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Chapter IV

Immunohistochemical analysis of inflammation in primary sclerosing cholangitis

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Chapter IV

SUMMARY

Objectives: There are only limited data about the nature of the mononuclear infiltrate surrounding the affected biliary canaliculi in primary sclerosing cholangitis (PSC). The aim of this study was to characterise the composition of the mononuclear infiltrate and to detect signs of activation/proliferation among the various subpopulations involved. Furthermore the putative role of the biliary epithelium as antigen presenting cells (APC) is assessed.

Methods: Liver biopsies of 14 PSC patients were analysed. Seven liver specimens of non-inflammatory liver disease (NIL) patients with hepatocellular carcinoma or metastasis from colorectal carcinoma as well as eight liver biopsies of primary biliary cirrhosis (PBC) patients, served as controls. Paraffin embedded material was stained with GB7, anti CD3, anti CD20, anti CD45RO. Deep frozen sections were stained with anti CD4, anti CD8, anti CD25, anti CD86, anti HLA-DR, anti IFNγ, anti IL4, anti ICAM1 and anti α4β7. Stainings were scored by 2 pathologists using a semiquantitative scale.

Results: The portal infiltrate was found to consists mainly of CD3+CD45RO+ cells. Few cells expressed activation or proliferation markers in any of the liver specimens. In the PSC-material, significantly more of the infiltrative lymphocytes were positive for the integrin α4β7, as compared to hardly any positive cells in the NIL-group (p<0.001) and < 10% in the PBC-specimens (p<0.01). Variable HLA-DR expression of the biliary epithelium was observed in all groups, however without co-expression of ICAM1 or B7.2.

Conclusions: The portal infiltrate in PSC liver histology specimens appears to consist mainly of non-activated memory T-lymphocytes, a substantial proportion of which express the gut-homing integrin α4β7. An antigen-presenting role for the biliary epithelium could not be demonstrated.

Key words: Primary sclerosing cholangitis; Immunohistochemistry; Histology.
INTRODUCTION

Primary sclerosing cholangitis (PSC) is a rare, chronic disease characterised by inflammation of the intra- and extrahepatic bile ducts leading to progressive sclerosis. Clinically, the disease is manifested by signs of intermittent jaundice, cholestasis, pruritus, fatigue, and sometimes cholangitis, ultimately leading to biliary cirrhosis. In approximately 10% of cases, the course of the disease is complicated by the development of cholangiocarcinoma.

The etiology and pathogenesis of PSC are unknown, but increasing evidence indicates that PSC is an autoimmune or immune dysbalance disorder. In the past 20 years, only a handful of papers regarding cellular immune abnormalities in PSC have been published. In their classic paper from 1981, Ludwig et al. described the intense lymphocyte infiltration in enlarged portal areas and around bile ducts (1). Whiteside and co-workers were the first to determine lymphocyte subpopulations by immunohistochemistry (2). The portal infiltrate appeared to consist for at least 80% of T-cells in their series of rather advanced cases of PSC. There was a slight preponderance of CD4 over CD8 positive T-cells (1.26±0.1 SE). Only few T-cells appeared activated, as determined by the expression of the IL-2 receptor or HLA DR.

PSC is often diagnosed in conjunction with ulcerative colitis, and less often with Crohn’s disease. In these inflammatory bowel diseases (IBD) T-lymphocytic infiltrates are a prominent feature and are regarded to play a key role in the pathogenesis. Normally, lymphocytes continually patrol the body for foreign antigen by recirculating from blood, through tissue, into lymph, and back to blood. T-memory cells acquire an ability to recirculate through the tissues in which they first encountered foreign antigen (3, 4).

A certain subset of T-memory cells that has specific gut-homing properties has recently been described (5). These CD45RO + lymphocytes express the integrin α4β7 (6) and their primary addressin is MAdCAM-1 (7, 8).

The presence of so-called 'gut-homing' lymphocytes in the hepatic inflammation in PSC has so far not been assessed.

Normal bile duct epithelium expresses HLA class-I antigens but not HLA class-II antigens (9). In two studies, HLA-DR expression was shown on biliary epithelial
cells from PSC patients (10, 11). Potentially, these HLA class-II molecules can present a putative allo- or autoantigen to class-II restricted T-lymphocytes, thus initiating an inflammatory response. However, it should be noted that HLA-DR was also found on bile duct epithelium in patients with extrahepatic obstruction (9, 10).

