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# Expression of antigens related to apoptosis and cell proliferation in chronic nonsuppurative destructive cholangitis in primary biliary cirrhosis

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**Abstract** The initial injury in primary biliary cirrhosis (PBC) is the destruction of portal bile ducts. Little information is available on apoptosis and cell proliferation in such bile ducts, so we used immunohistochemical techniques to locate molecules related to apoptosis [Fas antigen, Lewis Y antigen (BM1/JIMRO), and *bcl*-2 protein] and to cell proliferation (proliferating cell nuclear antigen, PCNA) in 21 patients with PBC. In addition, nick-end labelling was done to locate DNA fragmentation. The expression of these molecules in chronic nonsuppurative destructive cholangitis (CNSDC) was examined. Cell death and PCNA expression were both found in portal bile ducts affected by CNSDC in 7 of the 13 CNSDC patients examined. Fas antigen was found on the plasma membrane and rough endoplasmic reticulum of bile-duct cells with CNSDC in the frozen sections of all 6 patients with CNSDC out of the 9 patients inspected, and this antigen was found also in bile-duct cells without CNSDC in 2 of these 9 patients. It was not found in anatomically normal liver (from 2 patients with Gilbert's disease). The Lewis Y antigen was found in bile ducts with CNSDC and in proliferated ductules in all 16 patients examined. No *bcl*-2 protein was found in any bile-duct or ductule cells, but it was found in the cytoplasm of lymphocytes surrounding or invading CNSDC. DNA fragmentation was found in the nuclei of bile-duct cells with CNSDC by nick-end labelling. Our study indicated that Fas-mediated apoptosis might be involved in CNSDC, but that *bcl*-2 protein seems to participate less than Fas. Although the Lewis Y antigen was found in many bile ducts, the relationship between the antigen and apoptosis remains unknown because there was no evidence that this antigen mediates apoptosis.

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**Key words** Primary biliary cirrhosis · Fas antigen · *bcl*-2 protein · Lewis Y antigen · Proliferating cell nuclear antigen

## Introduction

In primary biliary cirrhosis (PBC), the initial injury is generally restricted to the interlobular and septal bile ducts, which are destroyed by chronic inflammatory cells, including lymphocytes, plasma cells, and macrophages [1, 32]. The destruction of bile ducts and dropping-out of hepatocytes lead to progressive fibrosis and ultimately to cirrhosis. Immunological mechanisms have been postulated to explain the occurrence of chronic nonsuppurative destructive cholangitis (CNSDC) [7, 24, 37, 40]. However, the exact aetiology of PBC is unknown. Four kinds of cell death involving biliary epithelial cells in CNSDC have been described: coagulative necrosis, lytic necrosis, apoptosis, and the detachment of several adjoining cells, often in association with lymphocyte infiltration [2, 6, 31]. There are many studies on necrosis of the affected biliary epithelial cells, but few on apoptosis and the antigens related to apoptosis. In addition, there are few reports on the proliferation of biliary epithelial cells after cell loss in the bile ducts of subjects with CNSDC. Such research seems to be hampered by the lack of detailed investigations on the antigens and antibodies related to apoptosis and by the lack of any satisfactory markers of cell proliferation. Recently, however, Fas antigen [29, 41, 45], Lewis Y antigen (BM1/JIMRO) [13, 25, 39], and *bcl*-2 protein [30, 42] have been found to be involved in apoptosis. Proliferating cell nuclear antigen (PCNA) [4, 9, 19], DNA polymerase-α [23, 36], and 5-bromo- and 5′-iododeoxyuridine [12] seem to be markers of cell proliferation. Therefore, we set out to examine biliary epithelial cells in PBC using monoclonal antibodies (MoAbs) related to apoptosis (Fas antigen, Lewis Y antigen, and *bcl*-2 protein) and cell proliferation (PCNA) to elucidate the significance of apoptosis and cell proliferation in CNSDC.

## Materials and methods

We examined 21 patients with PBC and 2 patients with Gilbert's disease (control livers) clinically and by laboratory and histological tests (Table 1). All patients with PBC were women, and their mean age was 50 years (range, 33–61 years). All were classified histologically into four stages, I–IV, according to the staging system of Scheuer [34]: 6 patients were in stage I, 9 in stage II, 4 in stage III, and 2 in stage IV. Anti-mitochondial antibodies (AMA, indirect immunofluorescence method for total AMA) were assayed in all patients, and were detected in 20 patients. All patients showed sustained high levels of serum alkaline phosphatase. All liver specimens were obtained with a Silverman needle under peritoneoscopy at our hospital. The specimens were fixed in 10% neutral formalin, and 5-μm serial sections were cut from paraffin blocks and processed for conventional staining including haematoxylin and eosin and Azan Mallory stains. Serial sections were prepared from specimens taken from the 16 patients (nos. 1–16, Table 1) for immunohistological staining for PCNA as a marker of cell proliferation, Fas antigen, Lewis Y antigen, and *bcl-2* protein as markers related to apoptosis, and for examination by terminal deoxyribonucleotidyl transferase-mediated deoxyuridine triphosphate-digoxigenin nick-end labelling (TUNEL) [11] assay for DNA fragmentation. Moreover, small portions of the biopsied specimens from 12 patients (nos. 10–21) were fixed in a mixture of periodate, lysine, and 2% paraformaldehyde (PLP), embedded in OCT compound (Miles Scientific, Naperville, Ill.), frozen in liquid nitrogen, and stored at –80°C until use.

Immunohistological staining for PCNA was done as described previously with minor modifications [19]. The anti-PCNA MoAb 19F4, a mouse IgG antibody, was purchased from Boehringer Mannheim/Yamanouchi (Tokyo). Sections were deparaffinized by treatment in a series of ethanol concentrations and washed in 0.01 mol/l phosphate-buffered saline (PBS). Next, hydrolysis was done by incubation for 30 min at 20°C in 2 mol/l HCl, and the sections were washed in two baths of 0.1 mol/l borax at pH 8.5 for 5 min each time. The sections were treated with 10% normal rabbit serum for 20 min, and a solution of MoAb 19F4 (5 μg/ml in PBS) against human PCNA or normal mouse IgG (control) was dropped on the sections, which were kept at 4°C overnight. After being rinsed with PBS, the sections were incubated with methyl alcohol containing 0.03% hydrogen peroxide for 20 min, rinsed with PBS, and treated with rabbit polyclonal Ab against mouse IgG, IgA, and IgM (Histfine kit, Nichirei, Tokyo). Then the sections were incubated with streptoavidin-biotin-peroxidase complex for 20 min. Peroxidase was treated with 3,3′-diaminobenzidine tetrahydrochloride (DAB), and the sections were counterstained with 10% methyl green.

Immunohistochemical staining for Lewis Y antigen was done as follows. Deparaffinized and dehydrated sections were treated with 10% normal rabbit serum for 20 min, and a solution of mouse MoAb to Lewis Y antigen (IgM, diluted 1:400 in PBS, Japan Immunoresearch Lab. Co., Takasaki, Japan) or purified mouse myeloma IgM (control, Zymed Lab. San Diego, Calif.) was dropped on the sections, which were treated as for PCNA staining in the remaining steps.

