Clin. exp. Immunol. (1989) **76** 210–215

Clonal analysis of liver-derived T cells of patients with primary biliary cirrhosis

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(Accepted for publication 1 March 1989)

SUMMARY

Lymphocyte infiltration in liver tissue is one important histological finding in primary biliary cirrhosis (PBC). So far, functional analyses of lymphocytes in PBC have focused on circulating lymphocytes, whereas lymphocytes at the involved site, the liver, have not been examined functionally. We have established interleukin 2 (IL-2)-dependent T lymphocyte lines (TLL) and clones (TLC) from liver biopsies of 14 patients with PBC. Phenotypic analysis using the monoclonal antibodies MT910 (CD2), MT811 (CD8), and MT151 (CD4) revealed that in nine of 14 TLL cytotoxic-suppressor T cells predominated ($CD8^+$: 52–84%; $CD4^+$: 14–48%), whereas in five of 14 TLL a preponderance of the $CD4^+$ subpopulation was found ($CD4^+$: 56–73%; $CD8^+$: 28–45%). From 10 patients 137 TLC were generated which phenotypically correlated to the TLLs. We have tested the cytotoxic potential of seven TLL and 43 TLC in LDCC (lectin-dependent cell-mediated cytotoxicity), NK (natural killing) and ADCC (antibody-dependent cell-mediated cytotoxicity) assays. All TLL and all but one $CD8^+$ TLC tested showed high activity in the LDCC assay, reflecting the cytolytic activity of cytotoxic T cells (CTL). $CD4^+$ clones with LDCC activity were rarely found. NK activity and K cell activity could only be found in two clones. For the first time TLC and TLL from liver tissue of PBC patients could be generated. The high cytotoxic activity displayed by T cells derived from the liver indicates an important role for this immunological mechanism in the tissue damaging process.

Keywords primary biliary cirrhosis immunopathology T cell clones

INTRODUCTION

Primary biliary cirrhosis is characterized by a progressing inflammatory process of small bile ducts, which leads to their injury and destruction (Popper & Schaffner, 1970). The etiology and pathogenesis of this process are still enigmatic. As the histologically demonstrable early event of the disease is an infiltration of lymphoid cells in intimate contact with the bile duct epithelial cells, tissue damaging immune processes are believed to play a crucial role (James, Vierling & Strober, 1981; James *et al.*, 1983). Thus attention has focused on the study of lymphocytes with cytotoxic capacity. So far, this analysis has been limited to peripheral blood lymphocytes (Vierling *et al.*, 1977; Geubel *et al.*, 1976; Dienstag & Bhan, 1980). Lymphocytes in liver tissue could only be defined phenotypically (Pape *et al.*, 1983). Many of these studies gave conflicting results. Therefore the analysis of the cytolytic capacity of liver-tissue-derived lymphocytes will be an important contribution to more profound knowledge of the immunological mechanisms leading to a complete destruction of bile ducts.

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MATERIALS AND METHODS

Patients

Fourteen patients (13 female, one male, aged 39–60 years, mean age 52 years) with PBC were studied. The diagnoses were based on the presence of a typical constellation, biochemical, serological and histological findings. Seven patients were in early disease stages (histologic stages I and II according to Popper & Schaffner, 1970) and seven patients fulfilled the histologic criteria of advanced stages of PBC (III and IV according to Popper & Schaffner, 1970). None of the patients studied received immunosuppressive therapy for at least 6 months before the biopsies were taken. As controls 14 patients with chronic hepatitis B, five patients with chronic hepatitis non-A, non-B (both groups contained patients with different degrees of cholestasis), one patient with autoimmune liver disease and two patients with acute hepatitis A (one with cholestasis, one without) were included.

Preparation of biopsied materials

Percutaneous biopsies of liver tissue from all 14 patients were obtained for diagnostic reasons with a needle (1·4 mm in diameter) according to the technique of Menghini. The biopsied

specimens were divided into three pieces; part of the material was fixed in formaline for conventional histological examination, another part was immediately snap-frozen for immunoperoxidase-studies, and a third part (about 0·5 cm in length) was transferred in RPMI 1640 (Gibco, Grand Island, NY) containing 2% glutamine and 1% penicillin-streptomycin (PS).

