Immunologic Studies in Patients with Chronic Active Hepatitis and Primary Biliary Cirrhosis
I. Cytotoxic Activity and Binding of Sera to Human Liver Cells Grown in Tissue Culture[1] (37407)

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(Introduced by Hans Popper)

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Although an immunologic reaction is thought to play a role in chronic active hepatitis and primary biliary cirrhosis, the significance of humoral antibodies and cell-mediated immunity has not been elucidated. A high incidence of antibodies to mitochondria, smooth muscle, and nuclei has been described in patients with these diseases; however, the pathogenetic importance of these antibodies is not yet clarified (1). The present work utilized tissue culture of liver cells to demonstrate that sera of patients with chronic liver diseases, although binding to liver cells, are devoid of cytotoxic properties.

Materials and Methods. Sera of patients were kept at —20°. Before use, sera were inactivated at 56° for 30 min; in three instances sera were used immediately after being drawn. Diagnosis of chronic active hepatitis and primary biliary cirrhosis was made from a correlation of clinical and histologic findings. As a source of complement, serum from a healthy person was obtained and used immediately.

Hepatitis B antigen (HB Ag) was tested for on all specimens by immunodiffusion, counterimmunoelectrophoresis (2), and complement fixation (3). Anti-HB Ag was obtained from a patient with hemophilia.

Cell lines. The following cell lines were utilized: Chang liver cells, human fibroblasts (WL-38, Gibco, Grand Island, NY, and human fibroblasts derived in our laboratory from foreskin), and conjunctiva (Wong-Kilbourne derivative of Chang conjunctiva clone 1-5c; American-type culture collection, Rockville, MD). These cell lines were kept in plastic bottles (Falcon Plastic, Los Angeles, CA), with RPMI medium 1640 or MEM containing 10 to 15% heat-inactivated fetal calf serum. Liver tissue was obtained from biopsy, minced in RPMI medium 1640, and cultured following the method of Demoise, Galambos, and Falek (4). Subcultures were prepared every 7 to 14 days.

The following six cell lines derived from human liver were utilized for this investigation: (a) L25, fifth serial subculture from liver biopsy of a patient with chronic active hepatitis; (b) L44, first subculture from liver biopsy of a normal liver from a patient with chronic cholecystitis; (c) L41, fifth serial subculture from liver biopsy of a patient with HB Ag positive viral hepatitis; (d) L38, fourth serial subculture from liver biopsy of a patient with chronic active hepatitis; (e) L38, second serial subculture from liver biopsy of a patient with chronic active hepatitis; (f) L6, fifth serial subculture from liver biopsy of a patient with chronic active hepatitis.

Cytotoxicity. In the early part of the investigation, Chang liver cells and fibroblasts WL-38 were labeled with ^{51}Cr following the method of Holm and Perlmann (5). After chromium labeling, 1 ml of cell suspension containing 5000 target cells was placed in a test tube and 0.1 ml of patient serum was added. In another set of test tubes, in addition to the patient serum, 0.1 ml of fresh human serum as a source of complement was added. Test tubes were incubated for 24 hr

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at 37° in air containing 5% CO_2. After that time, cytotoxicity was measured as percentage release of ^{51}Cr from target cells incubated with serum, minus spontaneous release (5).

In subsequent experiments, cytotoxicity was measured by the method of Takasugi and Klein (6). Cells were plated in the microwells of a microtest tissue culture plate (Falcon Plastics), with 5 μl of medium containing 15% inactivated fetal calf serum. After 24 hr the medium was removed and replaced with 2.5 μl of patient's serum, 5 μl of RPMI medium 1640, and 2.5 μl of fetal calf serum. In another set of experiments, serum was added immediately after trypsinization and plating of cells. Cells were incubated for 2 days at 37° with air and 5% CO_2. Plates were then stained with Giemsa stain, and number and morphologic appearance of cells were recorded.