To induce T-cell stimulation, HLA-DR expression on an antigen presenting cell (APC) is not enough. Binding of one or more co-stimulatory molecules such as ICAM-1 or B7.2 on the APC with their ligand on the T-cell surface is needed for effective stimulation.

The purpose of this study was to further characterise the mononuclear infiltrate in native liver biopsy specimens of PSC patients by using immunohistochemical stainings. In addition, the presence of gut-homing lymphocytes was determined. Furthermore, the putative role of the biliary epithelium as APC's was studied by determination of HLA-DR staining and the presence of the co-stimulatory molecules ICAM-1 and B7.2.

MATERIALS AND METHODS

Patients
Liver biopsy specimens (Quick Core 14 gage, William Cook, Bjaerverskov, Denmark) of 10 male and four female patients with a diagnosis of PSC established by endoscopic retrograde cholangiography and/or histology were used. Mean age was 40 ± 10 years, and 11/14 were known with IBD. The median duration of disease from diagnosis was 1 year (range 0-16). All patients had biochemical evidence of cholestasis. No patient had Child-Pugh B/C liver disease and none used ursodeoxycholic acid or other drug therapy for PSC. Histological staging was done according to the Ludwig scoring (1). Staging of the 14 PSC samples was as follows: stage I: n=5, stage II: n=0, stage III: n=7, stage IV: n=2.

Seven liver resection specimens of non-inflammatory liver disease (NIL) patients with hepatic carcinoma or metastasis from colorectal carcinoma, as well as eight biopsies from primary biliary cirrhosis (PBC) patients, served as controls. The PBC control group was made up of three stage II, two stage III, and three stage IV graded liver biopsies. None of these used immunosuppressants and five were on ursodeoxycholic acid therapy.

Immunohistochemistry
Liver specimens were stored embedded in paraffin or deep frozen at -80°C. Paraffin
slices were deparaffinized in xylene for 15 minutes and rehydrated in ethanol. Endogenous peroxidase was blocked by 0.3% H2O2 in methanol for 20 minutes. Table 1 shows the various antigens that were stained, as well as the main cell type they are expressed on, and a brief outline of their function. For CD3 staining, enzymatic pretreatment was performed using 0.25% pepsin in 0.01 M HCl for 15 minutes at 37°C. Granzyme B7 (GB7) slices were heated for 10 minutes at 100°C followed by immersion in 10 mM citrate buffer at pH 6.0 to unmask the antigen. The primary antibodies for the paraffin slices were antiCD3 (DAKO, Glostrup, Denmark), antiCD45RO (DAKO), GB7 (Monosan, Uden, The Netherlands), and antiCD20 (DAKO).

As secondary antibody to CD20, CD45RO and GB7 rabbit anti mouse, and for CD3 swine anti rabbit biotinylated streptavidin horse radish peroxidase were used. Detection was rendered by 3,3 diaminobenzidine, which yields a brown staining. Deep frozen slices were dried overnight and fixed for 10 minutes in acetone at room temperature. The primary antibodies antiHLA-DR (Becton & Dickinson, San Jose, CA, USA), antiCD4 (Becton & Dickinson), antiCD8 (Becton & Dickinson), antiCD25 (DAKO), antiCD86 = antiB7.2 (PharMigen, San Diego, CA, USA), antiICAM-1 (Monosan), antiInterferon-γ (anti-IFNy) (Genzyme, Cambridge, MA, USA), antiIL4 (Genzyme), and antiC4ß7 = Act-1 (Leukocyte, Boston, MA, USA) were incubated in phosphate buffered saline for 60 minutes. Endogenous peroxidase was blocked with 0.3% H2O2 in PBS for 10 minutes.

As secondary antibody rabbit anti mouse/HRP was used. Visualisation was obtained by amino-ethyl carbazole/H2O2, which results in a red staining.

One tonsillary biopsy served as positive control for the antiCD25 staining. All specimens were scored by two pathologists, using a semi-quantitative scale, ranging from 0 = no positive cells, 1 = < 10% staining cells, 2 = 10-50% positive cells, 3 = more than 50% positive staining cells, as percentage of the total population of mononuclear infiltrative cells or bile duct cells per portal tract in at least half of the portal tracts present in the slice. The relative contributions of CD4- versus CD8-positive cells were scored using two serial slices. The scoring was either CD4 < CD8, CD4 = CD8, or CD4 > CD8.