For immunohistochemical staining for Fas antigen, deparaffinized sections and 6-μm frozen sections rinsed with PBS were incubated with methyl alcohol containing 0.03% hydrogen peroxide for 20 min and treated with 10% normal goat serum for 20 min. Next, a solution of mouse MoAb against Fas antigen (IgM, diluted 1:1000 in PBS, Medical & Biological Lab., Nagoya, Japan) or purified mouse myeloma IgM (control) was dropped on the sections, which were left overnight at 4°C. After being rinsed with PBS, the sections were treated with goat Ab against mouse IgM conjugated with peroxidase (diluted 1:200 in PBS, TAGO, Burlingame, Calif.) for 2 h. After being rinsed with PBS, the sections were treated with DAB for 5 min and were counterstained with 10% methyl green. For electron microscopy done to examine the location of Fas antigen, 6-μm frozen sections were rinsed with PBS and treated with 10% normal goat serum for 20 min. Next, a solution of mouse MoAb against Fas antigen (IgM, diluted 1:200 in PBS) or purified mouse myeloma IgM (control) was dropped on the sections, which were left overnight at 4°C. After being rinsed with PBS, the sections were treated with goat Ab against mouse IgM conjugated with peroxidase (diluted 1:200 in PBS). After being rinsed with PBS, the sections were treated with DAB for 30 min and then in DAB with 0.03% hydrogen peroxide. The sections were fixed again with 2% osmium tetroxide, washed with PBS, dehydrated with graded concentrations of ethanol, and embedded in epoxy resin 812. Ultrathin sections were examined with an electron microscope (1200EX, JEOL, Tokyo) without being stained, or after double-staining with uranyl acetate and lead citrate.

The immunohistochemical study of human *bcl-2* oncoprotein was done as follows according to the manufacturer's instructions. Deparaffinized sections were immersed in a plastic jar filled with 10 mmol/l citrate buffer, pH 6.0, in a microwave oven (RE-M15, 600 W, Sharp, Osaka, Japan) twice for 5 min each, with the buffer being changed once. After being rinsed with PBS, the sections were treated with 10% normal rabbit serum for 20 min, and a solution of MoAb against *bcl-2* protein (diluted 1:100 in PBS, Dako, Glostrup, Denmark) or normal mouse IgG (control) was dropped on the sections, which were treated as for PCNA staining in the remaining steps.

**Table 1** Data on the 21 patients with primary biliary cirrhosis (*AMA* anti-mitochondrial antibody, *CNSDC* chronic nonsuppurative destructive cholangitis)

| Case no. | Age, years | Sex | AMA | Scheuer's classification | CNSDC |
|---|---|---|---|---|---|
| 1 | 60 | F | ×640 | II | Yes |
| 2 | 50 | F | ×80 | III | No |
| 3 | 38 | F | ×20 | III | Yes |
| 4 | 48 | F | ×40 | I | Yes |
| 5 | 48 | F | ×320 | I | Yes |
| 6 | 57 | F | ×80 | II | Yes |
| 7 | 47 | F | ×80 | I | Yes |
| 8 | 55 | F | ×640 | II | Yes |
| 9 | 56 | F | ×320 | I | Yes |
| 10 | 40 | F | ×320 | II | No |
| 11 | 61 | F | ×80 | II | Yes |
| 12 | 48 | F | ×320 | II | Yes |
| 13 | 58 | F | ×320 | IV | No |
| 14 | 55 | F | ×80 | III | Yes |
| 15 | 40 | F | ×160 | I | Yes |
| 16 | 33 | F | ×640 | IV | Yes |
| 17 | 40 | F | ×20 | II | Yes |
| 18 | 58 | F | 0 | III | Yes |
| 19 | 61 | F | ×320 | II | Yes |
| 20 | 59 | F | ×320 | I | Yes |
| 21 | 48 | F | x2560 | II | Yes |

**Table 2** List of the mouse monoclonal antibodies used in this study (*LM* light microscopy, *EM* electron microscopy)

| Monoclonal antibodies to: | Sources | Working dilution |
|---|---|---|
| PCNA (19F4) | Boehringer Mannheim Yamanouchi (Tokyo) | 5 μg/ml |
| Lewis Y antigen (BM-1) | Japan Immunoresearch (Takasaki, Japan) | 1:400 |
| Fas antigen | Medical & Biological Labs (Nagoya, Japan) | 1:1000 for LM<br>1:200 for EM |
| *bcl2* Oncoprotein | Dako (Glostrup, Denmark) | 1:100 |

DNA nick-end labelling was carried out as described by Schmitz et al. [35] with minor modifications. In brief, tissue samples were fixed in 10% neutral formalin and embedded in paraffin. After deparaffinization, 5-μm-thick serial sections were dehydrated with xylene and ethanol. Nuclei of the sections were removed by digestion of cellular protein with 20 μg/ml proteinase K (Boehringer Mannheim, Germany) for 15 min at room temperature and the slides were washed four times in distilled water for 2 min each time. Endogenous peroxidase was inactivated by covering of the sections with 2% hydrogen peroxide for 5 min. The sections were rinsed with distilled water and immersed in terminal deoxynucleotidyl transferase (TDT) buffer (30 ml of Trizma base, pH 7.2, 140 mmol/l sodium cacodylate, 1 mmol/l cobalt chloride). TDT and digoxigenin-deoxyuridine triphosphate in TDT buffer were then added to cover the sections, which were incubated in a humid atmosphere at 37°C for 60 min. The reaction was terminated by transferral of the slides to TB buffer (30 mmol/l sodium chloride and 30 mmol/l sodium citrate) for 15 min. The sections were rinsed with distilled water, covered with a 2% solution of bovine serum albumin for 10 min, rinsed with distilled water, and immersed in PBS. The slides were covered with anti-digoxigenin Ab conjugated with peroxidase for 30 min at 37°C, washed with distilled water, immersed for 5 min in PBS, and treated with DAB and nickel for 5 min at room temperature.

For double staining for Fas antigen and DNA fragmentation by TUNEL sections were fixed in PLP, frozen, cut 6 μm thick, and put on slides coated with 0.01% poly-(L)lysine. The slides were washed with PBS. Next, as described above for DNA nick-end labelling, the same procedure was performed up to the steps in which the slides were covered with anti-digoxigenin Ab conjugated with peroxidase, washed with distilled water, and immersed in PBS. The slides were then treated with 10% normal rabbit serum, covered with a solution of mouse MoAb against Fas antigen, and left overnight at 4°C. After being washed with PBS, the slides were treated with rabbit polyclonal Ab against mouse IgG, IgA, and IgM (Histfine kit) and incubated with streptoavidin–biotin-peroxidase complex for 20 min. Peroxidase was stained with a solution of 3-amino-9-ethylcarbazole (AEC substrate kit, Vector Lab., Burlingame, Calif.) for 3 min at room temperature according to the manufacturer's instructions.