Immunofluorescent investigations. To study the binding of antibodies to the target cells, liver cells derived from liver biopsy were plated in an eight-chambered tissue culture chamber/slide (Lab-Tek Products, Westmont, IL). After 2 to 5 days, the slides were washed with phosphate-buffered saline and fixed in acetone for 10 min. One set of slides was washed and used unfixed. Nine sera with smooth muscle antibodies, nine with mitochondrial antibodies, and five normal sera were studied. Each chamber was treated with the patient serum diluted 1:10, followed by fluoresceinated antihuman IgG (Behring Diagnostics, Inc., Woodbury, NY). Tests for the specificity of antisera, mounting of slides, and observation conditions with the fluorescent microscope have been described previously (7). Antibodies to nuclei, mitochondria, and smooth muscle were detected in the patients' sera by previously described techniques (8). Anti-Chang liver cell anti-

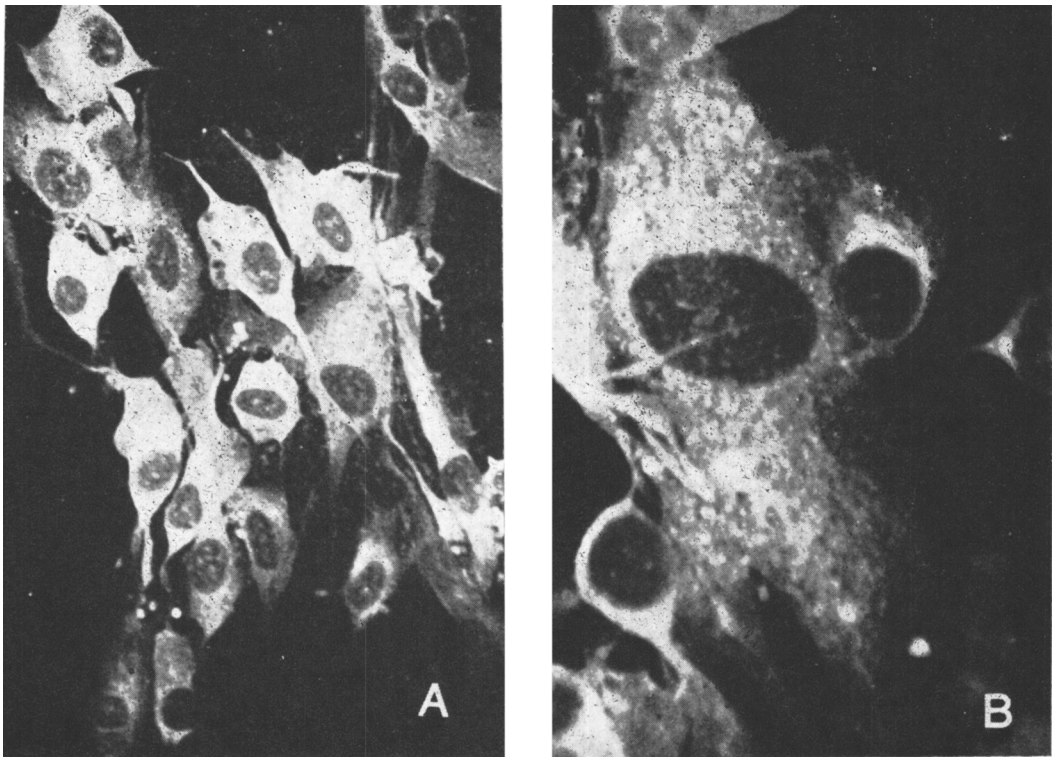

FIG. 1. Monolayer of liver cells (derived from a patient with chronic active hepatitis), treated first with 1:10 dilution of a serum with mitochondria antibodies followed by fluorescein-labeled human IgG antiserum. (A) Liver cells are diffusely stained (×250). (B) Higher magnification shows the finely granular staining of the cytoplasm (×560).

serum was produced in rabbits by three weekly injections of 10^7 washed Chang liver cells in 0.5 ml of physiologic saline and suspended in an equal volume of complete Freund's adjuvant.

Results. Using the indirect fluorescent technique, antibody from sera of patients with chronic active hepatitis or primary biliary cirrhosis, but not normal sera, bound to cells derived from human liver biopsies. Incubation of sera containing mitochondrial antibodies resulted in weak, diffuse, and sometimes granular staining of the cytoplasm of unfixed cells (Figs. 1A and B). The reaction with acetone-fixed cells was usually negative. Smooth muscle antibodies bound to the cultured cells in a filamentous pattern. The filaments were long, thin, and almost parallel, arranged along the major axis of the cells (Fig. 2A). Often they traversed the entire cell and overlapped the negative nucleus (Fig. 2B). The staining was similar on fixed and unfixed cells.

