T Cell-Mediated Cytotoxicity against HBsAg-Coated Chang Cells in Patients with Chronic Hepatitis: Evidence for Cytotoxicity Mediated by Delayed Hypersensitivity T Cell Reaction

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NONOMURA, A., OHMORI, K., OHTA, G., KATO, Y., KOBAYASHI, K., NISHIMURA, I. and SUGIOKA, G. T Cell-Mediated Cytotoxicity against HBsAg-Coated Chang Cells in Patients with Chronic Hepatitis: Evidence for Cytotoxicity Mediated by Delayed Hypersensitivity T Cell Reaction. Tohoku J. exp. Med., 1982, 138 (2), 139-150 — T lymphocytes from 7 (21%) of 34 patients with chronic hepatitis showed positive cytotoxicity against HBsAg-coated Chang cells. This positivity was observed in HBsAg-negative patients having positive blast transformation responses to HBsAg, as well as in patients convalescing and recovered from acute B hepatitis. Levels of S-GPT in these patients were not different from those showing no cytotoxicity. T cell-mediated cytotoxicity against HBsAg-coated hepatocytes in HBsAg-negative patients thus may have no significant pathogenetic role in destruction of hepatocytes and may represent anamnestic response of sensitized T lymphocytes to HBsAg. Positive cytotoxicity against HBsAg-coated Chang cells was also found in 3 of 17 patients with HBsAg-positive chronic hepatitis. All positive cases exhibited blast transformation responses to HBsAg and low HBsAg titers in their sera. Levels of S-GPT in these patients were significantly higher than in those showing no cytotoxicity, suggesting possible presence of T cell-mediated liver cell damage in these patients. T lymphocytes from asymptomatic HBsAg carrier showed no cytotoxicity to HBsAg-coated hepatocytes and no blast transformation responses of lymphocytes to HBsAg. From the results of the parallel occurrence of T cell-mediated cytotoxicity and blast transformation responses to HBsAg, and of presence of lymphotoxin in the supernatant co-cultured HBsAg and cytotoxicity-positive lymphocytes, it seemed likely that T cell-mediated cytotoxicity in our system might be mediated by lymphokine produced by T cells as a result of delayed hypersensitivity reaction in vitro. —— HBs-coated Chang cells; cytotoxic T cells; delayed hypersensitivity T cells; lymphocyte stimulation by HBsAg; lymphotoxin

Cellular immune responses specific to hepatitis B virus (HBV) or to antigens induced by it have been implicated in the pathogenesis of liver cell destruction in

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hepatitis B. Evidence of generation and participation of such cytotoxic effector mechanisms have, however, not been fully understood, because of inability to maintain HBV in cultured hepatocytes. In a human primary liver cancer cell line (PLC-PRF/5) producing HBsAg, this viral antigen is detected only in the supernatant medium but not in the cytoplasm or the cell membrane of cultured cells (Macnab et al. 1976). HBsAg-coated rabbit or chicken erythrocytes have been used as target cells for a lymphocyte cytotoxicity test that provides support to the hypothesis mentioned above (Alberti et al. 1977; Elsheikh et al. 1978).

Electron microscopy has revealed lymphoid cells in close contact with HBsAg-containing hepatocytes, the latter undergoing variable degrees of degeneration, the significance of this is, however, a matter of speculation (Karasawa and Shikata 1977).

A technique using HBsAg-coated hepatocytes as target cells was recently introduced by Warnatz et al. (1979), who tested both antibody-dependent cell-mediated cytotoxicity and spontaneous cell-mediated cytotoxicity to HBsAg-coated hepatocytes in patients with acute and chronic active hepatitis B.

The present experiments were designed to study T cell-mediated cytolysis against HBs-coated Chang cells in patients with HBsAg-positive, HBsAg-negative, HBsAb-positive and HBsAb-negative chronic hepatitis to obtain further knowledge concerning T cell-mediated cytotoxicity against HBsAg.

