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T lymphocytes from patients with primary biliary cirrhosis produce reduced amounts of lymphotoxin, tumor necrosis factor and interferon-γ upon mitogen stimulation

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Primary biliary cirrhosis (PBC) is considered an autoimmune disease characterized by destruction of small intrahepatic bile ducts by lymphocytes. Altered functions of these lymphocytes might reflect an abnormal immune response leading to tissue damage. We investigated lymphokine secretion by mitogen-stimulated T lymphocytes from the liver biopsies of patients with PBC and for comparison also peripheral blood. In PBC, diminished synthesis of lymphotoxin (TNFβ), tumor necrosis factor (TNFα) and interferon-γ (IFNγ) was found both in T-cell lines from liver tissue and in peripheral blood. The reduction was most prominent for TNFβ in early histological stages of PBC, and appeared to be a stable phenomenon when T cells were tested after long-term tissue culture. Analysis of mRNA levels indicates a possible link between reduced TNFβ production and a defect in interleukin-2 transcription. The data suggest that diminished lymphokine production in patients with PBC may play an important role in the immunopathogenesis of this disease.

Primary biliary cirrhosis (PBC) is a chronic and progressive nonsuppurative inflammation of the intrahepatic bile ducts, possibly caused by autoimmune reactions. Although much progress has been made in unravelling the autoantibody response and the nature of associated autoantigens, the etiology and pathogenesis of PBC are still unknown. Activated T lymphocytes infiltrating affected bile ducts are the histological hallmark of this disease. Thus, the contribution of T lymphocytes may play a pivotal role in the induction and progression of PBC.

Lymphokines are secreted by activated T lymphocytes as essential soluble transmitters of cell-to-cell communication in the immune system. They exert potent immunoregulatory functions, and control local and systemic immune responses. Moreover, recent work suggests that local lymphokine production may be necessary for the induction of anti-self T lymphocytes (1). In order to investigate functions of T lymphocytes at the site of inflammation, we established T-cell lines both from liver biopsies and from the peripheral blood of patients with PBC and measured the production of lymphotoxin (TNFβ), tumor necrosis factor (TNFα) and interferon γ (IFNγ) upon stimulation with mitogen.

Patients and Methods

Patients

Peripheral blood and liver tissue specimens from 35 female and four male patients with primary biliary cirrhosis, aged 32–64 years, were included in this study. Diagnosis of PBC was established by accepted clinical, biochemical and histological criteria. All patients had significant titres of antimitochondrial antibodies. Histologically, 20 patients had early disease (stages I or II

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according to Scheuer (2)), and 19 patients had advanced disease (stages III or IV). Sixteen healthy women and 15 healthy man aged 24 to 66 years volunteered to give blood for control experiments. Eight patients with acute and 41 patients with chronic hepatitis B, 16 patients with chronic non-A, non-B hepatitis, and ten patients with primary sclerosing cholangitis (PSC) were studied as disease controls.

Preparation of T cells

Peripheral blood mononuclear cells (PBMC) were purified from heparinized blood on a Ficoll-Paque density gradient (Pharmacia, Sweden) (3). T lymphocytes were propagated as T-cell lines in RPMI 1640 medium (2% L-glutamine, 1% penicillin/streptomycin and 10% AB serum) in the presence of 10^4 irradiated (4000 rad) autologous feeder cells, 0.125 μg/ml phytohemagglutinin (PHA; Sigma, F.R.G.) and 20 U/ml human recombinant interleukin-2 (IL2; kindly provided by Dr. F. Sinigaglia, Basel Institute for Immunology; Hoffmann-La Roche, Switzerland). By a similar technique T-cell lines were generated from small fragments of liver specimens obtained by the Menghini technique, as described in detail previously (4). All liver biopsies were taken for diagnostic reasons not related to this study.

Phenotypes of T cells were analyzed on a fluorescence activated cell sorter (FACSCAN, Becton Dickinson, U.S.A.) by indirect immunofluorescence (fluoroisothiocyanate-labeled goat-anti-mouse antiserum, Medac, Hamburg, F.R.G.) with monoclonal antibodies M-T310 (anti-CD4), M-T151 (anti-CD4) M-T811 (anti-CD8) and M-T122 (anti-CD8) (kindly provided by Dr. E.P. Rieber, Munich). Double-staining of cells was performed with phycoerythrin-coupled monoclonal antibodies 2H4 (anti-CD45R0) and 4B4 (anti-CD39) (Coulter Electronics, U.S.A.).