When sera of patients with antibodies to smooth muscle, mitochondria, nuclei, and HB Ag, as well as sera containing HB Ag, were applied to microwells containing liver cells, cytotoxic activity was not detected. Results did not change when sera were incubated with complement or when cells were used immediately after trypsinization. A typical experiment is illustrated in Table I.

In addition, sera of four patients with chronic active hepatitis containing antibodies to smooth muscle and nuclei were incubated for 2 days with autologous cell lines derived from liver biopsies. In no instance could morphologic alterations or reduction in number of cells be detected.

Cytotoxic activity could not be detected when cell lines other than liver cells (human fibroblasts, human conjunctiva) were utilized

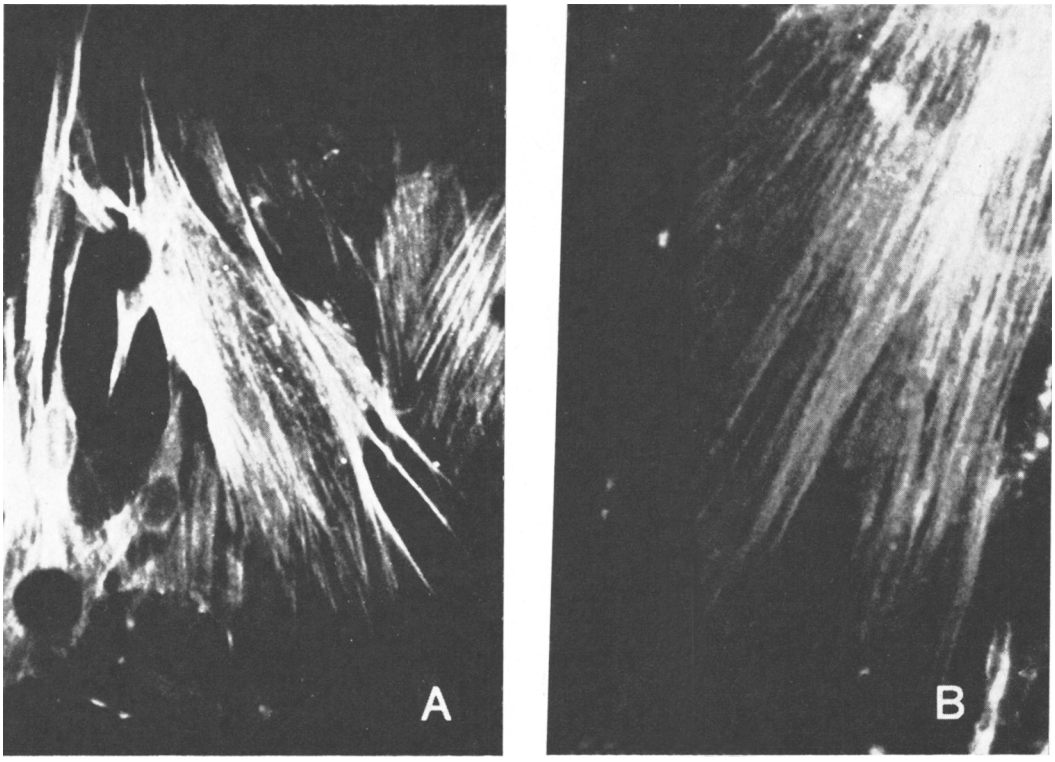

FIG. 2. Same monolayer as in Fig. 1, treated first with 1:10 dilution of a serum with smooth muscle antibodies followed by fluorescein-labeled human IgG antiserum. (A) Liver cells are stained in a filamentous pattern (×250). (B) Higher magnification shows the thin, almost parallel fibrils arranged along the major axis of the cell (×560).

TABLE I. Cytotoxicity of Sera from Patients with Primary Biliary Cirrhosis and Chronic Active Hepatitis Against Human Liver Cells Grown in Tissue Culture.

