 0.07$ $0.97 \pm 0.06$	$0.93 \pm 0.03$ $0.86 \pm 0.07$ $0.84 \pm 0.05$	>0.5 >0.7 >0.1	0.98 1.01 1.01	$\pm 0.04 \\ \pm 0.06 \\ \pm 0.05$	$0.90 \pm 0.0$ $0.89 \pm 0.0$ $0.84 \pm 0.0$	$\begin{array}{l} 7 &> 0.2 \\ 9 &> 0.2 \\ 4 &< 0.05 \end{array}$			

Abbreviations used: PBC, primary biliary cirrhosis; CH, chronic hepatitis.

\* Mean  $\pm 1$  SE of values for SCMC and MICC in patients expressed as a fraction of the values obtained concurrently in controls at an E:T = 100:1.

‡ Student's t test.

ing isolated lymphocyte subpopulations at several effector to target ratios are shown in Fig. 4. In each of the two patients with primary biliary cirrhosis as well as the two patients with chronic active hepatitis, the cytotoxic capacity of the macrophage-depleted lymphocytes was accounted for by the sIg- lympocyte fraction. Separation of this fraction based on the capacity of cells to bind to the Fc portion of antigenantibody complexes showed that SCMC was mediated exclusively by the sIg-, Fc+ lymphocytes but not by sIg-, Fc- lymphocytes. Lymphocytes bearing sIg did not mediate SCMC.

*MICC*. Representative results of MICC using PHA with lymphocyte subpopulations as effector cells are shown in Fig. 5. In contrast to SCMC, MICC was mediated by sIg-, Fc- lymphocytes in both the pa-



FIGURE 3 Comparison of SCMC against Chang cells from monolayer cultures harvested by repetitive trypsinization (multiple trypsin), single trypsinization (single trypsin) or scraping (spatula) and Chang cells from spinner cultures not exposed to trypsin. Controls (N = 2), primary biliary cirrhosis (N = 3), chronic hepatitis (N = 3). Values represent the mean  $\pm 1$  SE of SCMC against the monolayer cells expressed relative to the SCMC determined simultaneously against Chang cells in spinner culture at an E:T = 100:1.

tients with primary biliary cirrhosis and the patients with chronic active hepatitis. No cytotoxic capacity was evident for either sIg-, Fc+ or sIg + lymphocytes. In both primary biliary cirrhosis and chronic active hepatitis, sIg-, Fc+ lymphocytes caused <sup>51</sup>Cr release comparable to that seen in SCMC (Fig. 4) which suggests that SCMC mediated by sIg-, Fc+ lymphocytes occurs in the presence of PHA and that total <sup>51</sup>Cr release in MICC using peripheral blood mononuclear cells as effector cells represents the sum of SCMC and MICC releases.

It should be noted that each lymphocyte cell population may have undergone subtle changes as a result of the procedures used to isolate subpopulations and that such changes may have resulted in the cytotoxic potential of isolated cells not being identical to that of the same cells in unfractionated macrophage-depleted lymphocytes. For this reason comparisons of cytotoxicity data that include data obtained with isolated subpopulations of lymphocytes are qualitative rather than quantitative.

### DISCUSSION

Rational evaluation of immunologic function in chronic liver disease requires the study of well-defined and characterized patient populations. The observations that certain cases of chronic hepatitis are associated with hepatotoxic medications (12) or HBV infection (26) and that HB<sub>s</sub>Ag-positive and negative chronic hepatitis appear to differ in clinical and immunologic features (27–32) indicate that subgroups of chronic hepatitis exist which may differ in their mechanisms of pathogenesis. Whether or not an analogous situation exists in primary biliary cirrhosis is unknown since etiologic factors in this disease have not been identified. In the present study, we have investigated only patients who are HB<sub>s</sub>Ag-negative and have no history of exposure to medications associated with



FIGURE 4 Comparison of the capacity of lymphocyte subpopulations to mediate SCMC against Chang cells in chronic active hepatitis (A) and primary biliary cirrhosis (B). Cytotoxicity expressed as the mean  $\pm 1$  SE of the percentage of <sup>51</sup>Cr released in triplicate assays for a single patient with each disease.

chronic hepatitis. These criteria provided defined patient groups which are representative of a large proportion of cases of chronic hepatitis in the U.S.A. (33, 34) and the majority of cases of primary biliary cirrhosis (35). Further characterization of the patient groups was provided by the results of serologic tests for anti-HB<sub>c</sub> and anti-HB<sub>s</sub>. These showed a similar incidence of seroconversion to HB viral antigens in both disease groups and no evidence of HBV replication (16).

