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Primary Biliary Cirrhosis—Phenotypic Characterization of Immunocompetent Cells in Peripheral Blood and Liver Tissue

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ABSTRACT

Circulating T-cell subsets have been characterized in 18 patients with primary biliary cirrhosis (PBC) using a simple immunoenzymatic staining method on stored and prefixed cell samples. Furthermore, a double immunoenzymatic method was used to study T-cell subsets and their relationship to HLA-DR$^+$ cells in frozen sections of liver biopsies from 12 patients with PBC. In 10 cases blood and liver samples were taken simultaneously from the same patient.

Patients with PBC of early histological stage showed an elevated absolute number of helper/inducer T-cells (Leu 3a$^+$) and reduced relative percentages of suppressor/cytotoxic T-cells (Leu 2a$^+$) compared with healthy subjects resulting in an elevated helper/suppressor ratio.

Liver biopsies in PBC were dominated by helper inducer phenotype T-cells in all cases irrespective of histological stage, penicillamine treatment or the relative levels of circulating T-cell subsets. A relatively high amount of HLA-DR$^+$ cells were seen, several of these in close approximation to T-cells.

INTRODUCTION

Primary biliary cirrhosis (PBC) is a progressive liver disorder characterized histologically by bile ductular destruction, portal mononuclear infiltration with granuloma formation, and eventual fibrosis and cirrhosis. PBC is accompanied by immunological stigmata such as hypergammaglobulinemia (IgM), non-organ specific autoantibodies like antimitochondrial antibody (AMA) and circulating immune complexes (25). Cellular immune dysfunction including reduced responses to mitogens (8) as well as increased

proliferative and cytotoxic responses to hepatic biliary and extra-hepatic antigens have been demonstrated (16,17,19,20).

Taken together, these findings support the suggestion that PBC is an autoimmune disease, possibly with the bile duct expressing the primary target antigen(s) (24). However, exactly how these immunological abnormalities contribute to the pathogenesis of PBC is far from clear.

In recent years useful tools have become available for the analysis of lymphocytes through the development of monoclonal antibodies reacting with functionally discrete T-cell subsets ("helper/inducer" T-cells vs. "suppressor/cytotoxic" T-cells). Studies have already been carried out to quantitate these T-cell subsets in peripheral blood and liver tissue from patients with PBC. Routhier et al. (21) and Bhan et al. (2) found abnormal circulating helper/suppressor T-cell ratios in patients with PBC. When studying liver infiltrates, Eggink et al. (5) raised the question whether the pattern of lymphocyte subsets in peripheral blood is merely a simple reflection of immunological events taking place in the liver.

However, to our knowledge no study has been reported that compares the T-cell subsets in peripheral blood and liver tissue of individual patients.

We report here the T-cell subset distribution in the peripheral blood of 18 patients with PBC. Simultaneously obtained liver biopsies from 10 of these patients were also investigated using a double immunoenzymatic method enabling the recognition of cells expressing the HLA-DR marker together with cells expressing the T-cell subset markers. Liver biopsies but not the peripheral blood from another two patients were also analyzed.

The following questions were raised:

1. Is there any correlation between (a) the relative number of T-cell subsets in peripheral blood and liver infiltrate, (b) the relative number of T-cell subsets in peripheral blood and the histological stage of PBC, (c) the prominence and/or distribution of T-cell subsets in the liver infiltrate and the histological stage of PBC.
2. Are there any HLA-DR$^+$ cells present in the liver infiltrates and, if so, how is the relationship between these putative antigen-presenting cells and the T-cell subsets?

PATIENTS AND METHODS

Patients

20 cases (16 women, 4 men) with PBC were included in the study. The diagnosis was based on liver function tests indicative of cholestasis, positive mitochondrial antibody test, diagnostic or compatible liver histology and patent extrahepatic bile ducts on cholangiography. Age, duration of disease and drug treatment of the individual patients at the time of the study is shown in Table I.