**MATERIALS AND METHODS**

**Patients.** Studies were performed on 19 patients with chronic active hepatitis (CAH) (13 HBsAg-positive, 2 HBsAb-positive, 4 both negative), 15 with chronic persistent hepatitis (CPH) (4 HBsAg-positive, 3 HBsAb-positive, 8 both negative), 1 with acute hepatitis B, 2 with recovered acute hepatitis B, 5 asymptomatic HBsAg carriers, and 20 healthy subjects with normal liver function tests, no history of liver disease and seronegative for HBsAg and HBsAb. Two patients who had recovered from acute hepatitis B were medical doctors; one suffered from acute hepatitis B 6 years earlier but currently was negative for both HBsAb and HBsAg, and the other HBsAb seropositive suffered from acute hepatitis B 3.5 years earlier. A diagnosis was made by histological examination of needle liver biopsies, liver function tests, and the clinical course. Classification of chronic hepatitis was based upon Forgaty's histological criteria (Chalmers et al. 1974). Serum HBsAg was detected by a reversed passive hemagglutination (R-PHA) and serum HBsAb by passive hemagglutination. None had received corticosteroid or other immunosuppressive drugs.

**Target cells.** Chang cells coated with HBsAg were used as target cells. Coating was performed by slight modification of the method of Warnatz et al. (1979) using bisdiazotized benzidine reagent (BDB) prepared according to the technique of Campbell et al. (1970). Purified HBsAg (mixture of the subtypes adr and adw, at a concentration of 2,200 µg/ml) was kindly provided by Dr. H. Yoshizawa and Dr. M. Mayumi, Hepatitis Division, the Tokyo Metropolitan Institute of Medical Science. Chang cells (ATCC CL12, Flow Laboratories, USA) were cultured with Eagle’s Minimum Essential Medium (MEM) (Grand Island Biological Company, USA) supplemented with 10% heat-inactivated fetal calf serum (Microbiological Associates, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (MEM-FCS). The medium from the culture dish of 60–80% confluence of Chang cells was decanted, washed with MEM and added with fresh MEM-FCS containing 50 µCi/ml of 3H-proline (New England Nuclear, USA, sp. act. 20 Ci/mmol), then incubated overnight (18 hr) at 37°C. At the end of the labeling period, the medium containing radioisotope
was decanted and the cells were washed three times with MEM. Then the cells were
trypsinized and counted. 10⁶ Chang cells in 0.1 ml MEM were incubated with 0.2 ml
HBsAg preparation (50 μg/ml), 1.74 ml of 0.11 M phosphate buffer pH 7.4 and 0.7 ml of the
1:15 diluted BDB, for 30 min at room temperature. After washing, viable cells were
counted and suspended in MEM-FCS at a concentration of 5×10⁶/ml. Control cells were
prepared by coating Chang cells with human serum albumin (HSA) in the same manner.
Usually more than 94% of Chang cells coupled with either HBsAg or HSA were viable.
0.2 ml (1×10⁶ cells) of Chang cells coupled with either HBsAg or HSA were placed into
the wells (Falcon, Micro Test II Tissue Culture Plate, #3042) and incubated overnight at
37°C in a humidified incubator. In this period, the target cells adhered to the bottom
of the wells. The actual coating of each preparation of the target cells with each of them
was tested by indirect immunofluorescence. The concentration of HBsAg in the medium
after coupling was found to be reduced to 12.5% or less of the original HBsAg concentra-
tion.

Lymphocyte preparation. Macrophage-monocyte-depleted lymphocytes (less than 3%)
were obtained by Ficoll-Hipaque (F-H) gradient centrifugation after incubation of
venous blood with silica (1 hr at 37°C, 10% KAC-II suspension, Japan Immunoresearch
Laboratories Co., Ltd). (Böyum 1968). Lymphocytes forming rosettes with sheep
erthrocytes previously treated with 2-S-aminoethylisothiouronium bromide (AET) as
described by Kaplan et al. (1976) were separated by the method of Yata et al. (1973) using
F-H gradient centrifugation, followed by removal of lymphocytes from the bottom of the
tubes and then by lysis of erythrocytes with a 0.83% NH₄Cl in 20 mM Tris buffer (pH 7.4)
for 5 min. The cells were resuspended in MEM-FCS at a concentration of 1×10⁵ cells/ml
after washing with Hank's balanced salt solution (HBSS, pH 7.4). T cell fractions thus
obtained showed to contain 92–96% of T cells and 3–7% of EAC-rosette forming cells.