The assessment of cytotoxic function in these selected patients employed three distinct in vitro assays of cytotoxicity. In the assay of SCMC, the capacity of lymphocytes to mediate cytolysis of target cells in the presence of nutrient medium is measured. Although the precise mechanism of cytolysis in SCMC is unknown, it is presumably initiated by the interaction of effector cell receptors with constituents on the target cell surface. Recent investigations in this labora-



FIGURE 5 Comparison of the capacity of lymphocyte subpopulations to mediate MICC against Chang cells in chronic active hepatitis (A) and primary biliary cirrhosis (B). Cytotoxicity expressed as the mean  $\pm 1$  SE of the percentage of <sup>51</sup>Cr released in triplicate assays for a single patient with each disease.



FIGURE 6 Comparison of the capacity of lymphocyte subpopulations to mediate ADCC against Chang cells in chronic active hepatitis (A) and primary biliary cirrhosis (B). Cytotoxicity expressed as the mean  $\pm 1$  SE of the percentage of <sup>51</sup>Cr released in triplicate assays for a single patient with each disease.

tory have demonstrated that in normal humans, SCMC against Chang cells is mediated by sIg-, Fc+ lymphocytes that predominantly do not form SRBC rosettes (18) and are, therefore, not T lymphocytes (36). However, in the event the population of lymphocytes being studied were to contain cells sensitized to antigens on the target cell surface, the cytolysis in this assay could also be mediated by T-lymphocytes (37).

The in vitro assay of ADCC assesses the effector cell mediated cytolysis of antibody-coated target cells. Target cell specificity is determined solely by the specificity of the anti-target cell antibody. Cytolysis occurs after interaction of the Fc receptor of a nonsensitized effector cell with the Fc portion of the antibody bound to the target cell. The effector cell in ADCC has been termed the K lymphocyte (25). In normal humans, ADCC against Chang cells is mediated by cells within the same sIg-, Fc+ lymphocyte subpopulation which contains the effector cells for SCMC (18). This does not necessarily indicate, however, that the effector cells for ADCC and SCMC are identical.

In the assay of MICC, the capacity of effector cells to mediate target cytolysis in the presence of plant lectins is measured. MICC has been considered an in vitro model of the cytotoxic potential of in vivo antigensensitized lymphocytes in which the plant lectin serves to bypass the afferent sensitization process of the effector cell (38). The ability of mitogen to induce this form of cytotoxicity appears to be separate from its ability to induce blastogenesis (39). MICC against Chang cells is mediated in normal humans by a subpopulation of sIg- lymphocytes which form SRBC rosettes and do not bind to the Fc portion of antigenantibody complexes (18). The ability to form SRBC rosettes identifies these cells as T lymphocytes.

In the present study, we have demonstrated that SCMC, ADCC, and MICC are mediated by the same subpopulations of lymphocytes in primary biliary cirrhosis and chronic hepatitis as in normal individuals. Thus, the results obtained reflect the function (or dysfunction) of defined subpopulations of lymphocytes and allow direct comparison of these subpopulations in control and patient groups.

MICC in both primary biliary cirrhosis and chronic hepatitis was similar to that observed in control individuals. These results indicate that the capacity of sIg-, Fc- lymphocytes to mediate cytotoxicity is equivalent for controls and both patient groups. The integrity of the cytotoxic function of sIg-, Fclymphocytes in MICC for primary biliary cirrhosis patients contrasts with observations that primary biliary cirrhosis patients, including those reported here,<sup>2</sup> exhibit diminished lymphocyte proliferation responses to these same mitogens (40, 41). These results indicate that the cytotoxic and proliferative responses of lymphocytes induced by mitogen are to a certain degree independent.

