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Characterization of the inflammatory infiltrate in autoimmune cholangitis

A morphological and immunhistochemical study

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Abstract Autoimmune cholangitis (AIC) is characterised by clinical and/or laboratory features of cholestasis, the presence of antinuclear antibodies and the lack of antimitochondrial antibodies. Histologically, changes largely identical to those found in primary biliary cirrhosis (PBC) are typically found. It is not possible to differentiate between AIC and PBC on conventional morphological grounds, and we therefore wished to find whether there is a difference between these entities in the composition of the inflammatory infiltrate leading to bile duct destruction. In liver biopsies from ten patients with confirmed AIC and ten patients with PBC the inflammatory infiltrate was characterised with antibodies against CD 3, OPD 4 CD 8, GB 7, L 26, CD 56 and CD 57. In AIC, T cells were predominant in the portal inflammatory infiltrate in nine cases. Granzyme B-positive activated cytolytic T lymphocytes were found in the bile duct epithelium in five cases. All these five cases showed inflammatory bile duct destruction. No significant differences between the immunohistochemical findings in AIC and in PBC were found. We suggest that AIC is a subgroup of PBC, antimitochondrial antibody-negative type.

Key words Cholangitis · Autoimmune diseases · T-Lymphocyte subsets · Immunohistochemistry

Introduction

Autoimmune cholangitis is an inflammatory disease of the small intrahepatic bile ducts, which was first described by Brunner and Klinge [2]. It characteristically affects middle-aged women, showing typical clinical, laboratory, and histological features. Clinically, patients complain of pruritus and fatigue with an insidious onset. The laboratory findings reflect cholestasis with elevation of serum alkaline phosphatase and moderate increase of gamma glutamyl transferase.

In contrast to primary biliary cirrhosis antimitochondrial antibodies and elevated gamma globulins are not found, but antinuclear antibodies are present generally. Chronic destructive nonsuppurative cholangitis is the histological hallmark of autoimmune cholangitis [1, 2, 7, 10, 13, 18].

Owing to its similarities with primary biliary cirrhosis there is some controversy over whether this disorder should be regarded as a subgroup of primary biliary cirrhosis that lacks antimitochondrial antibodies [7, 10, 20], as a separate entity in liver disease [4, 18] or as an overlap syndrome with autoimmune hepatitis [1]. To investigate this disorder further and to find out about the probable mechanism of bile duct destruction, the inflammatory infiltrate from ten cases of autoimmune cholangitis was characterised immunohistochemically. The results were compared with those obtained in ten cases of primary biliary cirrhosis investigated simultaneously.

Materials and methods

Patients were selected for the study when serum levels of alkaline phosphatase and gamma glutamyl transferase had been elevated for more than 6 months, titres of antinuclear and/or smooth muscle antibodies were raised and antimitochondrial antibodies were negative. Patients with signs of infectious or toxic liver diseases were excluded.

All sera were studied for the presence of antimitochondrial antibodies (rodent kidney), antinuclear antibodies (Hep-2 cells; Kallestad, Houston, Tex, HeLa cells), smooth muscle and liver kidney microsomal antibodies (mouse stomach/kidney/skin; Kallestad), by indirect immunofluorescence microscopy. Results were considered positive at antibody dilutions of over 1:40. Antibodies to M2 autoantigens were detected by immunoblotting as described by Lindenborn and colleagues [11].

Liver biopsies were made according to the Menghini technique. Liver tissues were fixed in 7.5% buffered formalin. Sec-

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Table 1 Antibodies used to characterise the inflammatory infiltrate of autoimmune cholangitis and primary biliary cirrhosis.

Antibody	Manufacturer	Species	Dilution	Specificity
CD 3	Dako, Copenhagen/Denmark	Rabbit	1:400	T cells
OPD 4	Dako, Copenhagen/Denmark	Mouse	1:100	Helper/inducer T cells (T4)
CD 8	Dako, Copenhagen/Denmark	Mouse	1:50	cytolytic T cells
L 26	Dako, Copenhagen/Denmark	Mouse	1:200	B cells
Leu 7 (CD 57)	Becton Dickinson, San Jose, Calif.	Mouse	1:10	NK cells
NCAM (CD 56)	Santa Cruz, Santa Cruz, Calif.	Mouse	1:200	NK cells
GB 7	Monosan Burlingame, Calif.	Mouse	1:10	Activated cytolytic T cells
HLA DR CR3/43	Dako, Copenhagen/Denmark	Mouse	1:200	HLA class II expressing cells

tions 4 μm thick were routinely stained with haematoxylin & eosin, Masson trichrome and Perls' Prussian blue.

