IMMUNOLOGICAL RESPONSE TO INFECTION

Frequency analysis of proliferating and cytotoxic T cells in livers and peripheral blood of patients with chronic hepatitis B

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Summary. Frequencies of proliferating and cytotoxic lymphocytes from liver biopsy samples and peripheral blood of chronic hepatitis B (CHB) patients and control subjects were monitored by limiting dilution analysis. Precursor frequencies of proliferating T lymphocytes were not significantly different in the liver and peripheral blood compartments of patients and controls. Moreover, similar frequencies of natural killer cells and cytotoxic T lymphocytes were observed in the peripheral blood of patients and controls. A higher frequency of cytotoxic T cells (1 of 22) compared to NK cells (1 of 306) was observed in liver tissues of CHB patients. Dual colour flow cytometric analysis revealed the presence of both CD4⁺ HLA-DR⁺ and CD8⁺ HLA-DR⁺ T cells in the liver tissues. These results suggest that in livers of CHB patients not only activated CD8⁺ T cells but also activated CD4⁺ T cells may play a significant role in the pathogenesis of chronic hepatitis B.

Introduction

Hepatitis B virus (HBV) causes acute and chronic liver disease of variable severity by mechanisms that are thought to be largely immune-mediated. Abundant inflammatory cells, mainly T lymphocytes, are always present in close contact with damaged hepatocytes in liver biopsy samples of patients with active liver disease.^{1–4} The antigen specificity and function of these lymphocytes are still not clearly defined. Several investigators have demonstrated that HBV antigens, both core (HBcAg)^{5–7} and surface (HBsAg),^{8–10} may be the target antigens for immune attack. To gain a better insight into the functional repertoire of lymphocytes, in the present study, a novel approach was used to estimate the frequencies of occurrence of proliferating and cytotoxic lymphocytes, in both the peripheral blood compartment and liver tissues of patients with chronic hepatitis B (CHB) by the limiting dilution microculture technique.¹¹ To assess further the precise immune status of the patients, dual colour flow cytometry was used to analyse the phenotypes of lymphocytes in the livers that may contribute to hepatocellular injury.

Patients and methods

Patients

Studies were undertaken on 15 patients with chronic liver disease (age range 18–40 years; 13 male and two female). Informed consent was obtained from all the patients and the study was approved by the local ethical committee. Patients with liver disease were diagnosed by appropriate clinical, biochemical and histological criteria.¹² Serum markers for viral infection were determined by commercially available enzyme-linked immunosorbent assay kits (Organon Teknika, Turnhout, Belgium). All patients were sero-positive for HBsAg but negative for antibodies to hepatitis C virus and hepatitis delta virus. Twelve healthy sex- and age-matched individuals who were negative for HBsAg served as controls. For comparison, specimens from three normal livers were obtained from men who underwent biopsy for suspicion of liver disease but who were subsequently found to have histologically normal livers. These individuals were serologically negative for HBsAg.

Isolation of liver infiltrating lymphocytes (LIL) and peripheral blood lymphocytes

Liver tissues were obtained either by laparoscopy or percutaneous liver biopsy. Each specimen was pro-

cessed separately for histopathological examination and separation of lymphocytes. LIL were separated by a method described by Shimizu *et al.*¹³ with minor modifications. Briefly, liver tissues were washed thoroughly with RPMI 1640 medium to remove the peripheral blood adhering to the tissues. Subsequently, liver tissues were finely minced in sterile complete RPMI 1640 medium containing heat inactivated human AB serum 10% with a mixture of antibiotics and stirred gently with collagenase (Type 4, Sigma) 0.05% and DNAase (Type 1, Sigma) 0.002% for 1–2 h at 37°C. Lymphocytes from enzymically digested liver tissue suspensions and from peripheral blood were separated by Ficoll-Hypaque gradient centrifugation. The total number of mononuclear cells that could be isolated from the control liver biopsy samples ranged from 8×10^3 to 2×10^4 and that from liver samples of CHB patients ranged from 2×10^4 to 4×10^5 cells. T cells were enriched from peripheral blood (PBL-T) with nylon wool columns.¹⁴

Microcultures of LIL and PBL-T

A limiting dilution microculture system described by Moretta *et al.*¹¹ was used with minor modifications. Lymphocytes were seeded in 96-well round-bottomed microtitration plates (Nunc, Denmark) at 0.75, 1.5, 3, 6, 12, 25, 50 or 100 cells/well as 24 replicates of each dilution with irradiated (4000 R) allogeneic pooled PBL (2×10^4) as feeder cells in a final volume of 200 μ l of complete RPMI 1640. At the onset of culture, PHA-M (Gibco) 1% v/v was added. After 48 h, recombinant human interleukin 2 (rIL-2, donated by Hoffman-La Roche, Switzerland) 50 U/ml was added. Microcultures were supplemented weekly with irradiated feeder cells and rIL-2. A set of cultures containing irradiated feeder cells, PHA and rIL-2 served as controls. Plates were incubated at 37°C in a humidified atmosphere of CO₂ 5% in air.