Peripheral blood leukocytes (PBL)

Peripheral heparinized blood was diluted 2:3 with phosphate-buffered saline (PBS) at pH 7.4 and layered on Ficoll-Hypaque according to the technique of Böyum (3). The cells were washed three times in PBS and then suspended in PBS containing 0.1 % bovine serum albumin (BSA) at 10^6 cells/ml. Approximately 10 µl of the cell suspension was dropped into each well of a multiple well microscope slide. The slides were air-dried at + 37° C for one hour and then stored at - 70° C. After storage the cell preparations were immediately fixed in acetone diluted 1:1 with water (+4° C) for 1 min. followed by final fixation in 100 % acetone (+4° C) for 5 min. The slides were then air-dried for 1 min. and washed in PBS for 5 min. before staining (12).

Liver biopsies

Liver biopsy ad Modum Menghini (18) was performed in 12 cases. The tissue specimens were cut into 2 pieces. One was fixed in 10 % aqueous formaldehyde and used for histopathology. Liver histology was classified according to Scheuer (22) in stage I = florid duct lesion, stage II = ductular proliferation, stage III = fibrosis and stage IV = cirrhosis. The other piece of liver tissue was placed in Histocon medium (Histolab, Bethlehem Trading Ltd., Gothenburgh, Sweden) at +4° C until frozen in isopentane and stored at -70° C. Sections 6 um thick were cut in a cryostat at -20° C, fixed in acetone for 10 min., airdried for 1 hour and then stored at -70° C until used.

Antisera and other reagents

The rabbit antiserum against HLA-DR antigens (14) and against alkaline phosphatase (15) have been described earlier. Swine

Table 1

Clinical characteristics and relative number of T-lymphocyte subsets in peripheral blood and liver tissue in patients with primary biliary cirrhosis

Sex (M/F)	Age (yrs)	Duration of disease (yrs)	Histological stage	Treatment	Peripheral blood[1]				Liver tissue[2]		Mono[5]
					Leu1	Leu3a	Leu2a	Leu3a/Leu2a	Leu3a	Leu2a	
F	75	8	I	-	71	41	17	2.4	+++	+	2500
F	55	9	I	-	52	41	14	2.9	+++	++	2300
F	55	1	I	-	29	24	9	2.7	+++	++	2700
F	51	2	I	-	60	45	18	2.5	+++	+	3000
F	64	2	I	-	nd[3]	nd	nd	nd	+++	++	1500
M	68	4	I	-	64	44	20	2.2	nd	nd	2800
F	66	11	I	-	67	52	20	2.6	nd	nd	3900
F	57	5	I	-	61	45	18	2.5	nd	nd	2800
M	41	1	I	-	59	41	20	2.1	nd	nd	4800
F	71	4	III	pc-amine[4]	55	40	15	2.7	nd	nd	2800
F	42	3	III	pc-amine	72	45	33	1.4	++	+	4000
F	50	4	III	pc-amine	61	46	18	2.6	+++	+	2900
F	35	2	III	-	64	44	22	2.0	nd	nd	2300
M	42	3	III	-	75	69	24	2.9	+++	+	2400
F	46	7	III	-	nd	nd	nd	nd	+++	++	2100
F	59	8	IV	-	55	40	23	1.7	nd	nd	-
F	57	9	IV	-	62	38	25	1.5	+++	+	800
F	43	8	IV	pc-amine	43	29	15	1.9	++	+	1400
F	50	9	IV	pc-amine	53	36	16	2.3	++	+	800
M	64	6	IV	pc-amine	62	51	27	1.9	nd	nd	-

1) % of mononuclear cells
2) Estimated relative occurrence of $Leu3a^+$ and $Leu2a^+$ cells in portal mononuclear cell infiltrate (+ ——> +++)
3) nd = not done
4) pc-amine = penicillamine treatment
5) absolute numbers of monocytes/mm^3 in peripheral blood

anti-rabbit IgG was purchased from Dakopatts (Copenhagen, Denmark). Biotinylated horse anti-mouse IgG, avidin DH, and biotinylated horseradish peroxidase ("ABC" kit) were obtained from Vector Laboratories (Burlingame, Calif., USA). The 3-amino-9-ethylcarbazole, alkaline phosphatase type VII, naphthol-AS-MX buffer and Fast Blue reagents were from Sigma (St. Louis, Mo., USA) and levamizole (Levoripercol^R) was obtained from Leo (Helsingborg, Sweden).