Stimulation protocol

T-cell lines were left for 10 days without adding fresh feeder cells before the stimulation experiments. Lymphokine production was induced by the addition of 2 μg/ml PHA (Sigma, F.R.G.) to $2 \cdot 10^6$ T lymphocytes in 1 ml serum free RPMI 1640 medium. Control experiments without PHA were always run in parallel to check for inadvertant activation of T cells by the medium. To ensure that T-cell lines were free of functional macrophages, control stimulations with 1 μg/ml lipopolysaccharide (LPS; *Escherichia coli* serotype 055:B5, Sigma, F.R.G.) (5) were included. Based on our results concerning the kinetics of TNFα/β and IFNγ production upon mitogen induction, supernatants from stimulated T-cell lines as well as from freshly isolated PBMC were collected 3 days after PHA stimulation for determination of TNFβ, TNFα and IFNγ. As internal stimulation control T lymphocytes of two previously tested individuals were included in each analysis, resulting in a reproducible lymphokine response. For costimulation experiments recombinant human interleukin-4 (IL4), interleukin-6 (IL6) and the monoclonal IL2 antibody DMS-1 were purchased from IC Chemikalien, Munich.

Determination of lymphokines

Interferon-γ (IFNγ), tumor necrosis factor (TNFα) and lymphotoxin (TNFβ) were measured by ELISA as described previously (6). Monoclonal antibodies clone 69 (anti-IFNγ), 199-1 (anti-TNFα), and 9B9 (anti-TNFβ) were used as first layer bound to polystyrene microtitre plates. One hundred μl (50 μl for the IFNγ test) of an appropriately diluted test sample or recombinant lymphokine as external standard were incubated for 24 h at 4°C on the precoated plates. Bound lymphokine was detected by a horseradish-peroxidase-coupled monoclonal anti-IFNγ antibody (clone 123) or by biotinylated antibodies against TNFα (195-8) and TNFβ (polyclonal antiserum) followed by incubation with peroxidase-coupled streptavidin (BRL, U.S.A.). Tetramethylbenzidine (Serva, F.R.G.) in acetate/citric acid buffer (pH 4.9) was used as chromogenic substrate. Extinctions were read in an ELISA spectrophotometer at 450 nm (SLT, Austria). Results were determined as means from double determinations of at least two subsequent serial sample dilution steps. Reagents for determinations of IFNγ were kindly provided by Hoffmann-La Roche (Dr. H. Gallati), Switzerland. Sensitivity was 10 pg/ml for TNFα or TNFβ and 10 U/ml for IFNγ. ELISAs were not crossreactive below 100 ng/ml.

For RNA isolation $8 \cdot 10^6$ cells were harvested from parallel stimulation experiments with the same cell preparations. 10 μg/ml cycloheximide (Sigma, F.R.G.) was added 3 h prior to harvesting of cells. Cells were lysed in guanidium isothiocyanate buffer and sonified for 5 s to achieve breakage of DNA (Branson cell disruptor B15 with microtip, U.S.A.). RNA was prepared by CsCl gradient centrifugation, fractionated by formaldehyde/agarose gel electrophoresis and blotted to Hybond N membranes (Amersham, F.R.G.). A genomic 625 bp *Hin*dIII/*Xho*I fragment spanning exon 4/3′ UT was used as specific probe for TNFα. The 600 bp *Pst*I/*Pvu*II TNFβ-specific fragment was prepared of the corresponding TNFβ region. IL2 was probed with a 800 bp *Pst*I cDNA fragment (7). Fragments were radiolabeled according to Feinberg and Vogelstein (8). Hybridization and washing of the filters was performed with $5 \cdot 10^6$ cpm/ml according to Church and Gilbert (9).

TABLE 1

Production of lymphotoxin (TNFβ), tumor necrosis factor (TNFα) and interferon-γ (IFNγ) by PHA-stimulated T-cell lines in patients with inflammatory liver disease