<sup>&</sup>lt;sup>2</sup> Vierling, J. M. Unpublished observations.

In contrast to the results of tests of MICC, SCMC against Chang cells was markedly decreased in primary biliary cirrhosis compared to either normal controls or patients with chronic hepatitis. Paradoxically, ADCC was normal in primary biliary cirrhosis despite the fact that both SCMC and ADCC are mediated by the same sIg-, Fc+ lymphocyte subpopulation. Fundamental to the understanding of this selective dysfunction of cytotoxicity is the question of the composition of the sIg-, Fc+ subpopulation of lymphocytes. Studies showing that trypsin treatment of human effector cells diminishes SCMC but does not alter ADCC (42) have been confirmed using Chang cell targets<sup>3</sup> and suggest that the cytotoxic mechanisms of the effector cell(s) necessary to mediate SCMC and ADCC are different. Therefore, the defect in primary biliary cirrhosis could represent either a selective dysfunction in the capacity of a single cell type (K lymphocyte) to mediate SCMC while ADCC function remains normal or, alternatively, dysfunction or decreased numbers of a separate cell type within the sIg-, Fc+ subpopulation of lymphocytes which mediates SCMC but not ADCC.

The finding of similar ADCC against Chang cells at each of several E:T for both patients with primary biliary cirrhosis and normal controls suggests the presence of similar quantities of effector cells in both groups. In addition, preliminary results of direct quantitation of peripheral blood sIg-, Fc+ lymphocytes have shown no difference between values obtained for patients with primary biliary cirrhosis and normal controls.<sup>2</sup> However, these data are insufficient to differentiate between the two possible explanations for decreased SCMC in primary biliary cirrhosis.

Although recent evidence suggests that SCMC mediated by sIg-, Fc+ lymphocytes may be involved in tissue cytolysis in human disease (43), the biologic significance of diminished SCMC in primary biliary cirrhosis is not clear. This defect in cytotoxicity did not correlate with the histologic stage of the hepatic lesion, the duration of clinical illness, or serum biochemical indices of cholestasis. Also, despite the fact that corticosteroid therapy may diminish SCMC (9), the single patient receiving prednisone did not bias the primary biliary cirrhosis group result as her SCMC was identical to the group mean. Furthermore, the decrease in SCMC in primary biliary cirrhosis was not attributable to the fact that all patients with primary biliary cirrhosis were females since the mean value of SCMC in primary biliary cirrhosis was also significantly less than that for either female controls or female patients with chronic hepatitis.

Of the various explanations for decreased SCMC in primary biliary cirrhosis, it is pertinent to consider the

<sup>3</sup> Nelson, D. L. Unpublished observations.

possibility that it may be related to innate, immunologic characteristics of individuals which might serve as a marker for susceptibility. Such a possibility has been raised by the demonstration of an association between decreased SCMC in normal humans and the HLA haplotype A3,B7 (44). However, recent investigations of individuals with this haplotype have demonstrated decreased cytotoxic function only in males (45). The exclusive female composition of our primary biliary cirrhosis group and the presence of the A3,B7 haplotype in only one of seven patients<sup>2</sup> indicate that decreased SCMC in this study cannot be explained on this basis.

Although the pathogenic role of sIg-, Fc+ lymphocytes in primary biliary cirrhosis and chronic hepatitis remains speculative, the results obtained in the SCMC assay are germane to the possibility that the pathogenic mechanism in both diseases involves an antigenspecific cytotoxic reaction against hepatobiliary tissue that is mediated by T lymphocytes. This follows from the fact, alluded to previously, that the SCMC assay is potentially capable of measuring cytotoxicity mediated by T lymphocytes reacting against specific target cell antigens as well as cytotoxicity mediated by SCMC effector cells (sIg-, Fc+ lymphocytes). In this study, SCMC against Chang cells did not exceed control values for primary biliary cirrhosis or chronic hepatitis patients at either an optimum E:T of 100:1 or a lower E:T (Fig. 1). Furthermore, SCMC was not preferential for Chang cells when compared to the dissimilar EL-4 mouse sarcoma target cells. Although these results do not support an antigen-specific, T lymphocyte mediated process in either disease, the existence of such a process cannot be excluded since it remains possible that the antigens required for its demonstration are not present on the Chang cell.