Statistics

Statistical calculations were performed using the SPSS package version 6.1, (SPSS Inc., Chicago, IL, USA). Comparisons between groups were done using Mann-Whitney U testing and within groups applying Wilcoxon rank sum testing.
Table 1.
Reminder of human leukocyte differentiation antigens to which specific antibodies were directed

<table>
<thead>
<tr>
<th>antigen</th>
<th>main cell types</th>
<th>designation</th>
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<tbody>
<tr>
<td>CD3</td>
<td>T-cell</td>
<td>T-cell receptor (TCR)</td>
</tr>
<tr>
<td>CD4</td>
<td>T-helper cells</td>
<td>co-recognition of MHC-II with TCR</td>
</tr>
<tr>
<td>CD8</td>
<td>T-suppressor/cytotoxic</td>
<td>co-recognition of MHC-I with TCR</td>
</tr>
<tr>
<td>CD20</td>
<td>B-cells</td>
<td>B-cell activation/proliferation</td>
</tr>
<tr>
<td>CD25</td>
<td>activated T-cells</td>
<td>IL-2 receptor, binding leads to proliferation of T-cells</td>
</tr>
<tr>
<td>CD45RO</td>
<td>mature T-cells</td>
<td></td>
</tr>
<tr>
<td>CD86</td>
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<td>co-stimulatory molecule</td>
</tr>
<tr>
<td>IL4</td>
<td>T-helper2 cells</td>
<td></td>
</tr>
<tr>
<td>IFNγ</td>
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<tr>
<td>GB7</td>
<td>T-cytotoxic cells</td>
<td></td>
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<tr>
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<td>Macrophages</td>
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<td>ICAM-1</td>
<td>Endothelium</td>
<td>addressin</td>
</tr>
<tr>
<td>α4β7</td>
<td>memory T-cells</td>
<td>gut-homing lymphocyte integrin</td>
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RESULTS

All PSC biopsies contained an adequate number (i.e. at least three) of portal tracts, but in 22 out of 224 slices an assessment of a specific staining could not be made due to the absence of sufficient portal tracts in that specific slice. In two of eight PBC biopsies assessment of positive staining of biliary epithelial cells could not be made due to the absence of original bile ducts. The NIL material contained adequate numbers of portal tracts exhibiting no or slight infiltration.

All PSC specimens showed a mononuclear portal infiltrate of moderate to strong intensity in the hematoxylin-eosin staining. The control slices showed slight to moderate infiltration in the portal tracts. Figure 1 depicts the semi-quantitative distribution of the various cell types constituting the mononuclear portal infiltrate. In the PSC material the mononuclear infiltrative cells were almost all lymphocytes as ascertained with CD3 and CD20 stainings, with the vast majority being T-cells as compared to B-cells (mean rank 7.5 versus 0.0, p < 0.002, Wilcoxon). Almost all
lymphocytes stained positive for CD45RO, (Fig 2). There was a preponderance of CD4 lymphocytes over CD8 positive cells, though not significantly (mean rank 2.67 versus 2.00, p = 0.27, Wilcoxon).

There were no signs of activation of the lymphocytic infiltrate as indicated by the paucity of positive staining for CD25, GB7, IL4 and IFNγ. Although only a minority of the CD4 positive cells stained with antiIL4 and antiIFN-γ, there seemed to be a preponderance of T-helper1 versus T-helper2 lymphocytes (mean rank 4.5 vs 0.0, p < 0.01, Wilcoxon).

Fig 1. Composition of the mononuclear portal infiltrate in the PSC group. See text and table 1 for explanation of CD markers. Medians depicted by horizontal line. Comparisons by Wilcoxon signed rank test.
In seven out of nine assessable portal tracts in the PSC biopsies the Act-1 staining scored 10-50 % or more, compared to virtually nil in the NIL group (mean rank 11.89 versus 4.14, p<0.001, Mann Whitney U), and < 10 % in the PBC specimens (mean rank 11.89 versus 5.75, p<0.01, Mann Whitney U). (Fig 3). There was no apparent association between the presence or number of Act-1 staining lymphocytes and comorbidity of IBD, but numbers were actually too small to draw any conclusions.
The bile duct epithelium.

The biliary epithelium of all PSC specimens expressed variable degrees of staining with anti HLA-DR, as did the NIL-control material. However, hardly any expression of ICAM-1 and B7.2 was found on the biliary epithelium in the PSC specimens or the controls, (Fig. 4).

For the PBC-controls, too few original bile ducts were present to reliably assess the ICAM-1 and B7.2 stainings.
DISCUSSION

The etiopathogenesis of PSC is still largely unknown. One of the steps to unravel the pathogenesis of this disease is to closely characterise the cellular infiltrate around the involved bile ducts.