Histologically, the slides were evaluated for the characteristics of autoimmune cholangitis, such as bile duct lesions and bile duct loss. Bile duct lesions were diagnosed when intraepithelial lymphocytes, rupture of ductular basement membrane, and irregularity of bile duct epithelium (swelling and eosinophilia of the cytoplasm, necrosis/apoptosis of epithelial cells) were observed. Bile duct loss was determined, according to the criteria of Nakanuma and Ohta [16] when portal branches of the hepatic artery were not accompanied by bile ducts. The ductopenia index was calculated from the bile duct/artery ratio in each biopsy specimen. Bile duct loss was recorded when the index was <0.7.

For determination of the stage of disease the classification system of Scheuer et al. [17], primarily set up for primary biliary cirrhosis, was used. In this system, stage 1 is characterised by florid duct lesions, stage 2 by ductular proliferations, stage 3 by scarring and stage 4 by cirrhosis.

In addition, inflammatory activity in portal tracts and lobules was graded as absent (grade 0), minimal (grade 1), mild (grade 2), moderate (grade 3), and severe (grade 4) according to Ludwig [12].

Immunohistochemistry was performed on formalin-fixed, dewaxed, paraffin-embedded serial tissue sections using a panel of eight different primary antibodies as outlined in Table 1. Immunoreactivity was investigated by a three-step immunoperoxidase technique (ABC Elite, Vector) for all antibodies except CD3, for which the Super-Sensitive-Kit (Bio Genex) was applied.

In portal tracts the various subsets of lymphocytes were evaluated on serial sections. One representative portal tract was selected for each case, and within this immunostained lymphocytes were counted using a cross-hatched grid at 400× magnification (0.2 × 0.2 mm) as described by Mosnier et al. [14]. From the numbers of lymphocytes counted per portal tract a semiquantitative score (described in detail in Table 5) was used.

The presence or absence of intraepithelial lymphocytes in bile ducts was determined and recorded. HLA-DR staining intensity was evaluated semiquantitatively with regard to cytoplasmic and membranous reaction patterns, as outlined in Table 5.

Ten liver biopsies from patients with confirmed primary biliary cirrhosis and two specimens of "normal" liver tissue were evaluated in the same way, as controls. For the primary biliary cirrhosis group only patients with the M2 subset of antimitochondrial antibodies and without antinuclear antibodies were selected. Furthermore, all patients had elevated IgM levels with a mean of 614 ± 264 mg/dl.

As normal controls we used needle biopsy specimens obtained from patients in whom tumours were suspected but not found.

For comparison of the mean numbers of lymphocyte subsets infiltrating portal tracts, Student's *t*-test was applied.

Results

The ten patients with autoimmune cholangitis (f/m 9/1; mean age 45.5 ± 11.5 years; range 29–70 years) accounted for 8% of 121 patients with liver morphology of chronic nonsuppurative bile duct destruction seen at our institution over the last 10 years. The serological data for the patients with autoimmune cholangitis are outlined in Table 2. In two patients there were concomitant autoimmune disorders. Patient 9 had Sjögren's syndrome and patient 7, systemic lupus erythematosus. In the control group of patients with primary biliary cirrhosis all patients were female, with a mean age of 57 ± 12 years (range 34–69 years).

Table 2 Auto antibodies in patients with autoimmune cholangitis (*AMA*=antimitochondrial antibodies, *ANA*=antinuclear antibodies, *SMA*=smooth muscle antibodies)

Case	AMA	ANA titres	SMA
1	Neg.	1:640	1: 40
2	Neg.	1:1280	Neg.
3	Neg.	1:640	Neg.
4	Neg.	Neg.	1:320
5	Neg.	1:640	Neg.
6	Neg.	1:1280	Neg.
7	Neg.	1:160	Neg.
8	Neg.	1:800	Neg.
9	Neg.	1:640	Neg.
10	Neg.	1:160	Neg.

Histologically, in both the autoimmune cholangitis group and the primary biliary cirrhosis group all biopsy specimens showed an inflammatory infiltrate of the portal tracts composed of lymphocytes and macrophages (Table 3). In addition, a few granulocytes and plasma cells were occasionally seen. Only one case of autoimmune cholangitis (patient 4) was rich in plasma cells. Other morphological features held to be characteristic of autoimmune hepatitis [5], such as severe interface and lobular inflammation and extensive formation of pseudorosettes, were not observed.