The mouse anti-human monoclonal antibodies denoted Leu 1, Leu 2a and Leu 3a were purchased from Becton-Dickinson (Sunnyvale, Calif., USA). Leu 1 defines all peripheral T-cells (7), whereas Leu 2a defines the "suppressor/cytotoxic" T-cell subset and Leu 3a the "helper/inducer" T-cell subset (6).

Immunohistochemical stainings

The liver sections were investigated using a sensitive double immunoenzymatic staining technique enabling the simultaneous recognition of cells positive for one of the monoclonal antibodies Leu 1, Leu 2a or Leu 3a (peroxidase catalysed staining) together with cells binding rabbit anti-HLA-DR antibodies (alkaline phosphatase catalysed staining). This technique has been detailed elsewhere (19) and is essentially a modification of a double immunoenzymatic technique described by Mason and Sammons (15) except that we have made use of the avidin-biotin-peroxidase procedure. Three types of controls were used: (25) omission of the primary antibodies (mouse monoclonals and rabbit anti-HLA-DR antibodies), (8) replacement of the primary antibodies with normal rabbit antiserum (diluted 1/160) and (19) omission of the pre-incubation step with H_2O_2 and exclusion of levamisole from naphthol buffer.

The prefixed cell preparations from peripheral blood were investigated using the single peroxidase-catalysed staining procedure. Omission of the primary antibody was used as negative control. More than 200 cells were scored within each population stained.

RESULTS

The number of circulating T-lymphocytes (Leu 1^+, Leu $2a^+$ and Leu $3a^+$) in patients with PBC and healthy controls are shown in Table 1. When comparing the results from patient with early PBC (stage 1) with controls (n=12) there is a decrease in the relative numbers of both total T-cells (Leu 1^+) ($\bar{x}$ 57.9 % vs. 61.6 %) and

helper/inducer T-cells (Leu 3a$^+$) ($\bar{x}$ 41.6 % vs. 43.0 %). Absolute numbers of Pan-T and helper/inducer T-cells were increased compared with controls (controls mono $\bar{x}$ 1800).

The relative number of suppressor/cytotoxic T-cells (Leu 2a$^+$) was more markedly decreased ($\bar{x}$ 17.0 % vs. 23.4 %) leading to an increased helper/suppressor ratio (2.4 vs. 1.8).

In patients with stages III and IV PBC there was no clear difference in numbers of total T-cells (60.2 %) and helper/inducer T-cells (43.8 %). However, the relative number of suppressor/cytotoxic T-cells were also moderately decreased (21.8 %) resulting in a nearly normal helper/suppressor ratio (2.0). When values from untreated patients in the group with stages III and IV PBC (4/10) were calculated similar results were obtained as in the whole group. The mean relative number of total T-cells was 64.0 %, helper/inducer T-cells 47.8 % and suppressor/cytotoxic T-cells 23.5 % leading to a helper/suppressor ratio of 2.0.

The immunoenzymatic staining of liver biopsies (see Fig.) from 12 patients showed a variable amount of Leu 1$^+$ cells in both early and late stages of the disease.

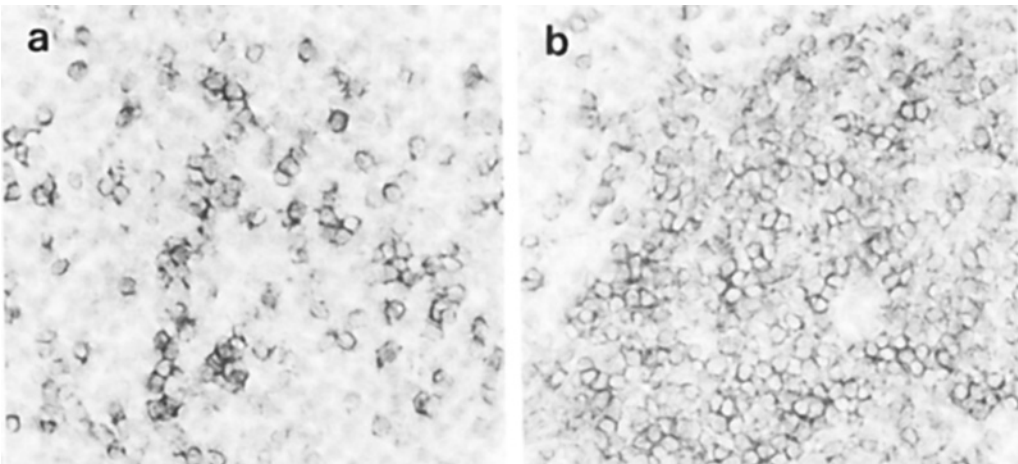

Fig.