	Lymphotoxin (pg/ml)	Tumor necrosis factor (pg/ml)	Interferon-γ (U/ml)
T-cell lines from liver biopsies			
PBC (early)	1810 ± 636 (9)[a,**]	793 ± 162 (9)[d,*]	42 ± 14 (6)[f]
PBC (late)	2714 ± 837 (11)[b]	1463 ± 461 (11)[e]	228 ± 100 (4)
H-B (chronic)	4755 ± 714 (23)	4514 ± 810 (23)	194 ± 33 (14)
HNANB	2713 ± 617 (10)	2678 ± 734 (10)	236 ± 96 (4)
PSC	6625 ± 2157 (4)	2730 ± 677 (4)	99 ± 6 (3)
T-cell lines from peripheral blood			
PBC (early)	925 ± 914 (4)[c,*]	1466 ± 793 (4)	134 ± 50 (4)
PBC (late)	5804 ± 2173 (14)	2237 ± 682 (14)	97 ± 24 (12)[g]
H-B (acute)	6253 ± 2802 (6)	3150 ± 325 (6)	167 ± 43 (3)
H-B (chronic)	6808 ± 1045 (11)	4813 ± 125 (11)	74 ± 23 (8)
HNANB	5675 ± 2630 (11)	1956 ± 102 (11)	262 ± 13 (3)
PSC	13719 ± 7359 (4)	2630 ± 115 (4)	201 ± 17 (2)
Controls	12272 ± 2536 (15)	2370 ± 448 (15)	294 ± 70 (13)

Protein levels (mean ± S.E.) of lymphotoxin (TNFβ), tumor necrosis factor (TNFα) and interferon-γ (IFNγ) in supernatants of PHA stimulated T-cell lines derived from liver tissue and peripheral blood. All culture supernatant (1 ml/well) was removed from $2 \cdot 10^6$ cells on day 3 after the addition of 2 μg PHA and frozen at −70°C until analysis by ELISA. The study groups consisted of patients with early (histological stages I and II according to (2)) and late (histological stages III and IV according to (2)) primary biliary cirrhosis (PBC), acute and chronic hepatitis B (H-B), chronic non-A, non-B hepatitis (HNANB), primary sclerosing cholangitis (PSC) and healthy controls. For ethical reasons only T-cell lines from peripheral blood were available in healthy controls and patiens with acute hepatitis B. The number of cell lines tested within each group is given in brackets. Results of the Kruskal Wallis test are indicated by * for $p < 0.05$ and ** for $p < 0.01$. Differences between groups were evaluated by the Mann-Whitney test as: [a]early PBC vs. chronic H-B: $p = 0.0160$, vs. PSC: $p = 0.0308$; [b]late PBC vs. chronic H-B: $p = 0.0489$, vs. PSC: $p = 0.0265$; [c]early PBC vs. controls: $p = 0.0051$, vs. acute H-B: $p = 0.0330$, vs. chronic H-B: $p = 0.0090$; [d]early PBC vs. chronic H-B: $p = 0.0053$, vs. HNANB: $p = 0.0549$, vs. PSC: $p = 0.0055$; [e]late PBC vs. chronic H-B: $p = 0.0489$; [f]early PBC vs. chronic H-B: $p = 0.0065$, vs. PSC: $p = 0.0196$; [g]late PBC vs. controls: $p = 0.0462$.

Statistics

Quantitative data are expressed as mean ± S.E. The *H*-test of Kruskal and Wallis was used to study whether the various patient groups originated in a common population. In addition, patient groups were compared in pairs by the Mann-Whitney test. $p < 0.05$ (two-sided) was considered to indicate statistically significant differences.

Results

Reduced lymphokine production in PBC

The cytokines TNFβ, TNFα and IFNγ could not be detected in unstimulated supernatants of T cells. According to our stimulation protocol, TNFβ, TNFα and IFNγ reached maximum protein levels 2–4 days after the addition of the mitogen. Thereafter protein levels declined.

The lymphokine production was analyzed in three T-cell compartments: in T-cell lines derived from liver biopsies and peripheral blood and in freshly isolated PBMC. In patients with PBC the activation of T-cell lines by mitogen resulted in lower production of TNFβ, TNFα and IFNγ compared to healthy controls and patients with acute and chronic hepatitis B, chronic non-A, non-B hepatitis and sclerosing cholangitis (Table 1). This result was obtained both with T-cell lines derived from peripheral blood or from liver biopsies. Moreover, a lower lymphokine production could be observed in patients with PBC, when TNFβ, TNFα and IFNγ were measured at each interval from days 2 to 6 after mitogen addition. Reduced lymphokine production by T lymphocytes from patients with PBC was a persistent phenomenon when T-cell lines were tested after longer periods of tissue culture (up to 6 weeks tested). Impaired lymphokine synthesis in PBC was detected irrespective of the dose of applied mitogen (0.5–8 μg/ml PHA per 10^6 cells). Generally the T-cell lines consisted of 63–72% $CD4^+$ and 31–33% $CD8^+$ lymphocytes. Furthermore, differences in lymphokine induction could not be attributed to interindividual alterations in the phenotypic composition of T-cell preparations. Moreover, lymphokine formation was not related to the degree of cholestasis of PBC as judged by serum bilirubin and alkaline phosphatase levels. Interestingly, lymphokine production appeared to be particularly, poor in patients with early histological stages of the disease (stages I or II). This observation was confirmed by stimulation experiments of