## Results

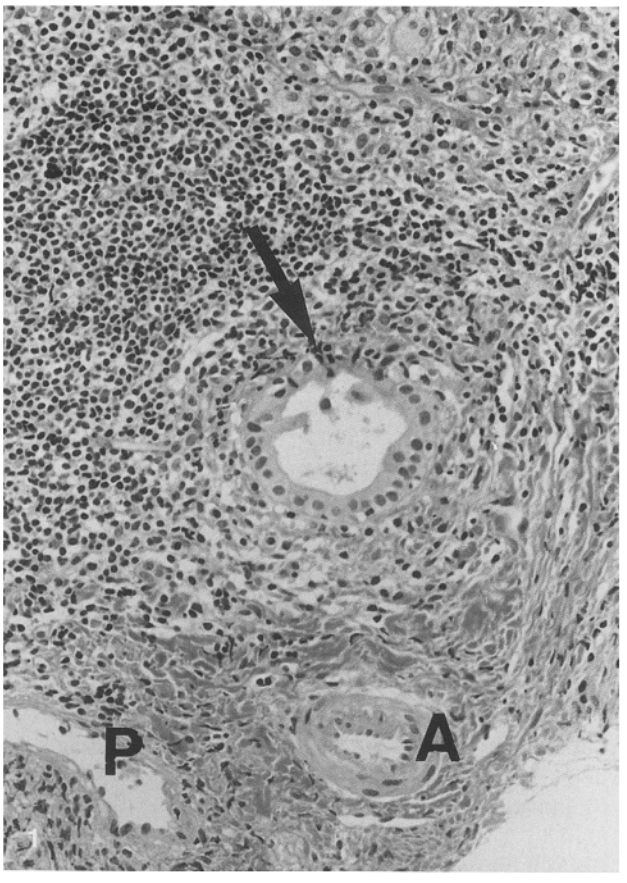
The nomenclature of Rubin et al. [32] is used to report the results. Definitions were as follows. Bile ductules were passages lined by cuboidal or flat epithelium that were not accompanied by very small branches of a hepatic artery. These flat or cuboidal-cell ductules could be further classified into typical, with well-defined lumina, and atypical, with ill-defined lumina and abnormal structure [32, 37]. Interlobular bile ducts were the smallest ducts to run next to a branch of the portal vein; they had a cuboidal or columnar epithelium with nuclei in the centre or toward the basal portion of the cell. More than one interlobular bile duct might appear in a portal tract, and each was accompanied by portal vein branches. Septal bile ducts, from which the interlobular ducts branched, were single ducts with high columnar epithelium and basal nuclei. As it is difficult to distinguish between interlobular bile ducts and septal bile ducts, we call them all simply bile ducts, except for the bile ductules.

The 21 patients included 18 who had CNSDC, and in sections obtained from the remaining 3 there were fewer bile ducts seen on haematoxylin and eosin staining. The lesions diagnosed as CNSDC were bile ducts composed of cuboidal or columnar cells. Chronic inflammatory cells surrounded these bile ducts, and the basement membrane of the affected ducts was disrupted. The individual bile-duct cells were swollen or contained abundant coagulative eosinophilic cytoplasm. Some of these cells had dropped out into the lumina of bile ducts (Fig. 1). Some bile-duct cells had become stratified. In portal areas expanded by lymphocyte infiltration and collagen accumulation, bile ductules that had proliferated were frequently found. Most of these ductules were atypical [32, 37].

Staining for PCNA was done on specimens from 16 of the patients (nos. 1–16 in Table 1). In bile ducts undergoing CNSDC, some bile-duct cells had nuclei stained for PCNA (Fig. 2). In control specimens treated with normal mouse immunoglobulin G (IgG) instead of the MoAb to PCNA staining for PCNA was not found, and in the specimens obtained from the 2 patients with Gilbert's disease such staining was not found in any biliary epithelial cell. The intensity of the staining for PCNA was different in different nuclei. The nuclei of cells that had dropped out into the lumina were not stained. Some inflammatory cells that surrounded bile ducts undergoing CNSDC were stained for PCNA. Some hepatocytes were stained for PCNA, but few or no epithelial cells in bile ductules that had proliferated were stained for PCNA (Fig. 3). In 7 of the 13 patients with CNSDC in this group bile ducts undergoing CNSDC were stained for PCNA, but in the remaining 6 patients examined staining was not found (Table 3). Most epithelial cells in bile ducts without injury were not stained for PCNA.

Staining for Lewis Y antigen was done on specimens from the same 16 patients. Lewis Y antigen was found in the cytoplasm of bile-duct cells but not in the nuclei. Hepatocytes were not stained for this antigen. Almost all biliary epithelial cells of bile ducts undergoing CNSDC expressed the Lewis Y antigen, as did those of bile ducts and ductules that had proliferated (Fig. 4). However, normal bile ducts and ductules generally did not express the antigen, although injured or proliferated bile ducts/ductules sometimes did. In specimens from 12 of the 16 patients examined, staining for the antigen was diffuse in the bile ducts and ductules and in specimens from three other patients, staining was focal. In the remaining specimen, staining was not found (Table 3). Some endothelial cells and the infiltrating cells in sinusoids and portal tracts expressed this antigen. In control specimens treated with normal mouse IgM instead of the MoAb to Lewis Y antigen staining for Lewis Y antigen was not found, but in the specimens from the 2 patients with Gilbert's disease staining was found focally in biliary epithelial cells of some bile ducts in one specimen.