Cytotoxicity test. The target cells were incubated with T cells in a ratio of 1:100
and incubated for 48 hr at 37°C in a humidified atmosphere of 95% air, 5% CO₂. The
final volume of medium in each well was 0.2 ml. At the end of incubation period the
microtest plates were washed three times with HBSS containing 5% FCS. They were then
inverted and allowed to dry. After the microplates were thoroughly dry, the well bottoms
were cut off, placed into vials together with 5 ml of scintillation fluid (toluene base with
0.5% PPO and 0.2 POPOP) and kept for 24 hr at 4°C. Radioactivity measurement with a
Beckman liquid scintillation counter was performed in quadruplicate and cytotoxic activity
was expressed in the following manner:

\[
\text{% cytotoxicity} = 100 \times \left(1 - \frac{\text{Mean }[^3\text{H}]\text{thymidine incorporation in the presence of effector lymphocytes}}{\text{Mean }[^3\text{H}]\text{thymidine incorporation in the absence of effector lymphocytes}}\right)
\]

Blocking experiments. The medium of the wells containing target cells was first replaced
by medium containing rabbit anti-HBs antibody (PHA titer 2560, kindly provided by Dr.
H. Yoshizawa and Dr. M. Mayumi) and incubated for 30 min at 37°C. After washing,
effector T lymphocytes were added and subsequent procedures were carried out as stated
before.

Lymphocyte stimulation by HBsAg. Whole lymphocyte fraction, obtained from
heparinized venous blood by F-H gradient centrifugation, was adjusted to 1×10⁶ cells/ml
in RPMI 1640 medium (Nissui Seiyaku Co., Ltd., Tokyo) supplemented with 10% fetal
calf serum, 100 units/ml of penicillin and 100 μg/ml of streptomycin (RPMI-FCS). The
lymphocytes of 0.5 ml aliquots were stimulated with 5 μg/ml of purified HBsAg, a
concentration of which was confirmed to result in optimal stimulation of the peripheral
lymphocytes. Control tubes received 0.5 ml of lymphocyte suspension only. All
experiments were performed in triplicate and incubated in a 5% CO₂, 95% humid
atmosphere at 37°C. After four days, 1 μCi of ^3H-thymidine (sp. act. 20 Ci/mmole, The
Radiochemical Centre, Amersham, England) was added to the wells, and incubation was
continued for an additional 24 hr before harvest. The amount of ^3H-thymidine in-
corporated into DNA by each triplicate sample was determined by a Beckman liquid
scintillation counter. The lymphocyte stimulation index (SI) was determined by
dividing cpm of HBsAg-stimulated cultures by cpm in the unstimulated cultures.

**Lymphotoxin assay.** Whole lymphocyte fraction was suspended in RPMI-FCS at a concentration of 1×10⁶ cells/ml. The lymphocytes were divided into two groups of 2 ml aliquots and incubated with HBsAg (5 µg/ml) (HBsAg-stimulated culture) or without HBsAg (HBsAg-unstimulated culture) for 5 days at 37°C. The supernatant of each culture (HBsAg stimulated and HBsAg-unstimulated supernatants, respectively) was collected after centrifugation at 500 × g for 5 min. To the HBsAg-unstimulated culture, HBsAg was added to yield a concentration of 5 µg/ml at the time of harvest.