Previous studies of lymphocyte cytotoxicity in primary biliary cirrhosis (6, 10, 11) and chronic hepatitis (6-11) have yielded variable results against a variety of target cells including Chang cells (8, 9), rabbit hepatocytes (6, 7), cultured autochthanous liver tissue (10), and fresh, autologous hepatic tissue obtained from individual patients by percutaneous liver biopsy (9, 11). In these studies, increased cytotoxicity compared to controls was observed for at least some of the patients evaluated. However, the significance of the results in these studies is unclear because: (a) no control target cells were used to assess the target specificity of the observed results; (b) data on the nature of the effector cells which mediate SCMC have been presented only for the rabbit hepatocyte; (c) methods used to prepare autologous hepatic tissue are likely to result in a heterogeneous mixture of distinct target cell types whose relative cytolysis cannot be defined; (d) the proportion of hepatocytes contained within cultures of

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autochthanous liver tissue has not been identified with precision; (e) the use of low effector to target cell ratios in some studies raises the possibility that variation in cytotoxicity does not reflect differences in the cytotoxic capacity of effector cells but rather reflects variation in the number of effector cells present in diluted cell suspensions.

These features as well as differences in the target cells used, patient populations studied, and the presentation and analysis of data, make it difficult to compare these studies with one another or the present investigation. It is, however, particularly relevant to compare our results with those of Wands et al. (8, 9). Using Chang target cells, these investigators found spontaneous cytotoxicity to be increased in four (8) and eight (9) chronic active hepatitis patients when compared to controls in an assay similar to our assay of SCMC. This finding contrasts with the findings reported here in which SCMC was found to be similar in patients with chronic hepatitis and controls. The difference between these results is not explained by Wands et al. use of Chang cells cultured in monolayers and harvested with trypsin rather than the use of untrypsinized Chang cells since our studies indicate that SCMC was not appreciably greater using trypsinized compared to untrypsinized Chang cells. Our inclusion of two patients receiving a low dose of prednisone is also unlikely to have caused the discrepancy in results since the value for SCMC in the chronic hepatitis group is not substantially changed when the prednisone-treated patients are excluded from the analysis. However, a possible explanation for the difference in results is the composition of the patient populations studied: HBV replication was not present in any patient in the current study, whereas the majority of patients evaluated by Wands et al. were positive for HB<sub>s</sub>Ag. The presence of HBV replication may be a determining factor for the magnitude of spontaneous cytotoxicity as recent data suggests that cytotoxicity against Chang cells is increased in HB<sub>s</sub>Ag-positive compared to HB<sub>s</sub>Ag-negative chronic hepatitis patients (46).

In summary, the present investigations of the in vitro cytotoxic potential of lymphocytes from selected patients with primary biliary cirrhosis and chronic hepatitis identify the Chang cell as a useful target cell for the assessment of the cytotoxic capacity of lymphocyte subpopulations in both of these diseases. Furthermore, these studies define the presence of a dysfunction of SCMC in primary biliary cirrhosis. The difference in SCMC against Chang cells in primary biliary cirrhosis and chronic hepatitis suggests that these disease groups differ in either the circulating numbers or the cytotoxic capacity of SCMC effector cells. To the extent SCMC function relates to pathogenesis in these diseases, this difference may reflect differences in the pathogenic mechanisms of primary biliary cirrhosis and chronic hepatitis. Finally, no evidence for specific T-lymphocyte-mediated cytotoxicity against Chang cells was noted for patients with primary biliary cirrhosis or chronic hepatitis. Future attempts to evaluate the possible existence of hepatobiliary antigen-specific cytotoxicity in primary biliary cirrhosis and chronic hepatitis will require the use of target substances or cells specific to these diseases.

#### ACKNOWLEDGMENT

The authors wish to thank Ms. Phyllis Cromwell for the rapid and accurate typing of the manuscript.

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