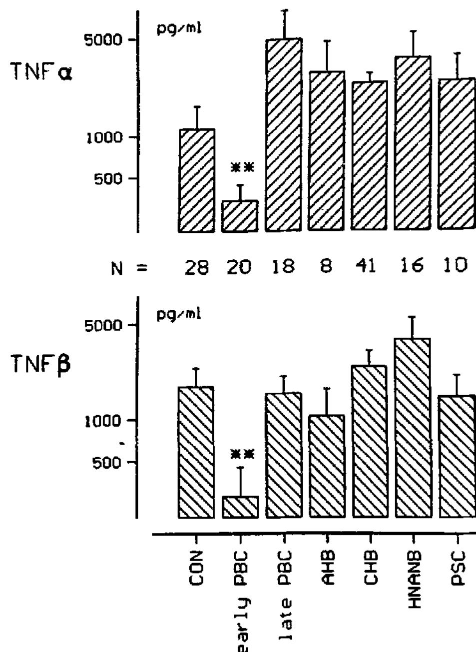

Fig. 1. TNFα and TNFβ production (mean ± S.E.) by freshly isolated PBMC obtained from patients with primary biliary cirrhosis (PBC), acute and chronic hepatitis B (AHB and CHB, respectively), chronic non-A, non-B hepatitis (HNANB), primary sclerosing cholangitis (PSC) and healthy controls (CON). Early PBC refers to histological stages I and II and late disease to histological stages III and IV (according to (2)). The number of patients studied in each group is given as '$N = \ldots$' between the two graphs. The stimulation procedure was identical to that used for T cell lines (see Material and Methods). **Indicates a level of significance $p < 0.01$ as calculated by the Kruskai-Wallis test. Differences between groups were evaluated as follows (Mann-Whitney test): (i) TNFα: early PBC vs. CHB $p < 0.0001$, early PBC vs. HNANB $p = 0.0020$; (ii) TNFβ: early PBC vs. controls $p = 0.0004$, vs. AHB 0.0252, vs. CHB $p = 0.0003$, vs. HNANB $p = 0.0204$, vs. PSC $p = 0.0072$ and late PBC vs. controls $p = 0.0184$.

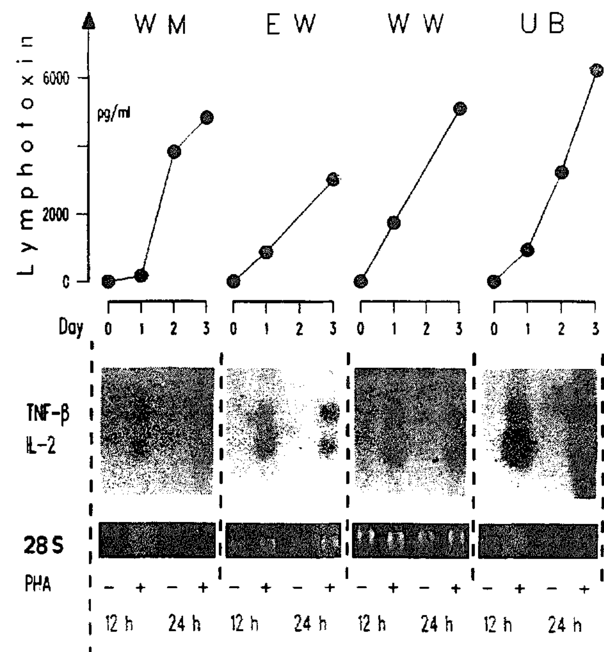

Fig. 2. TNFβ protein (pg/ml) and mRNA levels in PBMC from four healthy subjects (WM, EW, WW, UB) after PHA stimulation in correlation to the IL2 production. The TNFβ protein synthesis was determined in 1 ml culture supernatant ($2 \cdot 10^6$ cells) on days 0, 1, 2 and 3 of incubation with PHA. The amount of TNFβ mRNA (1.3 kb) and IL2 mRNA (0.9 kb) was analyzed after 12 and 24 h, respectively, with (+) and without (–) PHA by hybridization both with a TNFβ and IL2 specific probe in a Northern blot analysis of 8–10 μg total RNA prepared from parallel stimulation experiments. Cycloheximide (10 μg/ml) was added 3 h prior to harvesting of cells. Cycloheximide treatment increased IL2 and TNFβ mRNA signals, but did not change the relative character of mRNA inducibility. The ethidium bromide staining of the 28S ribosomal RNA is included as control for the amount of RNA loaded per lane. IL2 mRNA synthesis preceded TNFβ mRNA signals. The intensities of the hybridization signals for both lymphokines correlated with each other and with the amount of TNFβ protein.