Our findings indicate that in PSC the portal infiltrate mainly consists of T-cells with a slight preponderance of T-helper cells over T-suppressor/cytotoxic cells. This is in keeping with the results of Whiteside and co-workers (2). Surprisingly, we could not demonstrate signs of activation of these inflammatory cells by four different immunohistochemical markers for T-cell activation, not even in the 5 Ludwig stage I graded biopsies. This suggests that the inciting immunological noxious event may precede the histological damage by quite some time.

There is one paper in which the composition of the mononuclear portal infiltrate in children was studied (12). The results are in contrast to our findings in that up to 75
% of mononuclear cells expressed the IL-2 receptor (CD25). One possible explanation for this discrepancy is that these children may have had more active disease at the time of the liver biopsy, or that they may have been on the whole in an earlier phase of disease development.

Our finding that about 10-50% of the infiltrative cells stained positive for the gut-homing integrin α4β7 compared to only occasional staining in the NIL- and PBC-controls respectively, may be of particular interest. The link between IBD and PSC has up to now not been elucidated. If there is indeed an abnormal accumulation of gut-homing lymphocytes in PSC, this may point to a common pathophysiologic role for these T-lymphocytes belying both diseases. That α4β7-expressing T-cells may actually have clinical significance in IBD is highlighted by the study of Hesterberg et al., who demonstrated rapid resolution of chronic colitis in the cotton-top tamarin using an antibody to α4β7 (13).

Our study confirms the presence of HLA-DR expression on the biliary epithelium of PSC patients, as reported earlier (10, 11). HLA-DR molecules are capable of presenting allo- or autoantigens to class-II restricted T-helper lymphocytes, who in turn may initiate an immune response. For effective T-cell stimulation, presentation of an antigen attached to an HLA-DR molecule on an antigen presenting cell (APC) to the T cell receptor of a CD4 positive T-cell is not enough. One or more co-stimulatory bindings, such as between intercellular adhesion molecule-1 (ICAM-1) on the APC and lymphocyte function-associated antigen-1 (LFA-1) on the T-cell, or between B7,1/B7,2 on the APC and CD28/CTLA-4 on the T-cell, are necessary for induction of activation or proliferation (14).

Two previous studies have determined the amount of ICAM-1 expression on biliary epithelium of PSC patients. Adams et al. found an increased expression of ICAM-1 on bile ducts in end-stage PSC patients (15). In contrast, Broomé and colleagues found no ICAM-1 expression despite the presence of HLA-DR on bile ducts of PSC patients with moderately advanced disease (16). The discordance with the former study was explained by the differences in fibrotic and inflammatory activity. Leon and co-workers did not find B7.1 or B7.2 expression in liver sections from patients with PBC or allograft rejection (17). We found HLA DR expression of the biliary epithelium in our PSC samples, but could not demonstrate co-expression of the co-stimulatory molecules ICAM-1 and B7.2. Although we did not test for all the known co-stimulatory molecules (for example B7.1), these findings suggest that the
Biliary epithelial cells do not function as APC's. The HLA-DR expression should then be regarded as an epiphenomenon secondary to biliary obstruction, as has been suggested earlier (9, 10). Alternatively, interaction between MHC-II bound antigen with T-cells in the absence of co-stimulation may lead to subsequent unresponsiveness or apoptosis (14, 18). In this sense down-regulation of B7 expression on the biliary epithelial cell might provide a self-defence mechanism by which chronic activation of MHC-II restricted T-lymphocytes is circumvented. Although this remains speculative, it would also explain the apparent absence of markers of activation of the T-cellular infiltrate.

In conclusion, our data indicate that the mononuclear portal tract infiltrate in PSC patients mainly consists of mature T-lymphocytes, which are not activated. There appears to be abnormal presence of gut-homing lymphocytes in the portal infiltrate as compared to controls.

An antigen presenting role for the biliary epithelium could not be demonstrated.

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References


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biliary epithelial cells do not function as APC's. T cells may still recognize peptides derived from MHC class I antigens presented on APC's. These observations suggest that T cells in the liver are capable of recognizing both endogenous and exogenous antigens presented by APC's and that these antigens can be processed and presented by hepatocytes. The recognition of endogenous antigens by T cells in the liver may be important in the pathogenesis of autoimmune liver diseases such as autoimmune hepatitis and primary biliary cirrhosis.