Fas antigen was not detected in livers from any of the 16 patients (no. 1–16) when the sections were fixed with 10% neutral formalin. Therefore, we set out to examine the expression of Fas antigen in frozen sections fixed with a mixture of periodate, lysine, and 2% paraformal-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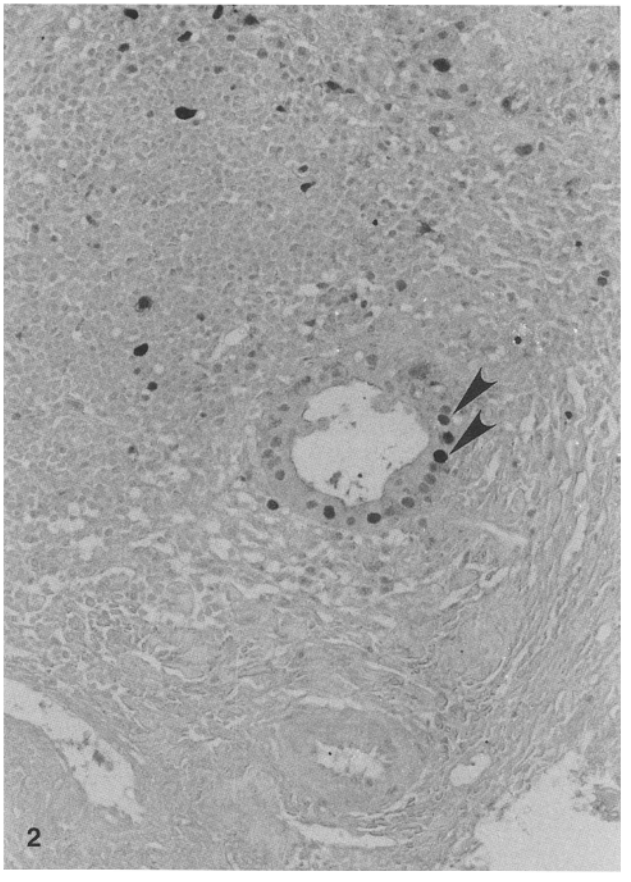
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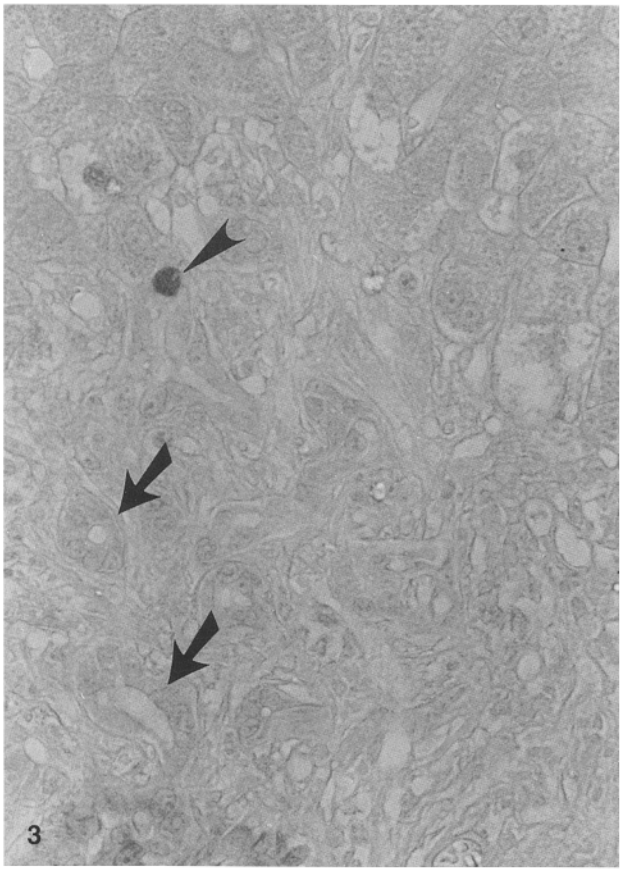
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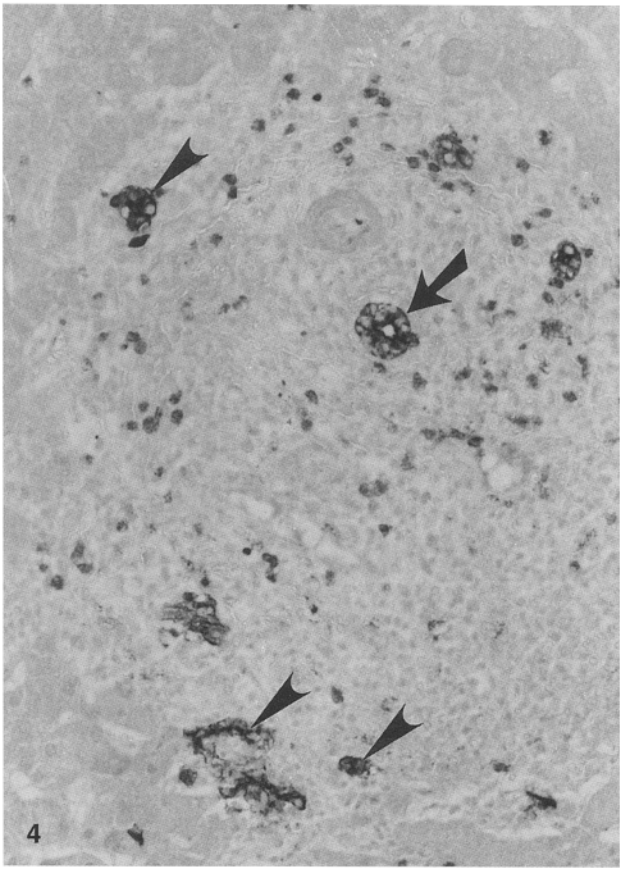
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**Table 3** Summary of results (*PCNA* proliferating cell nuclear antigen, *TUNEL* terminal deoxyribonucleotidyl transferase-mediated deoxyuridine triphosphate-digoxigenin nick-end labelling)

| | Expression site | | | |
|---|---|---|---|---|
| | CNSDC | Uninjured bile duct | Atypical bile ductule | Bile ductule |
| PCNA (19F4) | 7/13 (53.8%) | 3/11 (27.3%) | 2/16 (12.5%) | 5/14 (35.7%) |
| Lewis Y antigen (BM-1) | 13/14 (92.8%) | 2/9 (22.2%) | 14/16 (87.5%) | 11/16 (68.8%) |
| Fas antigen | 6/9 (66.7%) | 1/1 (100%) | 5/8 (62.5%) | 4/8 (50%) |
| *Bcl*-2 protein | 0/14 (0%) | 0/11 (0%) | 0/16 (0%) | 0/14 (0%) |
| TUNEL | 9/13 (69.2%) | 2/6 (33.3%) | 10/13 (76.9%) | 10/13 (76.9%) |

dehyde (PLP) from nine of the patients examined (nos. 13–21). CNSDC was present in 6 of the 9 patients. Staining for Fas antigen was strong in the cytoplasm of some biliary epithelial cells affected by CNSDC in all 6 of these patients with CNSDC (Fig. 5A, Table 3). The intensity of staining was different in different cells. The antigen was found also in the cytoplasm of biliary epithelial cells of seemingly normal bile ducts in portal tracts without inflammatory cell infiltration in 2 of the 9 patients. Fas antigen was found in epithelial cells of bile ductules surrounded by chronic inflammatory cells (Fig. 5B). In addition to the bile ductules, a few sinusoidal cells and some mononuclear cells around bile ducts and ductules in portal tracts were stained. In 3 of the 9 patients, some endothelial cells of arteries in portal tracts were stained for the antigen. In control specimens treated with normal mouse IgM instead of the MoAb to Fas antigen, staining was not found. In the specimens from the 2 patients with Gilbert's disease, staining was not found in many bile ducts or ductules, but was found along the luminal surface of one bile duct (Fig. 5C). In the specimens from the noncancerous portions of three livers resected for metastatic colon cancer, staining was not found in bile ducts or ductules. By electron microscopy, Fas antigen was found on the plasma membrane and rough endoplasmic reticulum of bile-duct cells undergoing CNSDC (Fig. 5D). Fas antigen was found mainly on basal and lateral surfaces, but seldom on apical surfaces. The cells expressing Fas antigen were neither apoptotic nor necrotic, and they had often some electron-dense biliary material in lysosomes, an endoplasmic reticulum with a slightly dilated cistern, mitochondria with a low-density matrix and curled cristae, and shortened microvilli facing the lumina (Fig. 5E). Many intercellular spaces were dilated. In the lumina of bile ducts undergoing CNSDC, much electron-dense biliary material and a few remnants of nuclei, were found, some condensed. These seemed to be from bile-duct cells (Fig. 5E, inset). Apoptotic bodies and apoptotic features of bile-duct cells were not found, but necrotic features were often present (Fig. 5F). Lymphocytes and polymorphonuclear leucocytes were sometimes found within the basement membrane of bile ducts affected by CNSDC.

