Steroid Receptors and Heat-shock Proteins in Patients with Primary Biliary Cirrhosis

ARTURO D. JORGE,[1] ARTURO O. STATI,[2] LAURA VARGAS ROIG,[2] GUILLERMO PONCE,[1] OLIVER A. JORGE[1] AND DANIEL R. CIOCCA[2]

[1]*Servicio de Gastroenterología, Hospital Español de Mendoza, and* [2]*Laboratorio de Reproducción y Lactancia (LARLAC), Centro Regional de Investigaciones Científicas y Tecnológicas, 5500 Mendoza, Argentina*

Primary biliary cirrhosis has a definite female preponderance. Increased estrogen levels have been found in patients with this disease; however no studies indicate the status of sex hormone steroid receptors in primary biliary cirrhosis patients. In this study the occurrence and distribution of estrogen receptors, progesterone receptors and androgen receptors in liver biopsy specimens from patients with primary biliary cirrhosis were examined and compared with these receptors in the normal liver. In addition, three heat-shock proteins associated with steroid receptors (90 kD, 70 kD and 27 kD) were examined. All of the receptor proteins were detected on immunocytochemical study using specific receptor antibodies; monoclonal and polyclonal antibodies were also used to detect the heat-shock proteins. Normal bile duct epithelial cells displayed low-to-moderate amount of estrogen receptors and abundant 90- kD, 70- kD and 27-kD heat-shock protein expression, whereas normal hepatocytes showed moderate estrogen receptor and 90-kD heat-shock protein and high 70-kD heat-shock protein expression. Expression of 70-kD heat-shock protein was due mainly to the constitutive form of this protein (hsc72). In patients with primary biliary cirrhosis, significant increases in estrogen receptor and 90-kD heat-shock protein content were seen in bile duct cells and in hepatocytes. Levels of 27-kD heat-shock protein were also increased in some of the primary biliary cirrhosis biopsy specimens. The expression of progesterone receptor and androgen receptor was very low in normal and primary biliary cirrhosis bile duct cells and hepatocytes. The high expression of estrogen receptor and associated heat-shock proteins in primary biliary cirrhosis patients indicates that estrogens and their receptors may play a role in this disease, a finding that may have therapeutic implications. (HEPATOLOGY 1993;18:1108-1114.)

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Address reprint requests to: Daniel R. Ciocca, M.D., Laboratorio de Reproducción y Lactancia (LARLAC), Casilla de Correo 855, 5500 Mendoza, Argentina.

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Several studies have implicated the immune system and infectious agents in the etiopathogenesis of PBC (1-8). However, several findings still confuse our understanding of the disease (e.g., PBC in the absence of antimitochondrial antibodies, the lack of a common identifiable infectious agent, cases of PBC with family clustering) (9). PBC has a definite female preponderance, and no clear studies indicate whether sex hormones play a role in the disease. In one recent communication, it was reported that increased estradiol levels have been found in postmenopausal women with PBC (10).

The liver is sensitive to hormone action; significant changes in liver function take place during pregnancy and during treatment with sex hormones (11, 12). The biological effects of steroid hormones on target cells are due to their interaction with specific nuclear receptors. The presence of estrogen receptors (ERs), progesterone receptors (PRs), androgen receptors (ARs) and glucocorticoid receptors (GRs) has been studied in normal and diseased liver biopsy specimens (13-17). We recently studied the expression of ERs and PRs in specimens from patients with hepatitis B virus infection. ER were seen in the nuclei of hepatocytes and bile duct epithelial cells (18). To advance our knowledge of the relationship of sex hormones and PBC, in this study we evaluated the expression of ERs, PRs, ARs and 90-, 70- and 27-kD heat-shock proteins (hsp90, hsp70 and hsp27, respectively) in liver biopsy samples from patients with PBC. These proteins were also studied because of their possible roles in the mechanism of steroid hormone receptor action and their roles as molecular chaperones (19, 20).