All biopsies showed bile duct loss and/or bile duct lesions (Table 3). Bile duct lesions were confined to interlobular bile ducts larger than 50 μm and were accompanied by epitheloid granulomas in three cases of autoimmune cholangitis and in five cases of primary biliary cirrhosis.

The mean numbers of the various subsets of lymphocytes per portal tract are outlined in Table 4. In all but 1 case of autoimmune cholangitis and primary biliary cirrhosis, T lymphocytes were predominant in the inflammatory infiltrate. Lymphocytes were generally scattered diffusely throughout the portal tracts with no preferential anatomical distribution.

Table 3 Morphological features comparing 10 cases of autoimmune cholangitis with 10 control cases of primary biliary cirrhosis. Portal (*PI*) and lobular (*LI*) inflammation were graded according to Ludwig [12], stage (S) was graded according to Scheuer [17], bile duct loss was considered when the ductopenia index (*DI*) was below 0.7. (*BDL* bile duct lesion, *IEL* intraepithelial lymphocytes, *EG* epitheloid granuloma, + present, – absent)

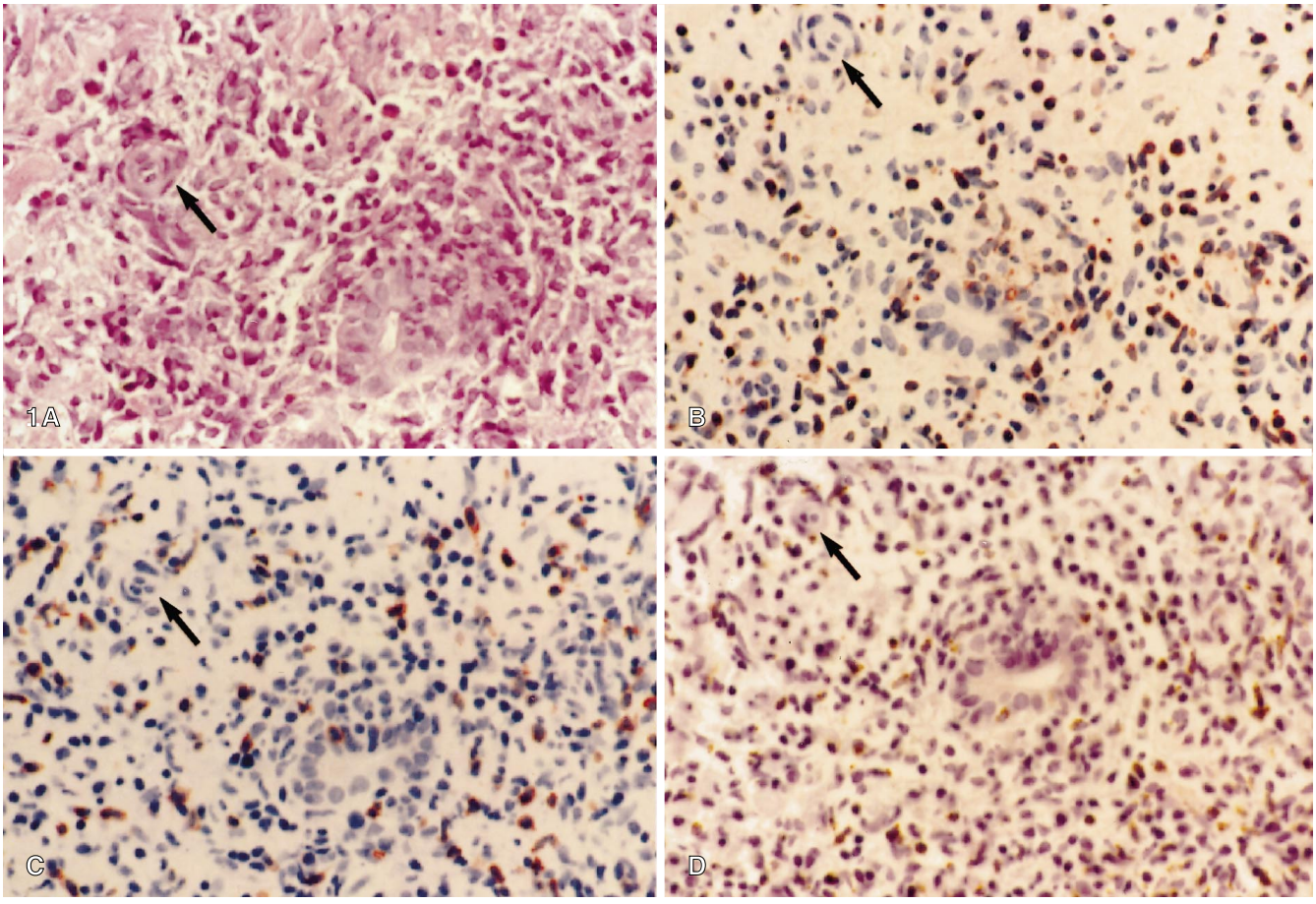
Case	PI	LI	BDL	IEL	DI	EG	S
Autoimmune cholangitis							
1	1	1	+	CD 3, CD 8, GB	0.60	+	2
2	2	1	+	CD 3, OPD 4, CD 8, GB	0.93	–	2
3	2	0	+	CD 3, OPD 4, CD 8, GB	0.50	–	3
4	2	2	+	CD 3, OPD 4, CD 8, GB, L26	0.84	+	2
5	1	0	–	CD 3	0,32	–	2
6	2	0	+	CD 3, GB	0.61	+	2
7	1	0	–	CD 3, CD 8	0.28	–	1
8	1	1	–	–	0.00	–	1
9	2	0	–	CD 3, OPD 4	0.55	–	2
10	1	0	–	CD 3, OPD 4, CD 8	0.66	–	1
Primary biliary cirrhosis							
1	2	0	+	CD 3, GB	0.75	–	2
2	1	0	+	CD 3, OPD 4, CD 8, GB	0.57	+	1
3	1	1	+	CD 3, CD 8, GB	0.83	+	1
4	2	2	+	CD 3, OPD 4, CD 8	0.80	+	2
5	2	0	–	CD 3, OPD 4, CD 8	0.28	–	4
6	1	1	+	CD 3, OPD 4, CD 8, GB, L 26	0.71	+	1
7	2	0	–	OPD 4, CD 8	0.50	–	3
8	2	0	+	CD 3, OPD 4, CD 8, GB, L 26	0.75	+	2
9	2	1	+	CD 3, OPD 4, CD 8, GB	0.70	–	3
10	2	1	+	CD 3, OPD 4	0.80	–	3