Quantitative assay of lymphotoxin was performed using mouse L cells (L-929, cultured in RPMI-FCS) as target. L cells were previously labeled with 50 µCi/ml of ³H-proline and dispensed into wells of microtest plates (Falcon, # 3034) in 200 µl volumes (1×10³ cells) as described above. After overnight incubation L cells were X-irradiated with 2000 R. The culture media were replaced with 0.2 ml of the test supernatants and incubated for 48 hr at 37°C in a humid atmosphere of 95% air, 5% CO₂. Then, supernatants were removed and washed three times with RPMI-FCS. All experiments were performed in triplicate. Radioactivity of L cells attached to well bottoms was determined as described above and lymphotoxin activity was expressed as percent cytotoxicity in the following manner:

\[
\text{% cytotoxicity} = 100 \times \left(1 - \frac{\text{Mean cpm of L cells in the presence of HBsAg-stimulated supernatants}}{\text{Mean cpm of L cells in the presence of HBsAg-unstimulated supernatants}}\right)
\]

**RESULTS**

**Cytotoxicity of T lymphocyte fractions**

The results are shown in Table 1. T lymphocytes from healthy subjects (n=20) exhibited a cytotoxicity of 3.47±6.34% (mean±s.d.) to HBsAg-coated Chang cells and 1.58±5.10% to albumin-coated hepatocytes. The upper limit of normal range was taken as 2s.d. above the mean in controls and each test (patient) result with a value higher than this was considered to be positive. Out of 34

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Number</th>
<th>Mean % cytotoxicity against:</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>HBsAg-coated Chang cells</td>
<td>Albumin-coated Chang cells</td>
</tr>
<tr>
<td>Patients with CAH total</td>
<td>19</td>
<td>6.04±11.57 (4)</td>
<td>4.85±4.40 (1)</td>
</tr>
<tr>
<td>HBsAg positive</td>
<td>13</td>
<td>6.88±10.80 (2)</td>
<td>4.39±4.94 (1)</td>
</tr>
<tr>
<td>HBsAb positive</td>
<td>2</td>
<td>20.67±0.69* (2)</td>
<td>7.95±1.84 (0)</td>
</tr>
<tr>
<td>Both HBsAg and HBsAb negative</td>
<td>4</td>
<td>-3.17±6.83 (0)</td>
<td>4.78±2.32 (0)</td>
</tr>
<tr>
<td>Patients with CPH total</td>
<td>15</td>
<td>8.30±19.96 (3)</td>
<td>3.85±5.15 (0)</td>
</tr>
<tr>
<td>HBsAg positive</td>
<td>4</td>
<td>5.32±14.13 (1)</td>
<td>5.80±3.00 (0)</td>
</tr>
<tr>
<td>HBsAb positive</td>
<td>3</td>
<td>16.34±10.95* (2)</td>
<td>2.03±7.15 (0)</td>
</tr>
<tr>
<td>Both HBsAg and HBsAb negative</td>
<td>8</td>
<td>6.77±7.04 (4)</td>
<td>3.56±4.76 (0)</td>
</tr>
<tr>
<td>Asymptomatic HBsAg carrier</td>
<td>5</td>
<td>5.81±3.39 (0)</td>
<td>0.26±3.68 (0)</td>
</tr>
<tr>
<td>Normal controls</td>
<td>20</td>
<td>3.47±6.34 (0)</td>
<td>1.58±5.10 (0)</td>
</tr>
</tbody>
</table>

CAH=chronic active hepatitis, CPH=chronic persistent hepatitis.
* Values significantly different from the control values (p<0.01).
† Values significantly different from the control values (p<0.05).
Parentheses indicate the number of patients showing the % cytotoxicity higher than mean±2s.d. of the controls.
patients with chronic hepatitis, 7 (21%) showed positive cytotoxicity against HBs-coated Chang cells, whereas in only 1 (3%) positivity was seen against albumin-coated Chang cells. The difference was statistically significant ($\chi^2=5.1$, $p<0.05$). Among the 7 patients showing positive cytotoxicity 2 were HBsAg-positive CAH (of 13), 2 HBsAb-positive CAH (of 2), 1 HBsAg positive CPH (of 4) and 2 HBsAb-positive CPH (of 3). The incidence of the positive cytotoxicity in HBsAb-positive chronic hepatitis (4/5) was significantly higher than that in HBsAg-positive chronic hepatitis (3/17) ($\chi^2=10.7$, $p<0.01$). In chronic hepatitis, mean % cytotoxicities of both HBsAb-positive CAH and HBsAb-positive CPH were significantly higher than those in normal controls ($p<0.001$ and $p<0.05$, respectively) (Table 1). No patients with asymptomatic HBsAg carrier showed positive cytotoxicity.