PBMC in a large collective resulting in a dramatically reduced TNFα and TNFβ production upon mitogen induction in patients with early PBC (Fig. 1).

Modulation of TNFβ production by interleukin-2

In PBC reduced lymphokine production in T cells of all three compartments was most prominent for TNFβ. Therefore, the synthesis of this lymphokine was further analyzed at the mRNA level. TNFβ protein levels were found to correlate closely to the amount of TNFβ mRNA transcripts when analyzed in parallel by stimulation of PBMC (Fig. 2). Moreover, TNFβ mRNA and protein appeared to be correlated also with the amount of IL2 mRNA. The association between low IL2 and a reduced TNFβ production was further demonstrated by dose-dependent increases of TNFβ protein when exogenous recombinant IL2 was added to the lectin stimulation (Fig. 3). Moreover, PBMC of PBC patients produced the same amounts of TNFβ as PBMC of controls when induced in the presence of 200 U/ml recombinant IL2. No comparable reconstitution of the TNFβ response was achieved by the addition of IL4, IL6, TNFα or IFNγ to PBMC. Thus, following mitogen stimulation IL2 appears to be a major modulating factor for TNFβ production. In supernatants obtained from T-cell lines or PBMC stimulated with 2 μg PHA, IL2 protein levels could not be measured by ELISA.

Discussion

Damage of intrahepatic bile ducts by activated T cells is assumed to be a key event in the pathogenesis of PBC (10). Since the antigenic determinants in PBC recognized by infiltrating T cells have not yet been identified, lectin stimulation by-passing antigen recognition has largely been used for studies of T-cell activation. This paper

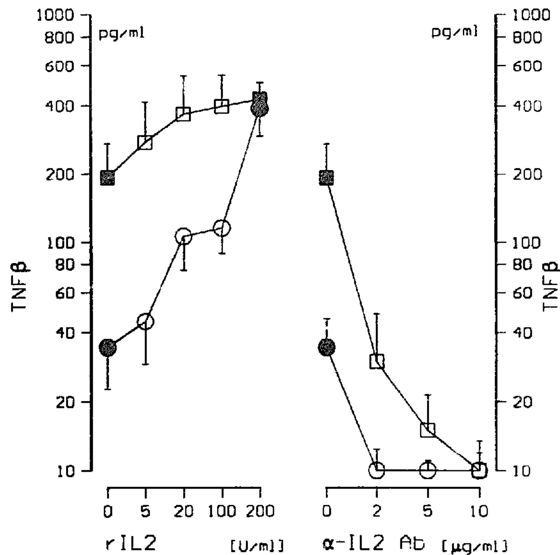

Fig. 3. Stimulation of TNFβ production by recombinant IL2. Left-hand side: TNFβ production (mean ± S.E.) in correlation to exogenous IL2. Increasing amounts (5–200 U/ml) of recombinant IL2 were added to $2 \cdot 10^6$ PBMC from patients with PBC (open circles, n = 7; black circles, n = 15) and healthy controls (open squares, n = 4; black squares, n = 7) during PHA stimulation. TNFβ was determined by ELISA in supernatants harvested 3 days after stimulation. Right-hand side: TNFβ production (mean ± S.E.) in the presence of anti-IL2 antibody (α-IL2 Ab). Two to ten μg/ml of the neutralizing monoclonal antibody DMS-1 were added to $2 \cdot 10^6$ PHA-stimulated (3 days) PBMC from patients with PBC (open circles, n = 5; black circles, n = 15) and healthy subjects (open squares, n = 3; black squares, n = 7). In the range used, DMS-1 was not crossreactive with TNFβ.

describes a defect in mitogen-induced production of TNFβ, TNFα and IFNγ by T lymphocytes specifically from patients with PBC. Reduced lymphokine secretion could be observed both in T-cell lines derived from peripheral blood and more importantly in T-cell lines from affected liver tissue. Several groups, including our own, have characterized T-cell lines from liver biopsies in PBC demonstrating that, at least phenotypically, liver-derived T-cell lines correspond to portal lymphocellular infiltrates (4,11,12). Provided that these T-cell lines also reflect the functional state of portal T lymphocytes, the results presented here indicate that lymphokine secretion and cell activation may be impaired in T lymphocytes as the site of tissue destruction. This reduced functional capacity is a stable characteristic of T cells in PBC, since defective lymphokine secretion was present in liver-derived T-cell lines kept in tissue culture for various periods of time (up to 6 weeks).