*bcl*-2 Protein was not expressed in the biliary epithelial cells of bile ducts or ductules, whether injured or not, in any of the first 16 patients. The antigen was found in the cytoplasm of mononuclear cells surrounding or infiltrating bile ducts or sinusoids (Fig. 6). Staining was not found either in the control specimens treated with normal mouse IgG or in the two specimens from the 2 patients with Gilbert's disease. Hepatocytes did not express the antigen.

TUNEL was done for the detection of DNA fragmentation in situ in 13 of the patients with CNSDC (nos. 1, 3–9, 11, 12, 14–16 in Table 1). Staining was found in some nuclei of biliary epithelial cells in bile ducts undergoing CNSDC in 9 of the 13 patients (Fig. 7). The staining was found in the nuclei but not in the cytoplasm. The intensity of staining was different in different nuclei, and darker in the periphery of the nucleus than in its centre in some cells. Some epithelial cells of atypical proliferated ductules were stained for the antigen (76.9%, Table 3). A few hepatocytes, sinusoidal cells, and mononuclear cells infiltrating in portal tracts also were stained. In specimens obtained from the 2 patients with Gilbert's disease, no nuclei with DNA fragmentation were found by TUNEL in the bile ducts.

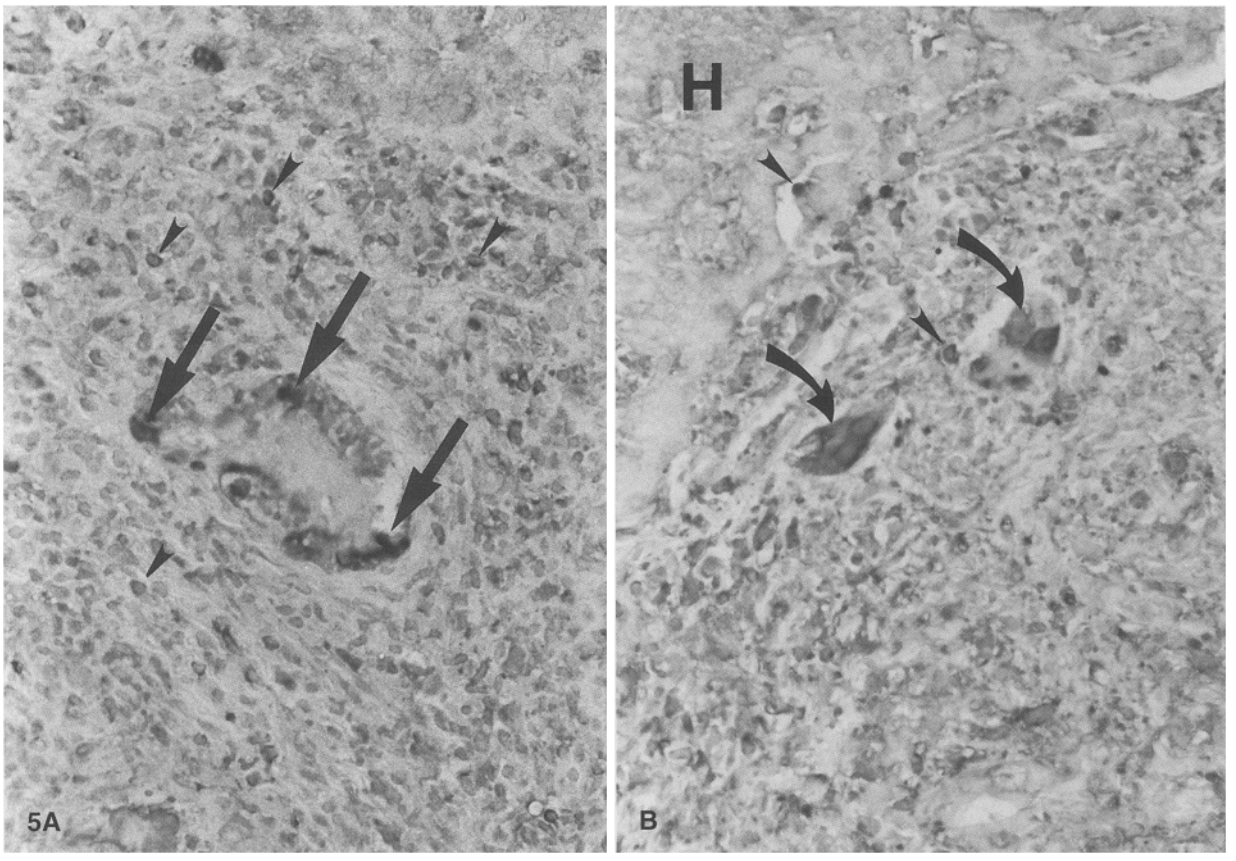
Frozen sections were prepared from the biopsied livers of 6 patients with PBC (nos. 14–16 and 19–21) for double staining for Fas antigen and DNA fragmentation by TUNEL. Of the 6 cases, CNSDC was seen in only 2, and these sections had bile-duct cells with nuclei showing DNA fragmentation and with cytoplasm stained for Fas antigen (Fig. 8). Nuclei with DNA fragmentation were stained light to dark brown and cytoplasm stained for Fas antigen was light to dark red. Some cells had stained nuclei or stained cytoplasm, but not both.

**Fig. 1** Interlobular bile duct showing chronic nonsuppurative destructive cholangitis (CNSDC). Some biliary epithelial cells have dropped out into the lumen. The basement membrane is disrupted (*arrow*). This bile duct is surrounded by many lymphocytes. *P* Portal vein, *A* artery. H&E, ×170

**Fig. 2** Proliferating cell nuclear antigen (PCNA) in interlobular bile duct showing CNSDC (section serial to that of Fig. 1). The nuclei (*arrowheads*) of some cells are stained for PCNA, but the cells that had dropped out into the lumen are not stained. Some cells surrounding the bile duct are stained. ×170

**Fig. 3** PCNA in atypical bile ductules that had proliferated (*arrows*). These ductules are not stained, but the nucleus (*arrowhead*) of one hepatocyte is stained. ×170

**Fig. 4** Lewis Y antigen (BM1/JIMRO) in a bile duct showing CNSDC (*arrow*) and bile ductules that had proliferated (*arrowheads*). The epithelial cells are diffusely stained for this antigen, but hepatocytes are not stained. Some infiltrating cells also are stained. ×170



