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EXPRESSION OF CO-STIMULATORY FACTOR B7-2 ON THE INTRAHEPATIC BILE DUCTS IN PRIMARY BILIARY CIRRHOSIS AND PRIMARY SCLEROSING CHOLANGITIS: AN IMMUNOHISTOCHEMICAL STUDY

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SUMMARY

Co-stimulatory factors B7-1 (CD80) and B7-2 (CD86) and their ligands, including CD28, are important for the efficient presentation and persistence of an antigen-specific immune reaction. Hitherto, there has been a paucity of data on the roles of such co-stimulatory factors in immune-mediated biliary diseases. In this investigation, the hepatic immunohistochemical expression of B7-1 and B7-2 has been studied, with emphasis on intrahepatic biliary epithelia, using wedge biopsies from 22 patients with primary biliary cirrhosis (PBC), seven with primary sclerosing cholagitis (PSC), and, as controls, eight cases of extrahepatic biliary obstruction, eight of chronic viral hepatitis C, and three histologically normal livers. In 10/22 (45 per cent) patients with PBC and 3/7 (43 per cent) patients with PSC, B7-2, but not B7-1, was expressed on the epithelial cells of small intrahepatic bile ducts and bile ductules. This expression was manifest as diffuse but variable cytoplasmic staining. Such B7-2-positive bile ducts were not seen in controls. Positive staining was found only in the early stage of PBC and PSC. In PBC and PSC, almost all lymphocytes in the portal tracts, including those around the damaged bile ducts, were positive for CD28, a ligand of B7-2. These results suggest that B7-2 expression on biliary epithelial cells is involved in antigen presentation and perhaps in bile duct destruction in PSC and PBC. © 1998 John Wiley & Sons, Ltd.

KEY WORDS—**co-stimulatory factor; B7-2; primary biliary cirrhosis; primary sclerosing cholangitis; intrahepatic small bile ducts**

INTRODUCTION

Primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) are both histologically characterized by progressive destruction or obliteration of intrahepatic bile ducts, probably mediated by autoimmune processes.[1–11] It is believed that antigen-presenting cells (APCs), including dendritic cells (DCs) or macrophages that infiltrate the bile ducts and periductal tissue in PBC, are also important in the recognition and presentation of bile duct-related antigen(s) to T cells.[10–12] In addition, an aberrant and increased expression of MHC class I and class II and intercellular adhesion molecule-1 (ICAM-1) are known to be present on damaged biliary epithelial cells.[7–11] This suggests that bile ducts are involved in antigen presentation and recognition and are attacked by cytotoxic T cells. These data strongly suggest that the bile ducts themselves may present autoantigen(s) to infiltrating T lymphocytes through contact between T-cell receptor (TCR) and MHC molecules.

Other co-stimulatory factors have been shown to promote antigen presentation and recognition by helper T cells.[13–15] In particular, CD28 and CTLA4 act as co-stimulatory signal receptors on T cells. The natural ligands for CD28 and CTLA4 are members of the B7 family, including B7-1 (CD80, BB1 or B7), B7-2 (CD86, B70), and possibly B7-3 which are all expressed on APCs.[13–16] Recently, Leon *et al.*[17] and Kaji *et al.*[18] reported that B7-2 was strongly expressed on dendritic cells, macrophages, and activated B cells around the damaged bile ducts of PBC. This finding was important, because earlier work had failed to demonstrate B7-1 on damaged bile ducts in PBC.[7] Because of this discrepancy, we studied, using immunohistochemistry, the expression of B7-1 and B7-2 and their receptor CD28 in liver samples from PBC, PSC, extrahepatic biliary obstruction, and chronic viral hepatitis and in normal livers.

MATERIALS AND METHODS

Liver specimens and tissue preparation

In a preliminary study, we determined that formalin-fixed, paraffin-embedded sections were suitable for the immunohistochemical detection of B7-1 and B7-2 after microwave treatment, using a standard avidin–biotin detection system. Only frozen sections were satisfactory for the immunostaining of CD28. In the case of B7-1 and B7-2, histological preservation was better and more readily visualized in formalin-fixed sections.