Immunohistological analysis

A part of each liver biopsy was immediately embedded in 'Tissue Tek O.C.T.' compound (Miles Laboratories, Inc., Naperville, IL) and snap-frozen in 2-methylbutane on dry ice. Frozen sections (4–7 μm) were stained by an indirect immunoperoxidase technique as described previously (Pape *et al.*, 1983; Spengler *et al.*, 1988). T cells were defined by the monoclonal antibody (MoAb) MT910 (CD2) (Rieber *et al.*, 1986) recognizing the T11 antigen. T cell subsets were defined by the MoAb MT811 (CD8; suppressor/cytotoxic T cell subset) (Lohmeyer *et al.*, 1981; Rieber *et al.*, 1981, 1984) and MT151 (CD4; helper/inducer T cell subset) (Rieber *et al.*, 1986). To detect T cells with NK cell function, the MoAb VEP13 (CD 16; Rumpold *et al.*, 1984) and NKH1 (Griffin *et al.*, 1983) were used.

Establishment of T cell lines and clones

Before starting the culture procedure, liver biopsies were kept in a test tube containing RPMI 1640 supplemented with 10% human AB serum and washed for several hours to remove adherent cells and tissue material. From the washed biopsies a single cell suspension was prepared by mechanical disintegration. One part of the cell suspension was seeded into a 96-well, round-bottomed tissue culture plate (Costar No. 3799) with 4×10^4/well irradiated (4000 rad) autologous peripheral blood mononuclear cells (PBMC), isolated according to the method by Böyum *et al.* (1968), in RPMI 1640 containing 2% L-glutamine, 1% PS, 10% human AB serum, 0·2% PHA-P (Difco, Detroit, MI) and 20 U/ml recombinant IL-2 (conditioned medium, (CM)) in a final volume of 0·2 ml. After about 10 days a sufficient number of cells had grown to perform the cloning procedure as described below. The second part of the cell suspension was centrifuged on a Ficoll-Paque (Pharmacia, Sweden) density gradient to remove contaminating liver cells. The interphase was washed three times and the resulting cells were cloned by limiting dilution in 96-well round-bottomed plates at 0·5 cells/well with subsequent visual control for the presence of single cells. Wells containing only one single cell were fed with 4×10^4 irradiated PBMC (4000 rad) in 100 μl of CM. After 4 days wells containing single cells were fed again with PHA. In some patients T cell lines were cloned additionally by the same procedure. Wells were observed daily with an inverted microscope to monitor growth of T cell lines and clones. Medium was changed every second day using 20 U/ml recombinant IL-2.

Determination of cell surface antigens

Phenotypic analysis was done by a rosetting technique using bovine erythrocytes coupled with MoAb according to a method described previously (Pechumer *et al.*, 1984). Briefly, 1 mg of purified MoAb was coupled to bovine erythrocytes, previously washed vigorously with 0·9% NaCl, by adding 1·6 mg $CrCl_3$. After 1h at 30°C the erythrocytes were washed extensively with 0·9% NaCl; the reagent was kept in pure FCS (Gibco, Grand Island, NY) and used at an erythrocyte concentration of $1–2 \times 10^9$/ml. About 500–1000 TLC cells were seeded per well of a 96-well round-bottomed microtitre plate, one drop of the erythrocyte suspension was added, centrifuged at 800 rpm for 2 min and kept on ice for at least 2 h. The pellets were resuspended, mounted on a slide, mixed with one drop of crystal violet (Merck, Art. 1408) and evaluated using a Leitz light microscope.