MATERIALS AND METHODS

Tissue Processing. Ten biopsy specimens from patients with PBC were studied (Table 1). Diagnosis of PBC had been made previously on the basis of clinical, biochemical, immunological and anatomopathological studies. Liver tissue was obtained by means of percutaneous transhepatic biopsy with a Menghini needle (1.8-mm diameter). Informed consent was obtained from each patient, and the study was approved by an ethic committee. Six normal liver specimens, obtained during cholecystectomies for cholelithiasis, were used as controls. The tissue samples were immediately fixed in picric acid–formal-

TABLE 1. Expression of steroid receptors and heat-shock proteins in bile duct epithelial cells

Type of liver	ER	PR	AR	hsp90	hsp70	hsp27
Normal	+	−	−	+	+ +/+ + +	+ +/+ + +
PBC	+/+ + +	−	−/+ +	+/+ + +	+/+ + +	−/+ + +

Evaluation of immunostaining: −, no staining; +, weak staining; + +, moderate staining; + + +, strong staining.

dehyde (PAF) solution (21) or in buffered formalin (10%) for 30 min at room temperature and then processed as previously described (18). This fixation-and-embedding protocol is useful in the detection of steroid receptors, with a sensitivity comparable to that seen in frozen sections (18, 22). To test the effect of fixation on hsp immunoreactivity, we fixed rat liver pieces in (a) PAF solution, (b) buffered formalin (10%) for 30 min or (c) buffered formalin (10%) for 24 hr. The best immunocytochemical results were seen with PAF or 30-min buffered formalin (10%) fixation, followed with a rapid embedding protocol (18). To avoid protein redistribution before fixation, we killed the female adult Wistar rats used in the study by decapitation and immediately placed small liver fragments in the fixatives described above. Liver fragments from two rats were immediately frozen (− 70° C) for Western blotting.

Immunocytochemical Procedures. Serial sections 6 μm thick were used for immunocytochemical study and for hematoxylin and eosin staining. The paraffin sections were mounted on 3-aminopropyltriethoxy-silane (Sigma Chemical Co., St. Louis, MO)–coated slides. The immunocytochemical procedure was performed as reported previously (23) with a highly sensitive peroxidase-labeled avidin biotin (LAB) detection system (24) (Dako Corp., Santa Barbara, CA). The antibodies used were (a) rat monoclonal antibodies H222 and D75 against ER from human breast cancer cells (MCF-7), (25) kindly provided by Dr. E. De Sombre (The Ben May Institute, University of Chicago); (b) rat monoclonal antibodies JZB39 and KD67 against PR from human breast cancer cells (T47D), (26) kindly provided by Dr. W.L. McGuire (University of Texas, San Antonio, TX); (c) rabbit polyclonal antibody PA1-110 against AR from fusion proteins, (27) purchased from Affinity BioReagents (Neshanic Station, NJ); (d) mouse monoclonal antibody C11 against hsp27 from MCF-7 cells, (28) kindly provided by Dr. W.L. McGuire and rabbit polyclonal antibody hsp25/27 against a hybrid protein containing part of the murine hsp25 and part of the human hsp27 (29), kindly provided by Dr. M. Gaestel (Institute of Molecular Biology, Berlin, Germany); (e) mouse monoclonal antibody N27 F3-4 against hsp70, which recognizes the constitutive and inducible forms of hsp70, (30) kindly provided by Dr. W.J. Welch (Lung Biology Center, San Francisco General Hospital, San Francisco, CA); (f) rat monoclonal antibody 1B5, specific against the constitutive (cognate) form of hsp70, termed hsc72 (31) kindly provided by Dr. A. Laszlo (Mallinckrodt Institute of Radiology, Washington University Medical Center, St. Louis, MO); and (g) mouse monoclonal antibody AC88, which has a broad spectrum of species cross-reactivity against hsp90, including human hsp90 (32, 33), kindly provided by Dr. D.O. Toft (Mayo Clinic, Rochester, MN). The tissue sections were incubated with the primary antibodies overnight at 4° C, in humidity chambers, at the following dilutions: H222 and D75, 20 μg/ml; JZB39 and KD67; 20 μg/ml; PA1-110, 1:150; C11, 5 μg/ml; hsp25/27, 1:1,200; N27 F3-4; 1:1,000; 1B5, 1:1,000; and AC88, 20 μg/ml. The second antibody was applied at a 1:10 dilution for steroid receptors and a 1:50 dilution for heat-shock proteins (45 min), and the LAB complex was applied at a 1:100 dilution for 45 min.