The cultures were scored microscopically for growth at regular intervals. Microcultures established from peripheral blood were analysed for proliferation and cytotoxicity after 18 days whereas those from liver were assessed after 24 days. The time points for the assays were decided after confluence of growth was observed in cultures containing the highest cell number plated.

Assays for proliferation and cytotoxicity

For assessing proliferation, the cultures were pulsed with tritiated thymidine (³H-TdR, Board of Radiation and Isotope Technology, India, specific activity 6–9 mCi/mmol) 0.5 μ Ci/well. The incorporated radioactivity was measured after 18 h by liquid scintillation counting. Cultures in which ³H-TdR uptake exceeded by > 3 SD the mean uptake detected in control cultures (irradiated feeder cells cultured with PHA and rIL-2) were defined as positive. Mean cpm and SD in control cultures ranged from 1157 SD 218 to 2252 SD 691.

Cytotoxic activities of individual microcultures were tested in a 4-h ⁵¹chromium (⁵¹Cr) release assay. Each microculture was split into two 100- μ l aliquots and tested for cytotoxicity against ⁵¹Cr (Board of Radiation and Isotope Technology, India, specific activity 50–150 mCi/mg) labelled targets (5×10^3 cells/well). To assess cytotoxic T lymphocyte (CTL) activity, a lectin-dependent cellular cytotoxicity (LDCC) assay was performed with concanavalin A (20 μ g/ml) and Raji cells (B lymphoblastoid cell line).¹⁵ Natural killer (NK) cell activity was determined with NK sensitive K562 cells (erythroleukaemic cell line) as targets. Percentage specific lysis was calculated by following formula.

Specific lysis (%)

$$= \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}}$$

Cultures in which the percentage specific release exceeded by > 3 SD the mean percentage specific release detected in control cultures (irradiated feeder cells cultured with PHA and rIL-2) were defined as positive. Mean percentage specific release and SD in control cultures ranged from 0.014 SD 1.7 to 0.45 SD 1.8.

Minimal estimates of the frequencies of proliferating and CTL precursors (PTL-p and CTL-p, respectively) were obtained by the minimum χ^2 method from the Poisson's distribution relationship between the responding cell number and the logarithm of the percentage of the non-responding cultures as described by Taswell.¹⁶ The statistical significance was analysed by Student's *t* test.

Phenotypic analysis of microcultures

Microcultures set up for estimating the precursor frequencies of proliferating and cytotoxic cells were also analysed for phenotypes of lymphocytes by single and dual colour flow cytometric analysis. Cells (5×10^5) were suspended in 100 μ l of phosphate-buffered saline (0.01 M, pH 7.5) containing fetal calf serum (Gibco) 1% and incubated with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-labelled monoclonal antibodies (MAbs) against CD3, CD4, CD8, CD56 and HLA-DR markers (Dako A/S, Denmark) for 30 min at 4°C. Single and dual colour flow cytometric analysis was performed on an EPICS 753 (Coulter Electronics, FL, USA) flow cytometer. Cells stained with mouse isotypes (all subclasses) conjugated to FITC and PE served as negative controls.

Results

Precursor frequencies of proliferating and cytotoxic T cells

The frequencies of proliferating and cytotoxic lymphocytes in the liver and peripheral blood were

Table. Frequencies of proliferating lymphocytes in peripheral blood and liver of controls and patients

Subject group	Mean PTL-p frequency (range) in	
	peripheral blood	liver
Controls	1 in 8 (1 in 14–1 in 6) n = 9	1 in 206 (1 in 338–1 in 155) n = 3
Patients	1 in 11 (1 in 37–1 in 4) n = 12	1 in 134 (1 in 405–1 in 64) n = 7

The differences in PTL-p frequencies between controls and patients were not statistically significant.

determined by limiting dilution analysis. As shown in the table, the precursor frequencies of proliferating T cells were not significantly different in the liver and peripheral blood compartments of controls and CHB patients. The proliferative capabilities of lymphocytes in the liver were always found to be lower (1 in 134, 1 in 206) than those observed in the peripheral blood (1 in 11, 1 in 8) of both patients and controls (table).

To study the cytotoxic potential of peripheral blood and liver infiltrating lymphocytes, two different assays (LDCC and NK cytotoxicity) were performed. In the absence of relevant target cells, lectin-mediated cytotoxicity can be employed to assess the cytotoxic potential of MHC-restricted CTL.¹⁵ The frequency analysis of cytotoxic cells in the peripheral blood compartment of controls and patients revealed no significant difference between CTL and NK cells (fig. 1a). In the liver tissues of controls and CHB patients, although frequencies of NK cells were similar, significant differences were observed in the frequencies of CTL (fig. 1b). A marked increase in the frequency of CTL (1 in 22) was observed in the livers of CHB patients as compared to controls (1 in 186). Com-

parison of cytotoxic cells in the liver tissues of CHB patients revealed significantly higher frequency of CTL (1 in 22) than NK cells (1 in 306).