Diagnosis	No. of sera	Sera with antibodies to: Nuclei	Smooth muscle	Mitochondria	HB Ag	Sera with HB Ag	Cytotoxicity[a]
Primary biliary cirrhosis	7	1	0	7	0	0	0
Chronic active hepatitis	13	7	13	3	0	3	0
Acute viral hepatitis	3	0	0	0	0	3	0
Hemophilia	2	0	0	0	2	0	0
Controls	12	0	0	0	0	0	0

[a] Target cells: human liver cell L38, originated from a patient with chronic active hepatitis. Method: microassay of Takasugi and Klein (6).

as targets in the cytotoxic test. Similar results were obtained when the cytotoxic method of ^{51}Cr release was utilized using fibroblasts and Chang liver cells as labeled target cells. Antiserum to Chang liver cells was, however, cytotoxic for Chang liver cells (Table II).

Discussion. Circulating antibodies to smooth muscle, mitochondria, and nuclei, which are frequently seen in sera of patients with chronic active hepatitis and primary biliary cirrhosis and are considered characteristic of these diseases, do not exhibit a direct cytotoxic activity against autologous or heterologous liver cells or other human cell lines (Chang liver cells, fibroblasts, and epithelial cell lines).

Although lacking cytotoxic activity, the sera still react with the target cells. The weak, diffuse reactivity of mitochondrial antibodies with liver cells is in keeping with the weak reactivity of liver mitochondria, in contrast to mitochondria of distal convoluted tubules of kidney, parietal cells of stomach, heart muscle, and parotid ducts (9). The filamentous binding of smooth muscle antisera to cells has previously been described by Farrow, Holborow, and Brighton (10), using chick embryo livers and human fetal lung cells, and was considered to represent binding to a component of cells related to smooth muscle actomyosin.

The nature of the cells derived from liver biopsy specimens has not been clarified in the present investigation. As described by Demoise, Galambos and Falek (4) and Le Guilly *et al.* (11), and confirmed by our observations, cells derived from liver biopsies are pleomorphic—some resemble epithelial cells; some are cells with granular cytoplasm; and others resemble macrophages and fibroblasts. Recent electron microscopic

TABLE II. Cytotoxicity of Sera from Patients with Primary Biliary Cirrhosis and Chronic Active Hepatitis.

	No. of sera	Sera with: Mitochondrial antibodies	Smooth muscle antibodies	Nuclear antibodies	Cytotoxicity[a] % release of isotope (mean ± SD)
Primary biliary cirrhosis	5	5	0	2	9.5 ± 3.7
Chronic active hepatitis	10	3	6	4	9.5 ± 1.9
Anti-Chang liver cells antiserum	4	0	0	0	62.7 ± 11.6
Normal controls	10	0	0	0	9.4 ± 1.3

[a] In presence of complement. Target cells: Chang liver cells; method: ^{51}Cr release from target cells [Holm and Perlmann (5)].

investigations indicate that these cells resemble hepatocytes or dedifferentiated hepatocytes (12), and are similar to hepatocytes in being rich in glycogen (11).

The possibility that the antibodies are cytotoxic by the formation of harmful immune complexes which may be deposited in the liver was not investigated in the present study. Circumstantial evidence suggests that these immune complexes, if formed, are not deposited in the liver since: (a) extracellular deposition of immunoglobulin and complement has not so far been described in the liver (13) except for the early stages of primary biliary cirrhosis (1). However, in some cases of Australia antigen positive chronic active hepatitis, immunoglobulin and occasionally trace amounts of complement have been reported (14); and (b) extracts of liver of patients with chronic active hepatitis and primary biliary cirrhosis are not anticomplementary (Vernace and Paronetto, unpublished data). Also not clarified is the possibility that humoral antibodies are protective against liver damage or might operate in synergy with a cell-mediated mechanism in inducing cell damage. Future studies on the cytotoxic effect of lymphocytes and on the protective or enhancing effect of antibodies might be rewarding from this point of view.

Summary. Sera from patients with chronic active hepatitis and primary biliary cirrhosis containing a variety of antibodies (smooth muscle, mitochondria, nuclei) as well as hepatitis B antigen failed to show a cytotoxic activity against autologous liver cells, heterologous liver cells, Chang liver cells or other cell lines of nonhepatic sources. Cytotoxicity was measured by the microassay technique using morphologic evaluation and counting of surviving cells, and by ^{51}Cr release from labeled cells.

Lack of cytotoxicity was observed in spite of reactivity of antibodies with cultured liver cells. Mitochondrial antibodies stained the cells diffusely; smooth muscle antibodies bound to the cells in a filamentous pattern.

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