For the demonstration of B7-1 and B7-2, wedge biopsy liver specimens from 22 patients with PBC and from seven patients with PSC were studied; 17/22 cases of PBC were classified as stage I or II (early stage) and the remaining 5/22 livers as stage III or IV (late stage) according to Scheuer's histological staging.[1] Four out of

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seven cases of PSC were classified as stage I or II (early stage) and the remaining 3/7 as stage III or IV according to Ludwig's staging.[19] Wedge liver biopsy specimens from eight patients with extrahepatic biliary obstruction (EBO), eight patients with chronic viral hepatitis type C (CVH-C), and three histologically normal livers were used as controls. The diagnosis in all cases was based on clinical and laboratory data and was confirmed histologically. Normal liver biopsies were obtained from patients with mild hepatic dysfunction during abdominal surgical procedures. All liver tissues were fixed in 10 per cent neutral buffered formalin and then embedded in paraffin. Approximately 10 sections, 4 μm in thickness, were cut from each paraffin block.

For the immunostaining of CD28, wedge biopsy specimens were used from six PBC patients, all early stage. As controls, wedge liver biopsy specimens from four patients with EBO, six with CVH-C, and two with histologically normal livers were used. All were embedded in optimal cutting temperature compound (OCT) (Miles Inc., Elkhart, IN, U.S.A.) and snap-frozen in liquid nitrogen. Several frozen sections, 5 μm in thickness, were cut with a cryostat and stored at -80°C until use.

Primary monoclonal antibodies and immunohistochemistry

A standard avidin–biotin detection system was used for the immunostaining of B7-1, B7-2, and CD28. As primary antibodies, we used a mouse monoclonal antibody against anti-human B7-1 (clone 307.4, Becton-Dickinson Inc., San Jose, CA, U.S.A.), a mouse monoclonal antibody against anti-human B7-2 (clone IT2.2, Pharmingen Inc., San Diego, CA, U.S.A.), and a rat monoclonal antibody against human CD28 (clone YTH 913.12, Serotec, Kidlington, U.K.). Optimal concentrations, and controls were used throughout.

Briefly, after standard microwave treatment[20] and incubation in normal horse serum[18] (Sigma Chemical, St Louis, MO, U.S.A.) the deparaffinized sections were incubated with primary antibodies, anti-BB1/B7 (1:20), or anti-B70 (B7-2) (1:5) overnight at 4°C. Then all slides were washed several times in Tris-buffered saline (TBS), followed by a 30 min incubation with biotinylated horse anti-mouse IgG (heavy and light chain) (Vector Lab., Burlingame, CA, U.S.A.; 1:200). Alkaline phosphatase-conjugated streptavidin–biotin complex (AB Complex/AP) (Dako, Glostrup, Denmark) was freshly prepared and applied to all sections for 30 min at room temperature. After washing with TBS, a solution of Vector Red or Vector Blue kit (Vector Lab) was applied and incubated for 10–15 min. Levamisole was added to the solution to block endogenous alkaline phosphatase activity. After three washes in distilled water, all sections were observed with a light microscope. Using the Vector Red Kit, the reaction products were visualized as red, while the reaction products were blue when the Vector Blue kit was applied.

The Vector Red product can also be visualized as a bright red fluorescent precipitate under a fluorescence microscope. These sections were viewed with the LSM 410 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany), using an argon laser (514 nm), a scanning speed of 8·05 s and final photography with a Polaroid camera (Camera Back L-III, Avio, Tokyo, Japan).

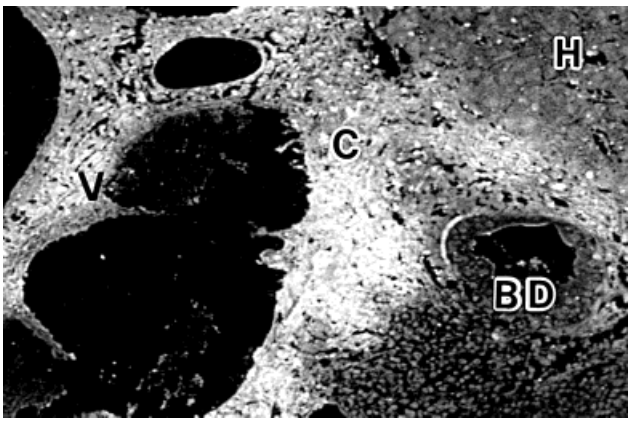

Fig. 1—Immunostaining for B7-1 in PBC viewed by confocal laser microscopy. B7-1 is detectable only in the connective tissue (C) of the portal tract, while it is not expressed on hepatocytes (H), mononuclear cells, bile duct epithelial cells (BD), or blood vessels (V). (AB Complex/AP method with the Vector Red kit); × 200, reduced to 77 per cent in printing