Table 4 Comparison of the means of various subsets of lymphocytes infiltrating portal tracts of autoimmune cholangitis, primary biliary cirrhosis and normal liver tissue (*AIC* autoimmune cholangitis, *PBC* primary biliary cirrhosis)

Lymphocyte subsets	AIC (mean number of infiltrating cells (*n*=10)	PBC (mean Number of infiltrating cells (*n*=10)	Normal liver tissue (mean number of infiltrating cells (*n*=2)
CD3	102.5±55.	96.9±56..	10.5±2..
OPD4	72.9±44.	81.5±38..	3.5±0..
CD8	43.2±24.8	40.4±26.0	10.0±7.0
GB7	14.3±8.2	13.4±9.5	2.5±0.7
L26	25.7±30.4	19.7±23.0	1±1.4
L26/CD3 ratio	0.2±0.4	0.1±0.2	0.1±0.1
OPD4/CD8 ratio	1.9±1.2	2.4±1.2	0.3±0.2

Table 5 Semiquantitative evaluation of lymphocyte subsets infiltrating portal tracts (+++ >70 lymphocytes per portal tract; ++ 21–70 lymphocytes per portal tract; + 1–20 lymphocytes) and semiquantitative evaluation of HLA-DR staining of bile duct epithelia (++ strong diffuse and membranous staining; + weak diffuse and membranous staining) with regard to the presence (+) or absence (–) of bile duct lesions (*BDL*). Comparison of the ten cases of autoimmune cholangitis with the ten control cases of primary biliary cirrhosis

Case	BDL	CD 3	OPD 4	CD 8	GB	L 26	Leu 7	HLA-DR
Autoimmune cholangitis								
1	+	+++	+++	+++	++	++	+	+
2	+	+++	+++	++	++	++	+	++
3	+	+++	++	+++	+	++	+	+
4	+	++	++	+	+++	+++	+	++
5	–	+++	++	++	+	+	+	–
6	+	+++	+++	++	++	+	+	+
7	–	++	+	+	+	–	–	–
8	–	++	++	++	+	+	+	–
9	–	+++	+++	+++	+	+	+	–
10	–	+++	+++	++	+	+	–	–
Primary biliary cirrhosis								
1	+	++	++	++	+	+	+	+
2	+	+++	+++	+++	++	++	+	++
3	+	+++	++	++	++	+	+	+
4	+	++	++	++	++	+	+	++
5	–	+++	+++	++	+	++	+	–
6	+	+++	+++	++	++	+++	+	+
7	–	++	++	+	++	–	+	–
8	+	+++	+++	+++	+	+	+	+
9	+	++	++	++	+	+	+	–
10	+	+++	+++	++	+	+	+	–