Diaminobenzidine (2 mg/ml)/hydrogen peroxide (0.001%) was used as chromogen substrate. After immunostaining, some of the slides were lightly counterstained with methylgreen (0.5%; 2 min) to visualize nuclei. To colocalize ER and hsp90 in the same cells, we applied a two-color immunostaining protocol (22). Once ERs were visualized with diaminobenzidine (brown reaction product) and photographed, the slides were unmounted (xylene) and the immunostaining was repeated for hsp90. In these cases hsp90 was visualized on incubation with the chromogen aminoethylcarbazole/hydrogen peroxide to obtain a red reaction product. We observed and photographed the slides with a Zeiss IM35 microscope (Carl Zeiss, Oberkochen, Germany), using differential interference contrast optics when it was required. We studied the tissue samples by assessing the site of staining, the proportion of cell staining (counting at least 1,000 cells/sample with × 100 lens) and the intensity of staining: weak (+), moderate (+ +) or strong (+ + +). At the same time the histological characteristics of the tissue sections stained with hematoxylin and eosin were recorded.

In the negative control slides (absence of immunostaining), the primary antibodies were replaced with normal mouse serum. Positive controls included human breast and endometrial biopsy samples sent for steroid analysis with ligand-binding assays (34). Small pieces of these tissues were used for immunohistochemical study and for Western blots to study the specificities and reactivities of the antibodies against the steroid receptors and heat-shock proteins used in this study. As a positive control for ARs we used biopsy samples from patients with prostate carcinomas.

Immunoblotting Procedures. The frozen tissues (human breast and endometrial biopsy samples used as positive controls, and rat livers) were pulverized, thawed in 3 vol of homogenizing buffer (5 mmol/L molybdic acid, 1.5 mmol/L EDTA, 500 mmol/L dithiothreitol, 10 mmol/L Tris-HCl; pH 7.4), and homogenized in an Ultra-Turrax homogenizer (IKA-WERK, Staufen, Germany) at 4° C. Cytosolic and pellet fractions were obtained after centrifugation at 140,000 *g* for 40 min at 4° C. Protein concentrations in the supernatant, SDS-PAGE and Western blotting were performed as described elsewhere (34). A 100-μg cytosolic sample protein was loaded on the stacking gel for SDS-PAGE, and the proteins from the liver pellets were resuspended with sample buffer and heated at 100° C for 5 min. The proteins, transferred to nitrocellulose papers, were detected immunoenzymatically with the LAB detection system. The dilution and time of incubation with the antibodies were the same as those for immunocytochemical study.

Statistics. Student's *t* test for independent data was used to establish whether the differences found in the biopsy specimens from normal and PBC patients were significant.

RESULTS

In the normal livers ERs were present in the ductules (Hering canals) and in the larger interlobular and septal bile ducts. These receptors appeared in the nuclei of several of the epithelial cells, with weak immunostaining

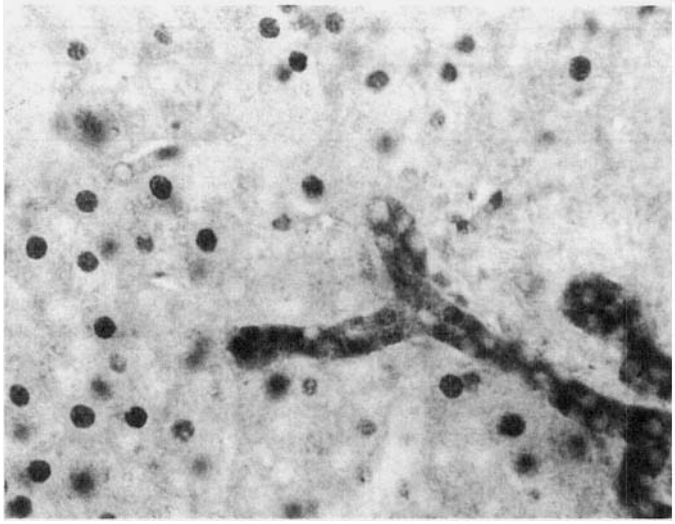

FIG. 1. Biopsy section from a normal liver immunostained to reveal hsp70. Note the presence of the heat-shock protein in the nuclei of hepatocytes and in the cytoplasm and some nuclei of bile ducts (original magnification ×280).