For the immunostaining of CD28, frozen livers were exposed to anti-CD28 antibody for 1 h at 4°C, after short fixation by acetone and pretreatment by 10 per cent normal horse serum. These sections were then treated and observed as above for the ABC/AP method for detection of B7-1 and B7-2.

Classification of the intrahepatic biliary tree

The intrahepatic bile ducts are classified as septal and interlobular bile ducts and bile ductules.[4] Septal bile ducts have an external diameter of more than 100 μm, while interlobular bile ducts are approximately 30–100 μm. Occasionally it is difficult to distinguish the former from the latter, and therefore they were called 'small bile ducts' in this study. The small bile ducts run parallel with hepatic arterial branches of equivalent size in portal tracts and are not contiguous with periportal hepatocytes. Bile ductules which are smaller than interlobular bile ducts are mainly seen at the periphery of portal tracts and frequently connect to the periportal hepatocytes.

Statistics

A chi-square test and Fisher's exact text were used for inter-group comparison. p values less than 0·05 were considered significant.[21]

RESULTS

Expression of B7-1

In all the specimens examined, B7-1 was preferentially detectable in the connective tissue of portal tracts (Fig. 1).

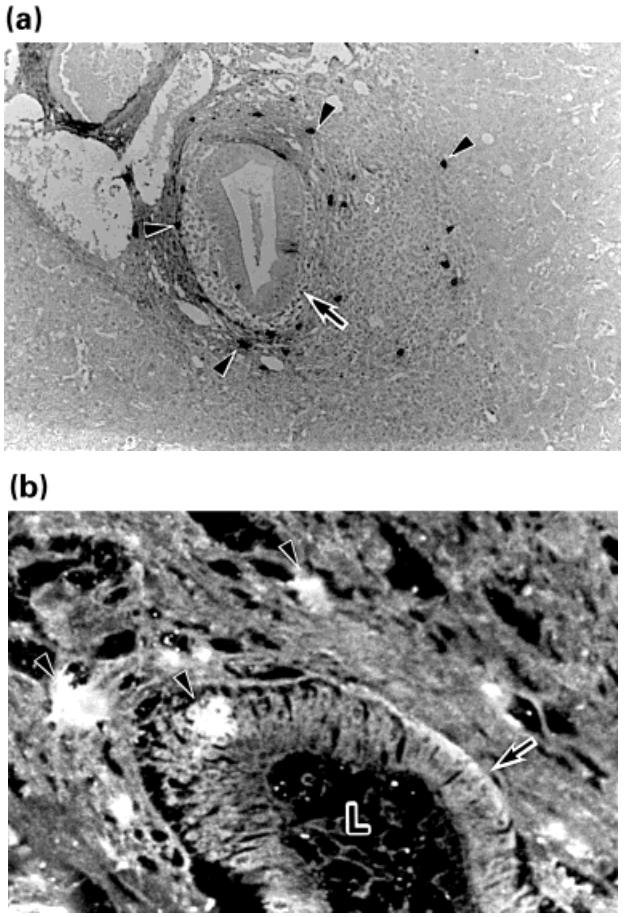

Fig. 2—(a) Immunostaining for B7-2 in PBC. There are many mononuclear cells positive for B7-2 (arrow-heads) in the portal tract, particularly around the interlobular bile duct (arrow). (AB Complex/AP method with the Vector Blue kit); × 200, reduced to 75 per cent in printing. (b) Immunostaining for B7-2 viewed by confocal laser microscopy in PBC. Several B7-2-positive non-epithelial cells with the shape of dendritic cells are seen in the biliary epithelial layer (arrow) of small bile ducts and periductal connective tissue (arrow-heads). L=lumen of septal bile duct. (AB Complex/AP method with the Vector Red kit); × 400, reduced to 75 per cent in printing

B7-1 was not expressed on mononuclear cells, bile duct epithelial cells, or blood vessels in the portal tract of any liver specimens examined. Hepatocytes, mesenchymal cells, and inflammatory cells including lymphoid cells were negative for B7-1.