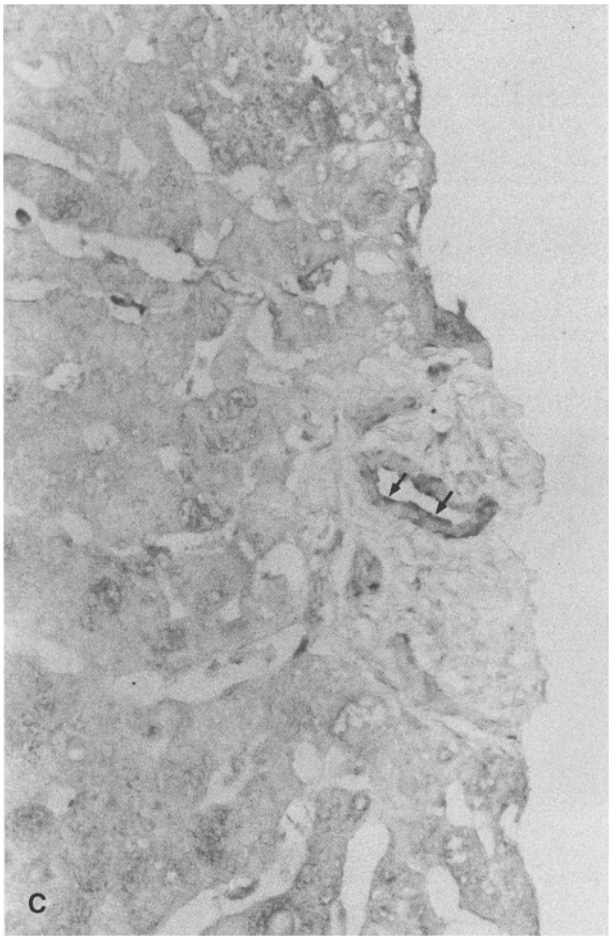




**Fig. 5** **A** Expression of Fas antigen. Some biliary epithelial cells (*arrows*) in a bile duct undergoing CNSDC are stained in an enlarged portal tract. Hepatocytes are not stained, but some mononuclear cells (*arrowheads*) are stained. ×170. **B** Expression of Fas antigen. Some biliary epithelial cells in bile ductules are stained (*arrows*) in a portal tract enlarged by infiltration of mononuclear cells and fibrosis. Some interstitial cells (*arrowheads*) are stained. ×170. **C** Expression of Fas antigen. The luminal surface of one bile duct is stained (*arrows*) in specimen from a patient with Gilbert's disease (control). This portal tract is well preserved. ×170. **D** Immunoelectron micrograph with staining for Fas antigen. Fas antigen is expressed on the cell membrane (*arrowheads*) of affected bile duct cells. *M* Mitochondrion. The same nucleus, marked with an asterisk, is seen in a different section shown in **E**. ×40 000. **E** Electron micrograph of CNSDC with double-staining of uranyl acetate and lead citrate. A section serial to that in **D**. Some bile-duct cells have much electron-dense biliary material in lysosomes (*arrows*), slightly dilated cisternae of their endoplasmic reticulum, and mitochondria with a low-density matrix. Many intercellular spaces are dilated. The lumen of a bile duct undergoing CNSDC is filled with numerous electron dense biliary material and some nuclei. Many mononuclear cells surround the bile duct. ×3800. *Inset*: higher magnification of the nucleus indicated by the *curved arrow*. This nucleus (*N*) has condensed. Bile pigment-like material (*arrow*) and an injured mitochondrion (*M*) are seen adjoining the nucleus. This nucleus seems to be derived from one of the bile-duct cells. ×35 000. **F** Electron micrograph of CNSDC in another section with double staining of uranyl acetate and lead citrate. A necrotic bile-duct cell has damaged cytoplasm (*arrow*). The basement membrane (*arrowheads*) and collagen fibres (*CF*) also can be seen. ×10 000

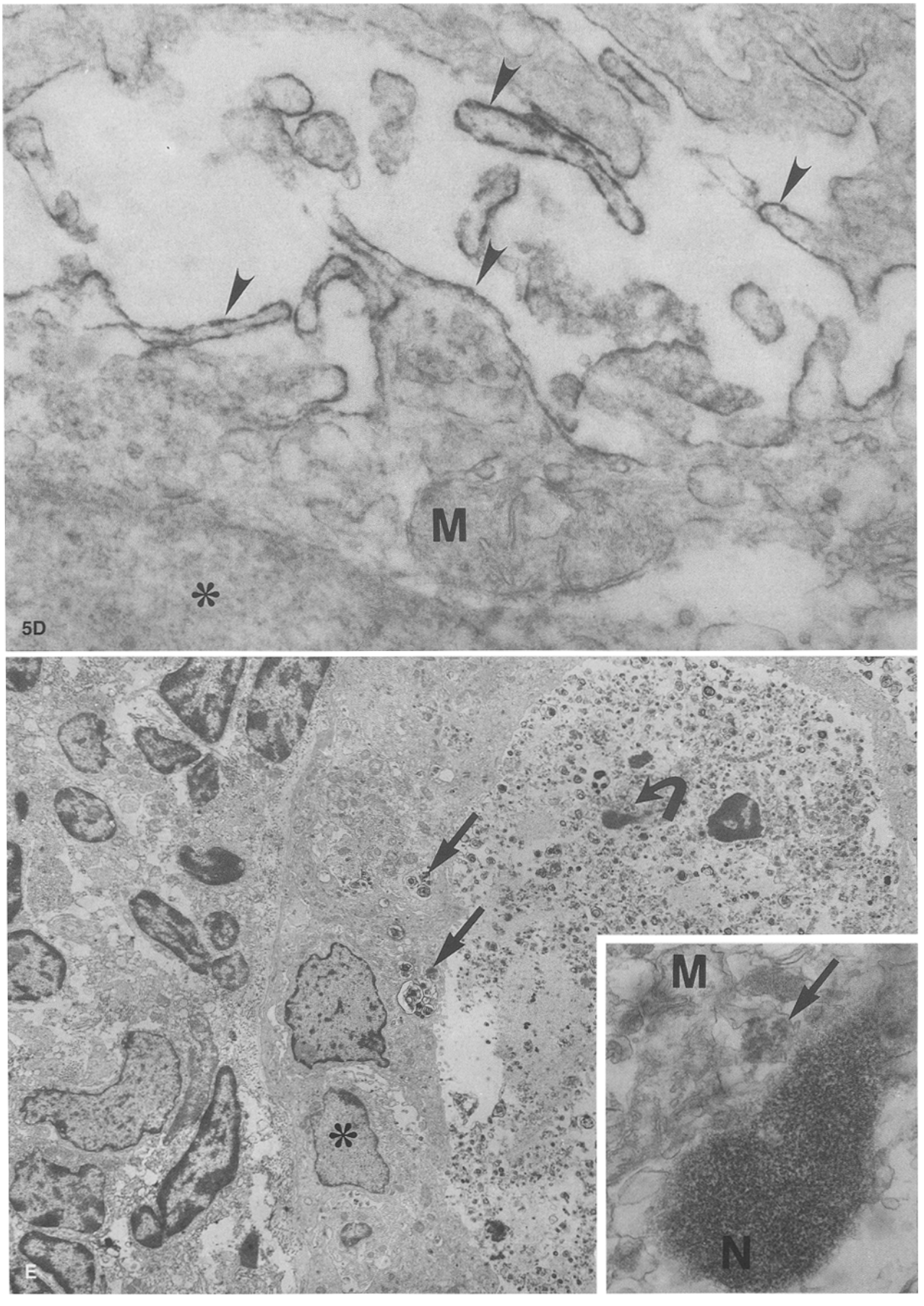
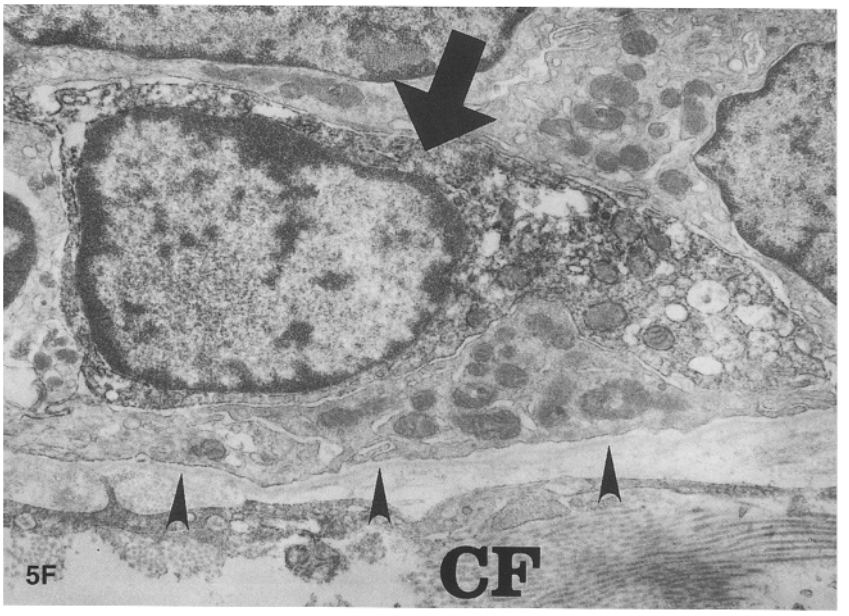