Impaired production of TNFβ, TNFα and IFNγ was particularly low in patients with early histological stages (stages I and II) of PBC. Inflammatory bile duct destruction mediated by immunological mechanisms is assumed to occur predominantly in the early stages of PBC, while fibrosis and cirrhosis and their consequences dominate the pathogenetic events in advanced PBC (12). Thus, it is likely that reduced production of TNFβ, TNFα and IFNγ may reflect the immunologically active stages of PBC rather than the consequences of cholestasis or cirrhosis. Although sequential testing of lymphokine synthesis has not yet been performed during the course of disease in individual PBC patients, the smaller decrease in lymphokine production in patients with advanced disease suggests that the apparent defect in mitogen induced lymphokine generation may be a transient phenomenon.

Reduced lymphokine production in PBC was most evident in the TNFβ response, and was also detected at the level of mRNA. Low amounts of TNFβ mRNA always corresponded with reduced TNFβ secretion. Moreover, the amount of IL2 mRNA correlated with TNFβ mRNA and TNFβ protein levels in healthy controls as well in PBC patients. Reduced IL2 activity in PBC has been reported by Saxena and his co-workers, when mitogen-stimulated lymphocytes from peripheral blood were tested in a bio-assay (14). Our results confirm the previous observations and demonstrate a tight linkage between the induction of IL2 and TNFβ transcription and TNFβ production. This linkage between IL2 and TNFβ production is further supported by the observation that addition of exogenous recombinant IL2 to PBMC of patients with PBC could specifically reconstitute TNFβ production in a dose-dependent fashion. Other cytokines like IL4, IL6 or IFNγ, which can also influence TNFβ production (15,16), showed no comparable enhancement of TNFβ synthesis. These results are in agreement with findings by Kasid and co-workers (17), who in a similar stimulation experiment identified IL2 and IL1 as major modulators of TNFβ, TNFα, and IFNγ production in human PBMC. However, it is not clear whether reduced generation of TNFβ and other lymphokines can be fully attributed to low IL2 activity in PBC. Allelic differences of the TNFβ gene linked with a differential TNFβ response cannot account for the reduced TNFβ production in PBC, since no association of the low responder allele was found for this disease (18).

Current evidence indicates that T cells require costimulatory activity in addition to triggering of the TCR complex for activation. Failure to provide sufficient costimulatory activity does not only prevent T-cell activation but instead results in a prolonged unresponsive state (19–21). The unresponsiveness in these cells is primarily the result of insufficient lymphokine secretion, particularly that of IL2 (21,22). In PBC, neo-expression of MHC class II antigens can be detected on biliary epithelial cells (23,24) which may thereby qualify for antigen presentation and triggering of the TCR complex. Since

these non-professional antigen presenting cells provide only little costimulatory activity (25,26), it is an intriguing speculation that in PBC the unresponsiveness of T lymphocytes with regard to TNFβ, TNFα, and IFNγ production might be the result of prior contact with parenchymal cells aberrantly expressing MHC class II molecules. As aberrant MHC class II expression is most prominent in the beginning of PBC (30), a considerably larger fraction of cells might be unresponsive in T-cell lines from patients with early stages of the disease.

Reduced production of IL2, IFNγ, TNFα, and TNFβ has been reported in a variety of human autoimmune diseases and in experimental models of autoimmune disease which are characterized by aberrant MHC class II expression (27–34). These experimental models further demonstrate that low levels of TNFα may be associated with tissue damage, whereas high amounts can exert protective effects (32). Replacement therapy with recombinant TNFα induced a significant delay in the development of autoimmune nephritis in (NZBxNZW)F1 mice (31), and could protect NOD mice and BB rats from developing autoimmune diabetes (32–34). TNFα could also prevent adoptive transfer of diabetes by splenic lymphocytes from diabetic NOD mice to irradiated nondiabetic NOD mice (34). The results obtained in these animal models propose, that the observed impairment in lymphokine synthesis by T lymphocytes of patients with PBC may be an important factor in the pathogenesis of autoimmunity also in this disease.

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