Phenotypic analysis of cultured LIL and PBL-T

As higher frequencies of CTL were observed in the liver biopsy samples of CHB patients (fig. 1b), these microcultures were phenotyped to analyse the percentages of activated T cells present. Immunophenotypic analysis revealed that lymphocytes cultured from livers of CHB patients showed dominant populations of CD3⁺ T cells (78–82%), whereas lymphocytes with CD56⁺ phenotype were present in a minor population (1–5%, data not shown). Dual colour flow cytometric analysis of the microcultures was performed with anti-CD4 MAb or anti-CD8 MAb conjugated to PE and anti-HLA-DR MAb conjugated to FITC. As shown in fig. 2 the total percentages of lymphocytes expressing CD4 and HLA-DR were 45% and 82%, respectively. The percentage of lymphocytes exhibiting dual expression of CD4⁺ HLA-DR⁺ phenotype was 31%. Similarly, the total percentage of CD8⁺ lymphocytes was 22% while the percentage of HLA-DR⁺ lymphocytes was 80%. CD8⁺ HLA-DR⁺ expression was seen on 21% of lymphocytes.

Discussion

The major hallmark of chronic hepatitis B is the histological appearance of inflammatory cells, mainly T cells, in close proximity to damaged hepatocytes.^{2–4} Most investigations so far have focused on immunohistological phenotypic analysis of liver-infiltrating lymphocytes. Although these studies showed variability in the lymphocyte subset patterns, they mainly focused on CD8⁺ T cells as being responsible for

Fig. 1. Frequencies of cytotoxic lymphocytes (CTL-p) in peripheral blood and livers of controls and patients. ▨, NK; ▩, CTL. * $p < 0.01$, statistical significance of CTL activity between controls and patients. ** $p < 0.01$, statistical significance between NK and CTL within the group.

Fig. 2. Dual colour flow cytometric analysis of lymphocytes from liver biopsies of CHB patients. MAbs used were PE-conjugated anti-CD4 (a) and anti-CD8 (b), and FITC-conjugated anti-HLA-DR. The percentages of cells showing dual markers were: CD4 and HLA-DR, 31 %; CD8 and HLA-DR, 21 %.

hepatocellular injury. However, the antigen specificity and function of LIL have not been clearly understood. In the present investigations, the pool sizes of proliferating and cytotoxic lymphocyte (NK, CTL) precursors from peripheral blood and liver tissues of patients with CHB, were quantified by a limiting dilution microculture technique. This method allows the extensive clonal proliferation of virtually all T cells.¹¹

Because of the practical difficulties in using autologous hepatocytes as targets in cytotoxicity assays, an alternative assay system (LDCC) was used to judge the intrinsic capacity of T cells to mediate cellular lysis. Lectins are known to mediate efficient effector-target cell interactions in the absence of specific antigen recognition.¹⁵ Our studies demonstrated that, although similar frequencies of CTL and NK were observed in the peripheral blood of controls and CHB patients, higher frequencies of CTL were observed in the liver tissues of patients. In the livers of CHB patients, the frequencies of CTL were significantly higher than NK. The low NK activity observed minimises the possibility that the lymphokine-activated killer cells may be involved in mediating lectin-dependent cytotoxic activity. These results are in agreement with immunohistological data reported by other investigators,²⁻⁴ who suggested a preponderance of CD8⁺ cytotoxic T cells compared to NK cells at the site of liver tissue injury. We believe that the higher frequencies of CTL observed in the liver tissues

of CHB patients could be contributed by both CD4⁺ and CD8⁺ CTL. The role of class II restricted CD4⁺ T cells in viral and bacterial infections has recently been clarified.¹⁷⁻²⁰

Our studies with dual colour flow cytometric analysis of LIL in CHB patients also demonstrated that both CD4⁺ and CD8⁺ lymphocytes were in an activated state (HLA-DR⁺). Recently, Barnaba *et al.*²⁰ reported that in chronic active hepatitis patients, cytotoxic CD4⁺ T cell clones with a Th1 cytokine secretion profile are compartmentalised in the liver and may play a pathogenic role in HBV infection. Several investigators have reported that HBcAg represents the major target antigen for T cell attack in chronic HBV infection.⁵⁻⁷ Van den Oord *et al.*² demonstrated that hepatocytes expressing HBV core antigen also expressed HLA-DR antigens. Their studies drew attention to the possibility that these hepatocytes may represent targets for cytotoxic class II restricted CD4⁺ T cells. Thus the results of the present study suggest that in livers of CHB patients, not only activated CD8⁺ T cells but also activated CD4⁺ T cells may play a significant role in the pathogenesis of hepatocellular injury. It would be interesting to investigate the antigen specificity of the CD4⁺ T cells in these liver biopsy samples further.

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