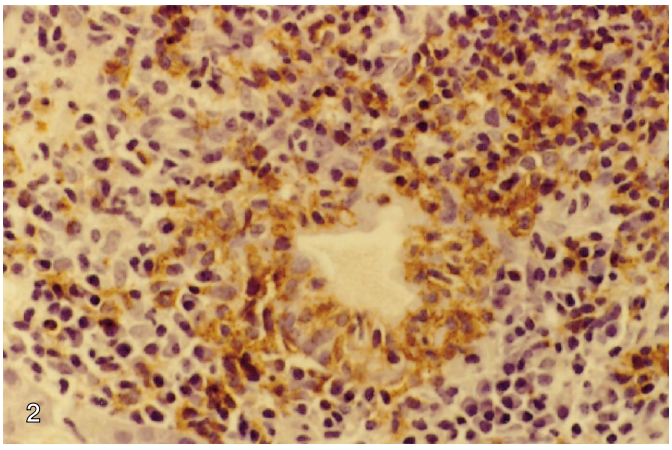


Fig. 1A–D Lymphocytes infiltrating portal tract and altered bile duct epithelium in a case of autoimmune cholangitis; serial sections at 400× magnification (*arrows* mark identical portal artery in each section). **A** H&E; **B–D** immunohistochemistry: **B** CD 3, **C** CD 8, **D** GB 7

Fig. 2 Bile duct lesion of patient 4: L 26 positive lymphocytes surrounding and infiltrating altered bile duct epithelium. ×400

Granzyme B7-positive lymphocytes were found in moderate numbers in all 20 cases of autoimmune cholangitis and primary biliary cirrhosis.

Generally, L26-positive B lymphocytes were seen in all cases as an element of the inflammatory infiltrate. In one case of autoimmune cholangitis (patient 4) a predominance of B lymphocytes, with a B/T ratio of 1.44, was observed. In this case B lymphocytes were concentrated around bile ducts, infiltrating the bile duct epithelium (Fig. 2). The density of the various subsets of lymphocytes within portal tracts is shown in Table 5.

Leu 7 (CD57)-positive NK/K cells were only seen in low numbers. No NCAM-positive NK lymphocytes were detected.

Within the bile ducts GB7-positive activated cytolytic intraepithelial lymphocytes were observed exclusively in association with duct lesions. In these cases CD3-positive pan-T lymphocytes, CD8-positive cytolytic and OPD4-positive T lymphocytes (Table 3, Fig. 1) were detected additionally within bile duct epithelia. In the absence of bile duct lesions GB7-positive intraepithelial lymphocytes were not detected. In these cases intraepithelial lymphocytes consisted mainly of CD3-positive

cells. In addition, in patient 4 in the autoimmune cholangitis group, L26-positive intraepithelial B lymphocytes were found (Fig. 2).

In no case did any of the intraepithelial lymphocytes show reactivity with Leu7 and NCAM antibodies directed against NK cells.

In autoimmune cholangitis and in primary biliary cirrhosis, HLA DR expression of the bile duct epithelia was seen exclusively in association with bile duct destruction and was confined to interlobular bile ducts of intermediate size and with membranous and diffuse cytoplasmic reaction patterns (Table 5).

The results recorded in the two cases of "normal" liver tissue are summarised in Table 4. No intraepithelial lymphocytes or bile duct lesions were observed at all.

With regard to the mean numbers of lymphocyte subsets infiltrating portal tracts no significant differences between autoimmune cholangitis and primary biliary cirrhosis were found.

Discussion

In 1987 Brunner and Klinge reported three female patients in whom clinical and morphological features resembled those of primary biliary cirrhosis, although they were negative for antimitochondrial antibodies and positive for antinuclear antibodies [2]. They termed this disorder "Immuncholangitis". Since then, similar cases have been reported and various other terms have been introduced such as autoimmune cholangiopathy [1, 3] primary autoimmune cholangitis [18] and autoimmune cholangitis [7, 10].