Cytotoxic activity after treatment of target cells with anti-HBs

The results are shown in Fig. 1. In all of 6 patients with chronic hepatitis (2 CPH, 4 CAH) who showed positive cytotoxicity against HBsAg-coated Chang cells, cytotoxic activity decreased sharply to normal or near normal values after treatment of target cells with anti-HBs antibody, indicating that cytotoxic reaction in this in vitro model may be directed against HBsAg.

T lymphocyte cytotoxicity in relation to serum HBsAg titer

As shown in Fig. 2, 3 patients with HBsAg-positive chronic hepatitis who had positive cytotoxicity showed R-PHA titer less than $2^{11}$, while 10 of 14 patients with negative cytotoxicity had R-PHA titer more than $2^{12}$.

T lymphocyte cytotoxicity in relation to serum transaminase levels

In HBsAg-positive cases, S-GPT levels of cytotoxicity-positive patients were

![Fig. 1. T cell-mediated cytotoxicity against HBsAg-coated Chang cells before and after treatment of target cells with anti-HBs antibody. CPH, chronic persistent hepatitis; CAH, chronic active hepatitis. Shaded area indicates mean±2s.d. of the control cytotoxicity against untreated target cells.](image-url)
significantly higher than those of cytotoxicity-negative patients (Table 2A, \( p < 0.001 \)), while in patients with HBsAg-negative chronic hepatitis, S-GPT levels of the two groups were not different (Table 2B, \( 0.7 > p > 0.6 \)).

**Relationship between T lymphocyte cytotoxicity and lymphocyte stimulation by HBsAg**

The T lymphocyte cytotoxicity test against HBsAg-coated Chang cells and the
lymphocyte stimulation test by HBsAg were performed simultaneously in 32 subjects (CAH 8 cases, CPH 10 cases, recovered acute hepatitis B 2 cases, convalescence stage of acute hepatitis B one case, and healthy subjects 11 cases). Control lymphocyte (n=18), stimulated by HBsAg, revealed SI of 0.71 to 1.63 with a mean of 1.083±0.242. The upper limit of normal range was taken as 2s.D. above the mean and each patient who showed the index higher than this value was considered to be positive. Both positive lymphocyte stimulation by HBsAg and positive lymphocyte cytotoxicity against HBsAg-coated Chang cells, as well as both negative ones, were parallel in 30 of 32 cases (Fig. 3). As shown in Fig. 4, both lymphocyte stimulation and cytotoxicity in a patient with acute hepatitis B were
not detected at the time when S-GPT levels returned to almost normal, but serum bilirubin levels were still high and serum HBsAg was present. One month later bilirubin levels returned to normal, HBsAg in the serum was negative, and both lymphocyte tests became positive. In two cases (medical doctors) completely recovered from acute hepatitis B, both tests were found to be positive, whereas lymphocytes from asymptomatic HBsAg carriers showed no blast transformation.
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responses to HBsAg nor cytotoxicity to HBsAg-coated hepatocytes (Fig. 5).

Lymphotoxin assay and lymphocyte stimulation by HBsAg

The lymphotoxin assay and lymphocyte stimulation by HBsAg were simultaneously performed in 10 patients with CAH, 2 recovered acute hepatitis B, and 4 healthy subjects. As shown in Fig. 6, lymphotoxin activity was observed in patients with positive lymphocyte stimulation by HBsAg (3.16±7.83 vs. 26.95±4.84, p<0.001).