Analysis of cytotoxic properties of TLC and TLL

For the assessment of cytotoxic properties three-different assays were performed: LDCC (lectin-dependent cell-mediated cytotoxicity) assays for the detection of cytotoxic T lymphocytes (CTL) irrespective of their specificity; NK (natural killing) assays; and ADCC (antibody-dependent cell-mediated cytotoxicity) assays for the detection of killer (K) cells. All three assays have been described previously (Pape *et al.*, 1979a, b; Moretta *et al.*, 1982, 1983a). Briefly, for the test of CTL activity (LDCC) TLC cells were transferred to V-bottomed wells of microtitre trays in a volume of 100 μl to which $2·5 \times 10^3$ ^{51}Cr-labelled murine P815 mastocytoma cells in a volume of 100 μl were added as target cells. PHA (10 μg/ml) (Sigma L-8754) was added to the labelled target cell suspension immediately before distributing the cells. The NK cell assay was performed by using ^{51}Cr-labelled K 562 cells as targets. K cell activity (ADCC) was determined in a ^{51} Cr-release assay using as targets P 815 cells sensitized with a 1 : 10 000 dilution of hyperimmune rabbit anti-P815 antiserum. All cell lines used were kept in continuous culture in the Institute of Immunology, Munich. In all three assays PBMC of a healthy donor with known cytotoxic activity were included as a positive control. The plates were incubated for 4 h at 37°C and 100 μl of the supernatant were removed for the measurement of ^{51}Cr release. Spontaneous release was determined in control microcultures prepared in the same manner as the experimental group without adding TLC. The percentage specific lysis was calculated using the equation: percentage specific lysis $= ((E-S)/(M-S)) \times 100$ where M is maximum release, S is spontaneous release, and E is experimental value determined for a particular effector to target cell ratio. Excess of more than 3 standard deviations of the mean spontaneous release was considered significant.

RESULTS

In-vitro growth of T cell lines and clones

In all cases in which there was evidence of lymphocyte infiltration in liver tissue T cell lines and clones could be established either by the direct cloning method or by limiting dilution from the T cell lines or by both methods. Growth of T cell lines and clones could be observed between days 6–15. For the expansion of the established clones autologous feeder cells (PBMC) and allogeneic feeders (PBMC and spleen cells) were used. Several lines and clones could be kept in continuous culture for more than 100 days. Cloning efficiency ranged from 29–86%. No difference was seen between the T cell clones obtained by the two different cloning methods.

Phenotypic analysis

Fourteen T cell lines derived from liver tissue of 14 patients with PBC, as well as 137 T cell clones of 10 patients, were analysed phenotypically. Table 1 depicts the histologic stage and the phenotypic distribution of the TLL of each individual patient.

Table 1. Phenotypic characterization* of T cell lines derived from liver biopsies of patients with primary biliary cirrhosis

Patient	Histologic stage	Monoclonal antibodies MT910 (CD2)†‡	MT811 (CD8)§	MT151 (CD4)¶
1	II	98	76	22
2	III	96	81	21
3	III	100	77	24
4	II	97	69	30
5	II	98	84	14
6	II	98	78	24
7	I	99	71	21
8	IV	95	61	40
9	II	97	52	48
10	IV	95	31	72
11	IV	97	38	63
12	II	99	33	64
13	III	97	45	56
14	IV	94	28	73

Results are expressed as percentage of positive cells.

* By rosetting technique (for details see *Materials and Methods*).

† Cluster of differentiation according to 1st and 2nd international workshops on leucocyte differentiation antigens.

‡ Monoclonal antibody (MoAb) detecting T11 antigen, thus defining all T cells.

§ MoAb detecting the T8 molecule, thus defining the cytotoxic/suppressor T cell subset.

¶ MoAb detecting the T4 molecule, thus defining the helper/inducer T cell subset (contains also cytotoxic cells).

Table 2. Phenotypic characterization of T cells cloned from liver tissue of patients with primary biliary cirrhosis

Patient	Number of clones	Monoclonal antibody MT910 (CD2)	MT811 (CD8)	MT151 (CD4)
1	2	2	2	0
2	4	4	3	1
3	3	3	3	0
7	29	29	10*†	19*†
8	29	29	19*	12*
9	9	9	6	3
10	17	17	7	10
11	15	15	5	10
12	20	20	6	14
13	9	9	3	6

Results expressed as percentage positive cells.