Immunohistochemical staining of lever biopsy sections in PBC (a,b). Single staining with Leu 2a antibodies (a) and Leu 3a antibodies (b) in adjacent sections. Nuclear counter-staining with haematoxylin.

The infiltrates were more concentrated to the central part of the portal area and were usually organized in clusters. In adjacent sections stained with Leu 3a antibodies, a similar pattern was observed as with Leu 1 antibodies.

The sections stained with Leu 2a antibodies revealed a different pattern with positively stained cells seen more scattered throughout the whole portal area.

Although the frozen section staining does not allow an exact quantitation, cells stained with Leu 3a antibodies appeared to dominate in all cases investigated. The dominance varied from moderate to extreme high. However, there was no correlation between the number of Leu $3a^+$ cells and the histological stage of PBC. When the double surface marker stainings were evaluated there was a diffuse weak staining with anti-HLA-DR antibodies over the whole portal area, but a relatively high amount of distinct HLA-DR^+ cells were present, some of these cells of dendritic morphology. The HLA-DR^+ cells were seen in the central part of the portal area intermingled in the T-cell clusters and surrounding the clusters. They were also seen in the periphery of the portal area but more scattered. Several T-cells (most of these with helper phenotype) were seen in close contact to the HLA-DR^+ cells.

The results on PBL and tissue sections from individual patients were also compared. No consistent relation was observed between the relative Leu 3a/Leu 2a ratio in peripheral blood and liver, although absolute numbers of Leu 3a in peripheral blood were generally increased in stage 1. The T-cell subset distribution in peripheral blood and liver infiltrate was also compared with liver function test (galactose), serum aminotransferases, bilirubin and titres of antichondrial antibody. No relation was however found.

DISCUSSION

In the present study we have made use of a sensitive immunocytochemical staining method to quantitate lymphoid cell subsets in peripheral blood and liver in patients with PBC. Using a double staining technique when staining liver tissue sections we were also able to study the relationship between HLA-DR^+ cells and the different T-cell subsets in liver specimens. In our hands this technology offers two important advantages over conventional

immunofluorescence; first, localisation of the marker-carrying cell in the tissue is frequently possible, and second, several preparations of peripheral cell smears and tissue sections can be stored in the frozen state for later staining under identical conditions and for comparison.

The number of circulating T-cell subsets in patients with PBC was heterogenous when compared to healthy control subjects. When the patients were grouped according to liver histology, two different patterns were observed. Cases with stage I PBC had in average low relative numbers of circulating suppressor/cytotoxic phenotype cells combined with slightly decreased numbers of helper/inducer phenotype cells. This resulted in an elevated helper/suppressor ratio. On the other hand, patients with more advanced PBC, stage III - IV,demonstrated an equal relative reduction of both subsets resulting in a Leu 3a/Leu 2a ratio not significantly different from that of the controls. We could not observe any clear-cut impact of penicillamine treatment on different cell subpopulations in this rather limited series of patients, neither was there any obvious correlation as to routine liver tests or titres of antimitochondrial antibodies. Our present findings on circulating T-cell subpopulations in PBC can be compared with only a few earlier reports. Routhier et al. (21) observed selectively reduced numbers of helper/inducer T-cells in the blood of PBC patients in stages I-II, whereas in more advanced disease both the suppressor and especially helper T-cells were reduced. The helper/suppressor ratios were low and uncorrelated with histological features. More recently, Bhan et al. (2) used the same immunofluorescence technique to find a relative decrease in helper T-cells in early PBC with a low helper/suppressor ratio, as opposed to late PBC where the suppressor population was reduced resulting in a high ratio. They also noted a correlation between the helper/suppressor ratio and bilirubin levels. The present findings of reduced relative levels of total T-cells, helper T- as well as suppressor T-cells in early PBC parallel the earlier observations of Bhan et al. (2) on later stage PBC. However, in the present study the helper/suppressor ratio in peripheral blood was reduced in only 2/18 patients with PBC (both in stage IV, and one of these on penicillamine treatment). It is conceivable that the diagnosis of PBC includes a whole spectrum of patients in continuous but variable progression, and the prognostic subclassi-

fication of PBC based on parameters like liver function, antimitochondrial antibodies and histology is difficult (4). This heterogenity could account for the discrepancies observed in circulating T-cell subsets, and pinpoint the importance to study individual patients prospectively.