**Fig. 5 D, E**

**Fig. 5 F**



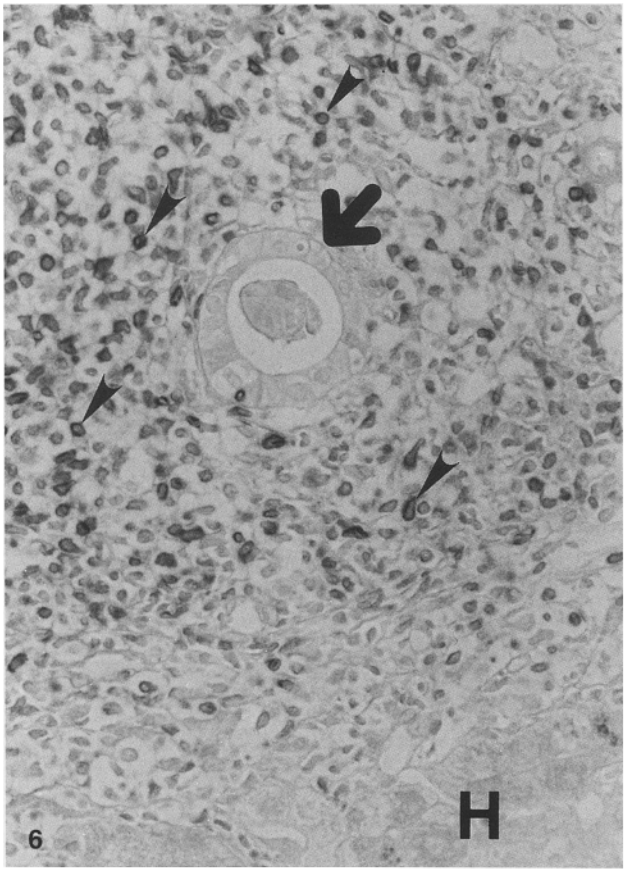




**Fig. 6** Expression of *bcl2* protein. Biliary epithelial cells in a bile duct undergoing CNSDC (*arrow*) are not stained. The cytoplasm of many lymphocytes surrounding the bile duct (*arrowheads*) are stained for this antigen and a lymphocyte infiltrating the bile duct is also stained. Hepatocytes (*H*) are not stained. ×170

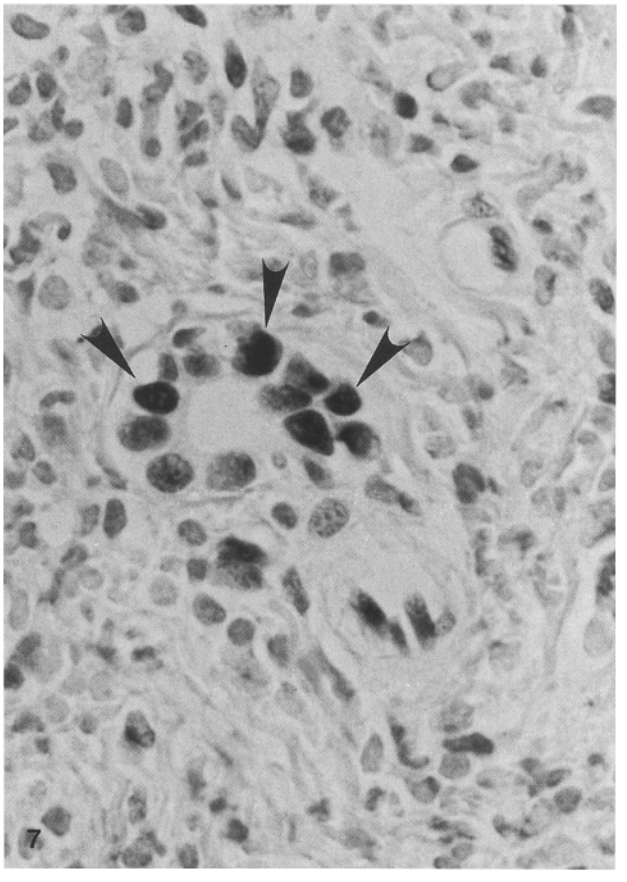



**Fig. 7** DNA fragmentation shown by terminal deoxyribonucleotidyl transferase-mediated deoxyuridine triphosphate-digoxigenin nick-end labelling (TUNEL). Some nuclei of bile-duct cells (*arrowheads*) undergoing CNSDC are stained. These cells do not look apoptotic in the light microscope. ×680