* Clones which showed coexpression of CD8/CD4.

† Clones without CD4 or CD8 expression.

In nine of 14 T cell lines $CD8^+$ T cells predominated ($CD8^+$: 52–84%; $CD4^4$: 14–48%), whereas in five of 14 T cell lines a preponderance of the $CD4^+$ subpopulation was found ($CD4^+$: 56–73%; $CD8^+$: 28–45%). In all T cell lines MoAb capable of detecting NK cells stained less than 5% of lymphocytes. A tendency towards increased numbers of $CD4^+$ cells within the intrahepatic T cells could be observed in advanced stages of the disease, but this observation was not statistically significant. Apart from stage of disease no other parameter, like age, clinical and/or biochemical markers, that might explain differences in the preponderance of a certain T cell population could be detected. Cholestasis did not affect the cloning efficiency. The phenotypic characterization of 137 T cell clones is given in Table 2. It should be mentioned that four T cell clones with a co-expression of CD4 and two clones with no CD4 or CD8 expression were found.

Table 3. Comparison between T cell subsets found in tissue sections and in liver-derived T cell lines in patients with PBC

Patient	Histologic stage*	CD8 cells (%) Immunohistology†	TLL *in vitro*
1	II	68	76
4	II	62	69
7	I	65	71
10	IV	35	31
13	III	42	45

* Histologic stage according to Popper & Schaffner (1970).

† See *Materials and Methods*; evaluation as described by Pape *et al.* (1983).

The phenotypic distribution of the established clones strongly correlated with the phenotypic distribution of the T cell lines in each individual patient. Furthermore, as is demonstrated in Table 3, the phenotypes of the clones and lines perfectly reflected the distribution of T cell subsets found *in vivo*, as determined by immunohistochemical stainings performed in parallel.

Cytotoxic activity of liver-derived T cell lines and clones

To study the cytotoxic capacity of T cell lines and clones three different assays were performed: LDCC, NK and ADCC. In the absence of a specific target cell line for CTL, we used the LDCC system for the assessment of CTL activity. Previous work has shown that this assay reflects well the cytotoxic capacity of MHC-restricted CTL (Spits *et al.*, 1982; Moretta *et al.*, 1983b; Gorman, Kane & Clarke, 1987; Miescher *et al.*, 1987). Seven T cell lines were tested in a LDCC and NK assay (see Table 4). All T cell lines tested showed cytotoxic activity in the LDCC assay. None of the T cell lines exhibited NK activity. In contrast, in two patients with acute A virus hepatitis (one biopsied for prolonged intrahepatic cholestasis secondary to hepatitis A (Gordon *et al.*, 1984), one without cholestasis biopsied because serological markers were reported falsely) the immunohistological examination showed a preponderance of $CD8^+$ cells (> 80%) and a moderate incidence of cells with markers for NK cells (21 resp. 24%). Clearly in TLL generated from these livers a low number of NK cells could be detected both by phenotypic analysis (18 resp. 22%) and functional testing. The results are given in Table 4. Furthermore, 43 T cell clones of nine patients (two TLC from patient 1; two TLC from patient 3; three TLC from patient 4; 12 TLC from patient 7; seven TLC from patient

Table 4. Cytotoxic properties of T cell lines derived from liver tissue of patients with primary biliary cirrhosis

		LDCC*		NK†	
Patient	Phenotype	E:T‡	^{51}Cr release (%)	E:T	^{51}Cr release (%)
2	CD8>CD4	10:1	79	10:1	8
4	CD8>CD4	10:1	51	10:1	7
5	CD8>CD4	10:1	81	10:1	3
10	CD4>CD8	10:1	56	10:1	3
11	CD4>CD8	10:1	83	10:1	12
12	CD4>CD8	10:1	31	10:1	3
13	CD4>CD8	10:1	74	10:1	3
Controls§					
HAV1	CD8>>CD4	10:1	72	10:1	46
HAV2	CD8>>CD4	10:1	79	10:1	34

* Lectin-dependent cell-mediated cytotoxicity; percentage ^{51}Cr release corrected for spontaneous release from P815 target cells in a 4-h cytotoxicity assay in the presence of 10 μg/ml PHA.