The distribution of T-lymphocyte subsets within liver infiltrates was similar in all twelve PBC patients irrespective of histological stage, penicillamine treatment or the relative levels of circulating T-cell subsets. The central part of the portal areas were dominated by T-cells staining with anti-helper T-cell antibody, whereas the relatively few suppressor/cytotoxic T-cells were equally scattered over the whole portal area. Within portal lymphocytic infiltrated clusters of T-cells (most of these cells with helper phenotype) were frequently seen in close contact with HLA-DR$^+$ cells.

Our present finding closely parallel those of Eggink _et al_. (5). The pattern of helper cell dominance is also similar to the patterns obtained in other immune disorders as Sjögren´s disease (1), rheumatoid arthritis (10,13) and thyroiditis (11) which all have auto-immune features.

Altogether, the present finding of a high helper/suppressor T-cell ratio and putative antigen presenting HLA-DR cells combined with the known presence of plasma cells within the portal areas of PBC patients argue for a local synthesis of autoantibodies. Such antibodies could then contribute to the destruction of bile ducts and parenchymal cells either directly, by activation of the complement system, or indirectly in antibody-dependent cell-mediated cytotoxicity. This is further supported by the _in vitro_ demonstration of a suppressor T-cell defect and a deficient autologous mixed lymphocyte reaction in PBC (9,26). However, it should be pointed out that the mere expression of the helper/inducer or suppressor/cytotoxic cell surface phenotype is not necessarily connected to the functional state of the lymphocyte. A mechanism similar to those operating in the classical delayed type of hypersensitivity (DTH) reaction could also be possible since the pattern with high numbers of helper T-phenotype cells in close relationship with HLA-DR$^+$ cells in liver infiltrates is similar to that found in acute DTH skin reaction caused by PPD (23).

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REFERENCES

1. Adamson, T.C., Fox, R.I., Frisman, D.M. & Howell, E.V.: Immunohistologic analysis of lymphoid infiltrates in Primary Sjögren's syndrome using monoclonal antibodies. J Immunol 130:203-208, 1983.

2. Bhan, A.K., Dienstag, J.L., Wands, J.R., Schlossman, S.F., & Reinherz, E.L.: Alternation of T-cell subsets in primary biliary cirrhosis. Clin exp Immunol 47:351-358, 1982.

3. Böyum, A.: Isolation of mononuclear cells and granulocyte from human blood. Scand J clin Lab Invest 21: Suppl 77:9-29, 1968.

4. Christensen, E., Crowe, J., Doniach, D. et al.: Clinical pattern and course of disease in primary biliary cirrhosis based on an analysis of 236 patients. Gastroenterology 78:236-246, 1980.

5. Eggink, H.E., Houthoff, H.J., Sippie Huitema, Gips, C.H. & Poppema, S.: Cellular and humoral immune reactions in chronic active liver disease. I. Lymphocyte subsets in liver biopsies of patients with untreated idiopathic autoimmune hepatitis, chronic active hepatitis B and primary biliary cirrhosis. Clin exp Immunol 50:17-24, 1982.

6. Engleman, E.G., Benike, C.J., Glickman, E. & Evans, R.L.: Antibodies to membrane structures that distinguish suppressor/cytotoxic and helper T lymphocyte subpopulations block the mixed leucocyte reaction in man. J Exp Med 153:193-198, 1981.

7. Engleman, E.G., Warnke, R., Fox, R.I., Dilley, J., Benike, C.J. & Levy, R.: Studies on a human T lymphocyte antigen recognized by a monoclonal antibody. Proc Natl Acad Sci USA 78:1791-1795, 1981.