Expression of B7-2

There were some B7-2-positive non-epithelial cells in the portal tracts in PBC and other diseases. These cells were dendritic in shape and were regarded as DCs (Figs 2a and 2b). They were more numerous in PBC and PSC than in control livers. Table I shows that 17 of the 22 PBC patients (77 per cent) and six of the seven PSC patients (86 per cent) had more than five B7-2-positive DCs in at least one portal tract. More than five B7-2-positive DCs in a portal tract were also found in two of eight patients (25 per cent) in EBO and one of eight patients with CVH-C. All histologically normal livers were negative for B7-2-positive cells in the portal tracts. Infiltration of B7-2-positive DCs was more frequent in PBC and PSC than in other groups ($p<0{\cdot}05$). In PBC and to a lesser degree PSC, these B7-2-positive DCs were found mainly around small bile ducts and some of them were actually located within the epithelial layer. In contrast, such cells were not related to the bile ducts and not found in the biliary epithelial layer in EBO and CVH-C.

In addition to these DCs, some, but not all, small bile ducts demonstrating variable periductal inflammation showed diffuse cytoplasmic staining for B7-2 in ten of the 22 PBC livers (45 per cent) and three of the seven PSC livers (43 per cent) (Fig. 3 and Table I). The number of B7-2-positive ducts was less than one per ten ducts in a given section. Some bile ductules were also positive for B7-2. All PBC and PSC cases with B7-2-positive bile ducts were in stage I or II. Histological differences between B7-2-positive and B7-2-negative bile ducts were not discernible in either PBC or PSC. There was no expression of B7-2 in the small bile ducts and bile ductules in the control livers examined.

Staining pattern of CD28

CD28 was expressed on the surfaces of almost all lymphocytes infiltrating the portal tracts in PBC, PSC, and control cases (Fig. 4). Not only in PBC and PSC,

Table I—Expression and frequency of B7-2 in liver disease

Disease	More than five B7-2-positive DCs in portal tracts (positive/examined cases)	Incidence of B7-2-positive small bile duct(s) (positive/examined cases)
PBC (total)	17/22 (77%)	10/22 (45%)*
(early)	15/17 (88%)	10/17 (59%)*
(late)	2/5 (40%)	0/5 (0%)
PSC	6/7 (86%)	3/7 (43%)*
EBO	2/8 (25%)	0/8 (0%)
CVH-C	1/8 (13%)	0/8 (0%)
Normal	0/3 (0%)	0/3 (0%)

%, percentage of positive cases.
*Significantly higher ($p<0{\cdot}05$) than other groups.

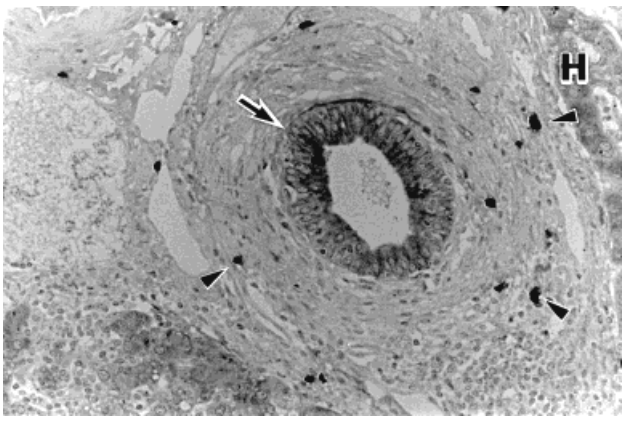

Fig. 3—Some small bile ducts (arrow) in PBC present a variable periductal inflammation. There is diffuse cytoplasmic staining for B7-2, while hepatocytes (H) are negative. There are also positive mononuclear cells (arrow-heads) in the portal tracts. Immunostaining for B7-2 (AB Complex/AP method with the Vector Blue kit); × 200, reduced to 75 per cent in printing