† Natural killing; percentage ^{51}Cr release corrected for spontaneous release from K562 target cells in a 4-h assay.

‡ Effector to target cell ratio.

§ Two T cell lines of patients with acute hepatitis A, HAV1 with cholestasis, HAV2 without cholestasis.

Table 5. Cytotoxic properties of T cells cloned from a liver biopsy of a patient* with primary biliary cirrhosis

Clone	Phenotype	LDCC	NK	ADCC†
E-D1	CD2,8	73	6	79
E-D4	CD2,8	76	−1	1
E-D5	CD2,8	77	2	−3
E-D6	CD2,—	59	2	1
E-D8	CD2,4,8	92	3	7
E-D7	CD2,4	84	77	81
E-D2	CD2,4	4	−3	9
E-D3	CD2,4	19	9	17
E-D11	CD2,4	6	−3	2
E-D12	CD2,4	5	1	1
E-D25	CD2,4	16	1	−1
E-D28	CD2,4	4	1	−3
Controls				
WY4E9‡	CD2,4	14	3	8
PBL H.R.§	—	33	37	31

Results are expressed as percentage ^{51}Cr release.

* Patient no. 7.

† Antibody-dependent cell-mediated cytotoxicity using P815 cells in the presence of rabbit anti-P815 antibodies in a 4-h ^{51}Cr release assay. The effector to target cell ration in all assays was 5:1.

‡ Noncytotoxic T cell clone derived from liver tissue of a patient with autoimmune liver disease; routinely negative control.

§ PBL of a healthy donor; routinely positive control.

8; three TLC from patient 10; two TLC from patient 11; eight TLC from patient 12; four TLC from patient 13) were tested in LDCC, NK and ADCC assays. Twenty out of 21 CD8$^+$ clones showed high CTL-mediated cytotoxicity (LDCC assay), whereas only one of 19 CD4$^+$ clones showed such activity. Natural cytotoxicity was displayed by only one of 43 clones (ED7, Table 5). K-cell-mediated cytotoxicity (ADCC) was exhibited by two out of 43 clones (ED1, ED7; Table 5). As controls unrelated PBL with known activity and a non-cytotoxic CD4$^+$ liver-derived clone of a patient with autoimmune liver disease were included in each experiment. Table 5 demonstrates the cytotoxic properties of T cell clones from liver tissue of one patient with PBC as a representative example.

DISCUSSION

Primary biliary cirrhosis is characterized by gradual destruction of intrahepatic bile ducts. Dense lymphocytic infiltrates around bile ducts are the histological hallmark of the disease. Previous immunohistological work has demonstrated CD8$^+$ cells in close contact with bile duct epithelial cells and has led to the hypothesis that CD8$^+$ cells may indeed be the culprits responsible for tissue damage (Pape *et al.*, 1983). This hypothesis was further supported by ultrastructural examinations from Yamada *et al.* (1986) who demonstrated that in PBC intraepithelial lymphocytes particularly carry the CD8 phenotype. Generally CD8$^+$ T cells are associated with cytotoxic functions. Thus the majority of authors agree that cytotoxic lymphocytes play a pivotal role in the pathogenesis of PBC. However, the relative contributions of different cytotoxic mechanisms to tissue injury are discussed controversially. 'Classical' T cell killing (by cytotoxic T lymphocytes, CTL) and antibody-dependent cell-mediated cytotoxicity (ADCC), as well as natural cytotoxicity (NK) have been implicated in the disease process (Geubel *et al.*, 1976; Vierling *et al.*, 1977; Dienstag & Bhan, 1980; Sandilands, Golbraith & Reich, 1980; James & Jones, 1985). Furthermore, in the meantime, it has become more evident that the phenotypic definition of lymphocytes need not necessarily reflect their functional capacity (Meuer, Schlossman & Reinherz, 1982; Flomenberg *et al.*, 1983; Krensky *et al.*, 1983; Strassman & Bach, 1984; Vidovic, Klein & Nagy, 1984). Therefore, the main issue of the present study was to investigate whether intrahepatic lymphocytes in PBC are cytotoxic and, if so, which kind of cytotoxicity they display. For that purpose, T cell lines and T cell clones directly derived from liver biopsies were generated.