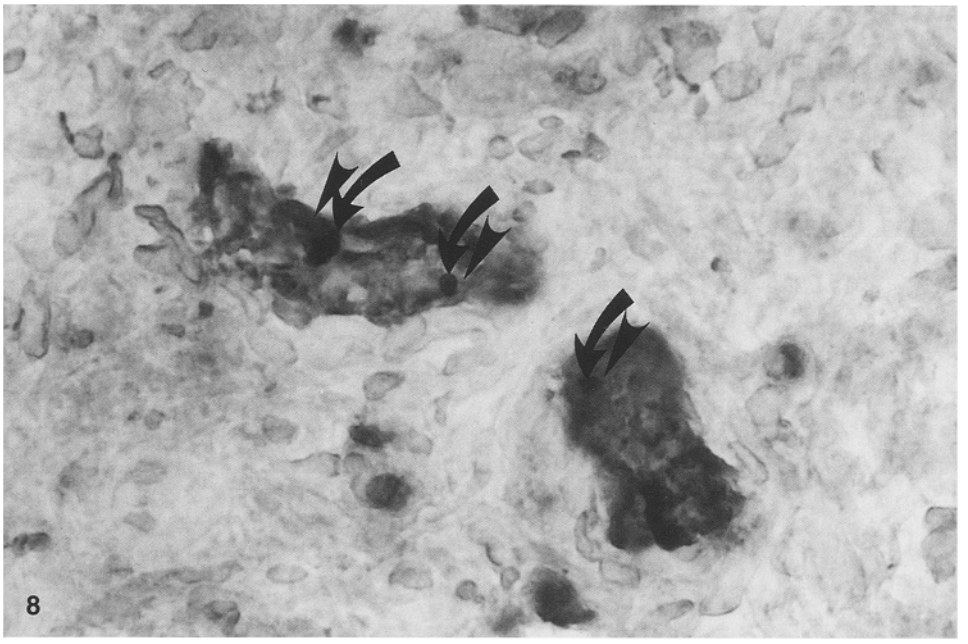



**Fig. 8** Double staining for Fas antigen and DNA fragmentation by TUNEL. Granules stained for Fas are seen in the cytoplasm (*arrowheads*) of bile-duct cells, which also have granules stained by TUNEL (*arrows*). Some bile-duct cells are stained for Fas, but not by TUNEL. Frozen section, fixed with PLP. ×680

## Discussion

In PBC, the basic injury is CNSDC. In addition, bile ducts disappear in areas of periductal oedema and periductal fibrosis, as seen in serial sections [28]. CNSDC was the main lesion in most of our cases of PBC. Generally, cell death takes different forms, such as necrosis and apoptosis [20, 43]. PCNA was identified as the auxilliary protein of 36 kDa of DNA polymerase-δ [4] and is a marker of cell proliferation [9, 19]. Our finding that some biliary epithelial cells in bile ducts undergoing CNSDC expressed PCNA indicated that cell death and cell proliferation were simultaneous in the same affected bile duct and that cell death was faster than cell proliferation. Irrespective of the type of cell death (necrosis or apoptosis), bile-duct cells had proliferated. Few biliary epithelial cells expressed PCNA in normal bile duct or in atypical bile ductules that had proliferated. Epithelial cells in atypical bile ductules were not proliferating when examined, as in our previous study of other liver diseases [37].

Recently, a number of reports on Fas-mediated apoptosis have been published [18, 29, 41, 45]. In liver tissue of subjects with chronic hepatitis and positive for hepatitis C virus antigens, Fas antigen is expressed by some epithelial cells of bile ducts, some hepatocytes, and infiltrating lymphocytes in areas of piecemeal necrosis [14]. However, APO-1 is expressed in hepatocytes and bile ducts in human livers and cloning of APO-1 and the Fas antigen has shown that the sequence of the APO-1 molecule is identical to that of the Fas antigen [18, 21, 45]. In our study, a number of biliary epithelial cells in bile ducts with or without CNSDC expressed Fas antigen, but most hepatocytes did not express this antigen. Fas antigen therefore seems to be important in the death of biliary epithelial cells in PBC. By electron microscopy, condensed nuclei were found in the lumina of bile ducts undergoing CNSDC. These nuclei seemed to be from biliary epithelial cells because electron-dense material was seen in their perinuclear cytoplasm. These condensed nuclei might be apoptotic. We could not find unmistakably apoptotic cells or apoptotic bodies, perhaps because apoptotic bodies disappear rapidly [22]. However, Bernuau et al. [2] have clearly demonstrated that apoptotic cell death does occur in CNSDC. By TUNEL, some nuclei of the epithelial cells of bile ducts undergoing CNSDC were stained, and since TUNEL discloses DNA fragmentation apoptosis is clearly present [10, 35]. It has been suggested, however, that TUNEL also shows the presence of necrosis [11, 27], but in any case, staining by TUNEL identifies injured nuclei. To judge from the electron micrographs of Bernuau et al. [2] that demonstrated apoptotic bodies, the apoptotic process surely occurs in CNSDC. The high percentage of staining in the TUNEL reaction in the proliferated ductules (76.9%) may indicate that these ductules have been destroyed by apoptosis and that thereafter fibrosis has developed. Bhathal et al. [3] have reported that hyperplastic biliary epithelial cells have been deleted by apoptosis.

In our light microscopic study, the patterns of staining for Fas antigen in specimens from patients with Gilbert's disease and from those with CNSDC were different. In normal bile ducts, Fas antigen was either not expressed or was expressed along the luminal surface of a bile duct, but in the injured bile ducts the antigen was expressed diffusely and strongly in the cytoplasm. By electron microscopy, Fas antigen was found on the cell membrane and rough endoplasmic reticulum of bile-duct cells, suggesting that the antigen is synthesized in the rough endoplasmic reticulum and transferred to the cell membrane. In double staining for Fas antigen and DNA fragmentation, the sections of CNSDC had bile-duct cells with nu-

clei showing DNA fragmentation and with cytoplasm stained for Fas antigen. Therefore, Fas antigen may participate in the apoptotic cell death of bile ducts undergoing CNSDC. In fact, apoptosis mediated by Fas antigen has been demonstrated in the livers of mice [29] and in both normal cells and malignant cell lines [8, 26].

Cell death caused by infiltrating lymphocytes (cytotoxic T cells) might also contribute to the destruction of bile ducts [44]. Hiraishi et al. [13] and other researchers [25, 39] reported that Lewis Y antigen might be involved in apoptosis, although this suggestion has not been confirmed. In our study, many abnormal bile ducts and ductules were stained for the antigen, but fewer normal bile ducts and ductules expressed the antigen. This finding was similar to findings reported by others [33]. However, the pattern of staining by Lewis Y antigen was different from that for the Fas antigen. It has not been proved that Lewis Y antigen mediates apoptosis, so it remains unclear whether it participates in the apoptosis of bile duct cells. Moreover, the other molecules related to apoptosis may be discoverd in the future.

Apoptosis in mammals can be inhibited by *bcl*-2 protein [17]. In B lymphocytes, a high apoptotic rate is related to the absence of *bcl*-2 [15, 16] and the *bcl*-2 protein is generally thought to have the opposite effect to Fas antigen in relationship to apoptosis. In our study, *bcl*-2 protein was not detected immunohistochemically in bile ducts with or without CNSDC, although it was found in lymphocytes infiltrating or surrounding bile ducts with CNSDC. This finding suggests that *bcl*-2 protein may not participate in apoptosis in PBC. However, Charlotte et al. [5] reported that the *bcl*-2 protein was expressed by epithelial cells lining bile ductules and small interlobular bile ducts in normal and diseased livers, but not by cells lining larger bile ducts in normal livers. Because the initial injury is restricted to the larger interlobular and septal bile ducts, the *bcl*-2 protein may not participate in CNSDC. Although we used the streptoavidin-biotin-peroxidase complex method and Charlotte et al. used the enzymatic alkaline phosphatase anti-alkaline phosphatase method, we cannot explain the discrepancy in the attitude of staining for the *bcl*-2 protein in the bile ductules and small interlobular bile ducts. Further examination may be needed.

Our immunohistocytochemical study with MoAbs related to cell proliferation and apoptosis has shown that apoptosis may be involved in the destruction of portal bile ducts in PBC, mediated by the Fas antigen.

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