8. Fox, R.A., Dudley, F.J., Samuels, M., Milligan, J. & Sherlock, S.: Lymphocyte transformation in response to phythaemagglutinin in primary biliary cirrhosis.The search for a plasma inhibitory factor. Gut 14:89-93, 1973.

9. James, S.P., Elson, C.O., Waggoner, J.G., Jones, E.A. & Storber, W.: Deficiency of the autologous mixed lymphocyte reaction in patients with primary biliary cirrhosis. J clin Invest 66: 1305-1310, 1980.

10. Janossy, G., Duke, O., Bofill, M., Poulter, L.W. & Goldstein, G.: Rheumatoid arthritis: a disease of T-lymphocyte/macrophage immunoregulation. Lancet 2:839-843, 1981.

11. Jansson, R., Tötterman, T.H., Sällström, J. & Dahlberg, P.A.: Thyroid-infiltrating T lymphocyte subsets in Hashimoto's thyroiditis. J clin Endodrinol Metab 56:1164-1168, 1983.

12. Karlsson-Parra, A., Forsum, U., Klareskog, L. & Sjöberg, O.: A simple immunoenzymatic batch staining method for the enumeration of peripheral human T-lymphocyte subsets. J immunol Methods 64:85-90, 1983.

13. Klareskog, L., Forsum, U., Wigren, A. & Wigzell, H.: Relationships between HLA-DR-expressing cells and T lymphocytes of different subsets in rheumatoid synovial tissue. Scand J Immunol 15:501-507, 1982.

14. Klareskog, L., Trägårdh, L., Lindblom, J.B. & Peterson, P.A.: Reactivity of a rabbit anti-serum against highly purified HLA-DR antigens. Scand J Immunol 7:199-208, 1978.

15. Mason, D.Y. & Sammons, R.: Alkaline phosphatase and peroxidase for double immunoenzymatic staining labelling of cellular constituents. J clin Pathol 31:454-460, 1978.

16. McFarlane, I.G., Wojcicka, B.M., Tsantoulas, D.C.: Leucocyte migration inhibition in response to biliary antigens in primary biliary cirrhosis, sclerosing cholangitis and other chronic liver disease. Gastroenterology 76:1333-1340, 1979.

17. McFarlane, I.G., Wojcicka, B.M., Tsantoulas, D.C.: Cellular immune response to salivary antigens in autoimmune liver disease with sicca syndrome. Clin Exp Immunol 25:389-395, 1976.

18. Menghini, G.N.: One-second biopsy of the liver-problems of its initial application. New Engl J Med 283:582-585, 1970.

19. Miller, J., Smith, M.G.M., Mitchell, C.G., Rees, W.D., Eddelston, A.L.W.F., Williams, R.: Cell-mediated immunity to a human liver-specific antigen in patients with active chronic hepatitis and primary biliary cirrhosis. Lancet 2:296-297, 1972.

20. Paronetto, F. & Vernance, S.: Immunological studies in patients with chronic active hepatitis: cytotoxic activity of lymphocytes to chthonus liver cells grown in tissue culture. Clin exp Immunol 19:99-104, 1975.

21. Routhier, G., Janossy, G., Epstein, O. et al.: Effect of cyklosporin A on suppressor and inducer T lymphocytes in primary biliary cirrhosis. Lancet 2:1223-1226, 1980.

22. Scheuer, P.J.: Primary biliary cirrhosis. Proc R Soc Med 60: 1257-1260, 1967.

23. Scheynius, A., Klareskog, L. & Forsum, U.: In situ identification of T lymphocyte subsets and HLA-DR expressing cells in the human skin tuberculin reaction. Clin exp Immunol 49:325-330, 1982.

24. Sherlock, S. & Sheuer, P.J.: The presentation and diagnosis of 100 patients with primary biliary cirrhosis. N Engl J Med 289:674-678, 1973.

25. Wands, J.R., Dienstag, J.L., Bhan, A.K., Feller, E.R. & Isselbacher, K.J.: Circulating immune complexes and complement activation in primary biliary cirrhosis. N Engl J Med 289: 233-237, 1978.

26. Zetterman, R.K. & Woltjen, J.A.: Suppressor cell activity in primary biliary cirrhosis. Digestive Diseases and Sciences 25:104-107, 1980.

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