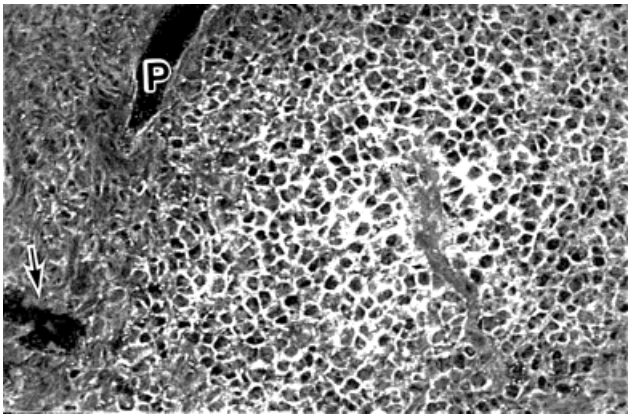

Fig. 4—CD28 expression in the portal tracts of PBC. A small bile duct (arrow) is negative for CD28. P=portal vein. (AB Complex/AP method with the Vector Red kit); × 400, reduced to 76 per cent in printing

but also in EBO and other controls showing periductal inflammation, lymphoid cells were seen in the vicinity of bile ducts. All such cells were positive for CD28.

DISCUSSION

In addition to the specific interaction between the TCR complex with its co-receptor (CD4 or CD8) and MHC class I or II combined with antigenic peptide, non-specific bindings, including adhesion molecules and their ligands, are required for the activation, proliferation, and function of antigen-specific T cells.[7–11] Recently, CD28 on lymphocytes and B7 molecules on APCs have been shown to interact and stimulate the antigen-specific reaction between helper T cells and APCs via intracellular signal transduction. The B7 family consists of two B7 molecules, B7-1 and B7-2, both presumably acting as co-stimulatory ligands for CD28 expressed on infiltrating lymphoid cells. Recent data suggest that B7-2, not B7-1, is the primary costimulatory molecule responsible for initiating antigen recognition by T cells and it provides the stimulus for specific B-cell proliferation and antibody production.[22–27]

In this study, we found that B7-2 was detected on DCs in portal tracts, particularly around the damaged bile ducts in PBC and to a lesser degree in PSC. This is compatible with the findings that DCs constitutively express B7-2.[17,18] Thus, it seems likely that these B7-2-positive DCs play an important role in immune recognition of target tissue, particularly bile ducts. These B7-2-positive DCs were constantly negative for B7-1 molecules.

More importantly, it was found that B7-2 was also expressed in the cytoplasm of biliary epithelial cells of small bile ducts in patients with PBC and PSC, but not other liver diseases. Such aberrant expression of B7-2 on bile duct epithelial cells was focal in a given specimen and was exclusively found in the early stage of both PBC and PSC (Table I). This focal expression of B7-2 is in accordance with the observation that the bile duct lesions of PBC and PSC are discontinuously and focally distributed along the biliary tree and that disease progression is heterogeneous in the liver.[6]

In the initial events of bile duct destruction in PBC and PSC, B7-2-positive periductal or intraepithelial DCs may be presenting antigen(s) to helper T cells infiltrating the periductal tissue. In addition, small bile ducts may also present antigens produced by themselves or absorbed from bile. Several peptides such as PDC-E2,[6–8] heat shock protein,[6] and biliary antigens are speculated to be autoantigens. It is also likely that cytokines secreted by mesenchymal and lymphoid cells,[28] and also biliary cells themselves, enhance the expression of B7-2 on small ducts. In addition, almost all infiltrating T cells around bile ducts express CD28 molecules on their surface. Through the interaction of B7-2-positive DCs or bile ducts and CD28-positive T cells, helper T cells may become activated to secrete specific cytokines. Recent data on the cytokine network in PBC support this hypothesis.[28] B7-2 transfectants preferentially activate Th2-type cytokines in human T cells[22–27] and thus play an important role in the recognition and progression of bile duct injury.

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