According to the literature, the diagnosis of autoimmune cholangitis is made in patients with clinical and laboratory features of cholestasis and a histology of chronic destructive nonsuppurative cholangitis in the absence of antimitochondrial antibodies [1, 2, 7, 10, 13, 18]. Circulating antinuclear and/or smooth muscle autoantibodies confirm the diagnosis of autoimmune cholangitis serologically. In our study all the cases investigated fulfilled these diagnostic criteria.

As it is not possible to differentiate between autoimmune cholangitis and primary biliary cirrhosis on conventional morphological grounds [7, 10, 13, 18], our intention in this study was to find whether there is a difference between the two entities in the composition of the inflammatory infiltrate leading to bile duct destruction.

Basically, several immune mechanisms are known to be able to mediate bile duct injury [20]. For primary biliary cirrhosis and hepatic allograft rejection T-cell-mediated cellular cytotoxicity is commonly postulated as the responsible mechanism of epithelial destruction. This is based on the facts that T cells are usually predominant in the lymphocytic infiltrate [9, 20], that CD 8+ cytolytic T lymphocytes infiltrate biliary epithelia in a manner analogous to the piecemeal necrosis seen in chronic active hepatitis [21] and that bile duct epithelia aberrantly express HLA class II molecules on the cell surface [20]. CD3-positive T lymphocytes have been found as the predominant lymphocyte subset surrounding bile ducts in a rodent model of immune-mediated cholangitis [19].

In the present analysis predominance of T cells in portal tracts and intraepithelial CD3 + pan-T lymphocytes were found in almost all biopsies from patients with autoimmune cholangitis. In addition, HLA class II molecules (HLA DR+) were expressed by bile duct epithelia in association with bile duct destruction.

Antibodies directed against Granzyme B7 were used to differentiate between activated and inactive cytolytic T lymphocytes possibly involved in bile duct destruction. Granzyme B is a neutral serin protease found in the granules of activated cytolytic T cells, natural killer cells, and lymphokine-activated killer cells. Heusel et al. have shown that granzyme B has a critical role in the induction of target cell DNA fragmentation and apoptosis by activated cytolytic T cells [8]. Therefore, in addition to CD8 antibodies, antibodies against granzyme B are helpful in differentiating between inactive and activated cytolytic T cells. In our series granzyme B-positive cells were found within portal tracts and within bile duct epithelia, particularly in cases with bile duct lesions. These results suggest a possible T-cell-mediated immune response as the main mechanism responsible for bile duct destruction. The finding that Leu7- or NCAM-positive NK/K cells were never seen among intraepithelial lymphocytes further supports this idea.

In addition, predominance of T lymphocytes within the inflammatory infiltrate and the presence of macrophages transforming into granulomas are thought to be characteristic of type IV delayed-type hypersensitivity reactions [6]. In these reactions predominance of CD 4-positive T cells over CD8-positive lymphocytes is reported. By using OPD4 antibodies to identify CD4 helper/inducer cells, we found that the OPD4-positive lymphocytes outnumbered CD8-positive cells in all cases of autoimmune cholangitis. Although this antibody is not entirely specific in identifying CD4 subsets, it clearly indicates that helper/inducer cells prevail over cytolytic T lymphocytes.

In patient 4 the inflammatory infiltrate was rich in plasma cells, with predominance of L26-positive lymphocytes. Serologically, smooth muscle antibodies were detected. Plasma cell predominance is one of the morphological features held to be characteristic of autoimmune hepatitis, so that an overlap syndrome with autoimmune hepatitis had to be ruled out. However, the IgG levels were normal and no other morphological features suggestive of autoimmune hepatitis were observed. Furthermore, the patient did not respond to a steroid trial. The observations in this patient rather indicate that humoral immune mechanisms might contribute to bile duct destruction, as already found in some cases of primary biliary cirrhosis [15].

In the present study no significant differences were found between the immunohistochemical findings in autoimmune cholangitis and in the cases of primary biliary cirrhosis. Therefore, it is suggested that the same mecha-

nisms of T-cell-mediated cellular cytotoxicity are responsible for bile duct destruction in both diseases, and that the presence of antinuclear antibodies in the absence of antimitochondrial antibodies remains the only difference between these two forms. The fact that antinuclear antibodies can also be found in up to 50% of cases of primary biliary cirrhosis might indicate that autoimmune cholangitis merely represents a subset of primary biliary cirrhosis, namely an antimitochondrial antibody-negative type.

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