This study shows for the first time that T lymphocytes with cytolytic function (CTL) represent the vast majority of cytotoxic cells, which can be grown in tissue culture as cell lines or clones from a biopsy. With only one exception these cells belong to the CD8 cluster. In the absence of a specific antigen the LDCC system was used to measure CTL activity. Previous work has shown, that LDCC is the appropriate test to measure CTL of unknown specificity (Spits *et al.*, 1982; Moretta *et al.*, 1983a; Gorman *et al.* 1987; Miescher *et al.*, 1987) and that NK and K cells lack such activity (Phillips & Lanier, 1986). Natural killer cells as well as K cells are not the cytotoxic effectors in liver tissue. This finding is noteworthy, since Moretta *et al.* (1983b), applying the same test systems used here, have shown that up to 50% of CD8$^+$ lymphocytes from peripheral blood with LDCC

activity also exhibit NK activity. Nevertheless natural cytotoxicity of peripheral blood lymphocytes may be of importance in the disease process: James and Jones (1985) have speculated that the inflammatory process in the liver may at least partly be a consequence of diminished surveillance function due to natural killer cell deficiency in peripheral blood.

The phenotypic composition of the cell lines derived from PBC biopsies was in full agreement with the immunohistochemical phenotyping of lymphocytes in all patients. This held true even in those biopsies in which $CD4^+$ lymphocytes outnumbered $CD8^+$ lymphocytes. The latter is in contrast to our findings in chronic hepatitis B, where $CD8^+$ cells predominated in all lines, clones and tissue sections investigated (Hoffmann *et al.*, 1986). A good correlation of phenotypes between immunohistology and *in-vitro* culture was also found in five patients with chronic hepatitis non-A, non-B and two patients with acute hepatitis A. In these controls an effect of cholestasis on cloning efficiency or selection of certain phenotypes could be excluded, since these patients showed various degrees of cholestasis. In acute hepatitis A some intrahepatic T cells with markers of natural killer cells were found. The TLL and some TLC established showed NK activity. These data strongly argue against a selection of certain phenotypes or T cells with certain functions by the culture system used to generate T cell lines and clones. Repeated examinations of several lines and clones demonstrated an intriguing stability of phenotypes and functions during the culture period in our system. It is well known that TLC may acquire cytotoxic functions when kept in long-term culture. Such an induction, however, has been reported to be characteristic especially for $CD4^+$ TLC after multiple restimulations with specific antigens and after long periods of time in culture (Chen *et al.*, 1986; Fleischer & Wagner, 1986). The role of $CD4^+$ lymphocytes in the liver in PBC is not yet clear. Some of them may have cytolytic capacity, as we have demonstrated in our investigation. They may belong to the subset of $CD4^+$ lymphocytes, which recognize their target via class II molecules (Meuer *et al.*, 1982). An aberrant expression of HLA-DR and HLA-DQ has been demonstrated on bile duct epithelial cells (Ballardini *et al.*, 1984; Spengler *et al.*, 1988). Taken together our studies strongly suggest that tissue damage during the course of PBC is due to the cytotoxic effect of $CD8^+$ cytolytic T lymphocytes (CTL). But as long as specific target antigens expressed on the lysed target cells in PBC remain undefined and test systems that allow the assessment of liver-derived MHC-restricted CTL in PBC are lacking, other mechanisms of CTL-mediated killing have to be considered. Following activation cytolytic T cells are able to lyse innocent bystanders *in vitro* (Fleischer, 1986). Thus, tissue damage in PBC could, at least partially, also result from a bystander killing mechanism of activated cytolytic T cells.

ACKNOWLEDGMENTS

This work was supported by the Wilhelm Sander Stiftung 83.o15.2Pa.

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