DISCUSSION

In the absence of a suitable in vitro culture model of HBV-infected hepatocytes the major pathogenetic events responsible for hepatocellular injury initiated by HBV infection have not yet been identified. Available information suggests that HBV itself has no cytopathic effect on hepatocytes and that host immune responses to HBV are responsible for the pathogenesis of hepatocyte destruction. The most likely candidate for a HBV-derived target in the immunopathologic reactions is HBsAg or related antigens expressed on hepatocyte surface membranes. We used HBsAg-coated Chang cells as models of in vivo HBsAg-expressed hepatocytes and T lymphocyte cytotoxicity against these cells was studied. Positive cytotoxicity was observed in 3 of 17 patients with HBsAg-positive chronic hepatitis, less than what we had expected. Positive cytotoxic reaction throughout the experiments was correlated well with positive lymphocyte stimulation by HBsAg and was blocked by anti-HBs antibody, suggesting that cytotoxic reaction in this in vitro model is mediated by T lymphocytes specifically sensitized to HBsAg.

There are at least two distinct pathways by which immune T lymphocytes can destroy target cells; one involves secretion of a nonspecific soluble factor, i.e., lymphotoxin, and the other requires intimate contact between the plasma membranes of the target and killer cells (Gately et al. 1976). These cytotoxic reactions have been considered to be mediated by two distinct T cell subsets (Henny 1977; Vadas et al. 1976); one mediated by cytotoxic T lymphocytes, and the other mediated by delayed hypersensitivity T lymphocytes. It is known, in a study of mouse, that cytotoxic T cell-mediated cytotoxicity to virus-infected cells is of H-2-restricted type (Zinkernagel and Doherty 1974). The same situation has been reported in human beings (McMichael and Ting 1977). Our present study suggests that the HLA system does not appear to be involved, since the target cell killing was found in as many as 20% of patients with chronic hepatitis. The fact that cytotoxic reactions were clearly correlated with lymphocyte stimulation by HBsAg and to lymphotoxin activity in the supernatants of lymphocytes co-cultured with HBsAg indicates that the cytotoxicity in our system is probably mediated by lymphokine produced by delayed hypersensitivity T cells.

Several recent investigations reported that cell-mediated reactions to HBsAg were observed in about half of the patients with chronic hepatitis, using a lymphocyte stimulation test, macrophage migration inhibition test and leukocyte migration
inhibition test (Dudley et al. 1972b; Irwin et al. 1974; Lee et al. 1975; Tong et al. 1975; Ohta et al., 1976 Frei et al. 1978). These suggest that the cell-mediated immune response to HBsAg may be responsible for elimination of this antigen from the liver. However, cell-mediated immune response to HBsAg neither simply indicates nor is correlated with the presence of hepatic injury, as evidenced by positive cytotoxicity and blastoid transformation found in our patients who recovered from acute hepatitis B and in those with HBsAb-positive, HBsAg-negative chronic hepatitis. These may probably reflect anamnestic T cell response to HBsAg. In HBsAg-positive cases, however, S-GPT levels of patients with positive cytotoxicity were significantly higher than those in cytotoxicity negative cases and serum concentrations of HBsAg were significantly lower in the cytotoxicity-positive cases. Thus, it appears that, in cases of both positive cytotoxicity and HBs-antigenemia, T cell immune responses to HBsAg may cause destruction of infected hepatocytes, reflected by high S-GPT levels in comparison to those in cases of negative cytotoxicity. The presence of low serum HBsAg levels in the former may be due to inefficient clearance of HBV from the livers, as suggested by Dudley et al. (1972a). The fact that the number of patients with HBsAg-positive chronic hepatitis, applicable to the situation of T cell destruction of liver cells, is rather small suggests that active T cell-mediated cytotoxicity is not detected in the blood from many patients because they may be sequestered to the livers or blocked by some serum factors (Elsheikh et al. 1978). Other mechanisms of liver cell destruction may be more important in the pathogenesis of CAH, such as cellular immune reaction to human liver-specific membrane antigens (Meyer zum Büschenfelde et al. 1974; Vogten et al. 1978), antibody-dependent cell-mediated cytotoxicity (Eddleston and Williams 1974) or natural killing (Dienstag and Bhan 1980).

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