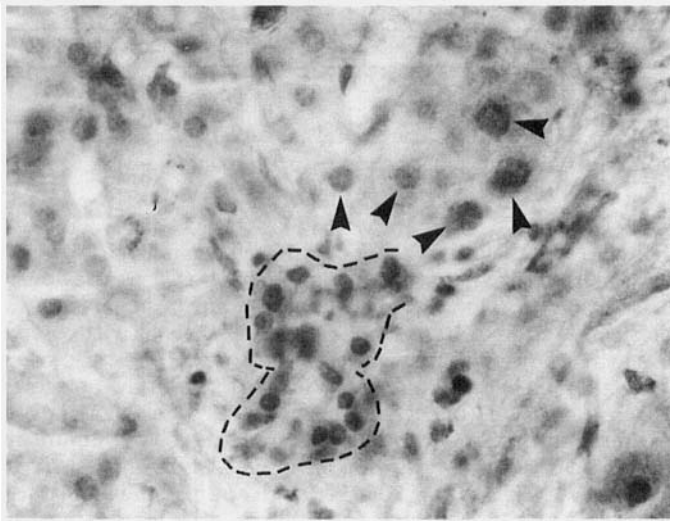

FIG. 2. Biopsy section from a patient with PBC (stage 2) immunostained to reveal ERs. The H222 ER monoclonal antibody was used here, but an identical staining pattern was seen with the D75 ER antibody. The receptor protein can be seen in the nuclei of several bile duct epithelial cells (- - - -) and in the nuclei of several hepatocytes *(arrowheads)* (original magnification ×280).

intensity. In the bile ducts it was not possible to quantitate the proportion of cells expressing the receptors and heat-shock proteins studied because of the small biopsy specimens and the scarcity of these structures. The expression of the steroid receptors and heat-shock proteins in the bile ducts is summarized in Table 1. High hsp70 content was detected in normal bile ducts, mainly those of small caliber (Fig. 1). This protein was seen mainly in the cytoplasm of the epithelial cells, but it also appeared in some of the nuclei. The hsp90 was also present in several of the normal bile duct epithelial cells, in the cytoplasm of the cells and also in some nuclei, but with weak immunostaining intensity. High hsp27 expression was observed in the normal bile ducts, but only in the cytoplasm of the cells. This heat-shock protein also appeared in the endothelium and smooth muscle of blood vessels of the septal areas.

Specimens from patients with PBC showed higher ER and hsp90 contents in bile ducts (Table 1). Histopathological appearance of PBC lesions was evaluated according to established criteria (35-37). Figure 2 shows bile ducts from a patient with grade 2 PBC immunostained to reveal ERs. The expression of this receptor protein decreased in the more advanced stages of disease (grades 3 and 4). PRs were absent in the bile ducts from normal and PBC patients, whereas ARs were seen weakly in some of the bile duct epithelial cells of PBC patients. Expression of hsp70 and hsp27 in bile ducts from PBC biopsy specimens was lower than that observed in the normal livers.

The expression of the steroid receptors and heat-shock proteins in normal hepatocytes and in hepatocytes from patients with PBC is shown in Table 2. Normal hepatocytes displayed moderate expression of ERs, both in staining intensity and proportion of stained cells. It is important to mention here that the staining for ERs in the liver was always lower than that observed in endometrial and breast cancers (positive controls). PRs and ARs were absent or weakly observed in the normal hepatocytes. In all cases the receptor proteins appeared in the nuclei of the hepatocytes.

Normal hepatocytes showed moderate hsp90 expression and high hsp70 nuclear content but almost no hsp27. We noted that hsp90 and hsp70 were mainly expressed in the nuclei of hepatocytes (Figs. 1 and 3). In the normal livers, Kupffer cells showed weak ER content, moderate hsp70 expression and high levels of hsp90 (Fig. 3). Hepatocytes from PBC biopsy specimens showed significantly increased ER expression compared with normal hepatocytes ($p < 0.001$). This increased ER expression was independent of the stage of the disease; the ER staining was nuclear (Fig. 2). The other significant change observed in hepatocytes from PBC patients was increased hsp90 content ($p < 0.001$). This heat-shock protein remained predominantly nuclear. Because in the biopsy specimens from patients with PBC the proportion of ER-stained hepatocytes was very similar to the proportion of hsp90-stained hepatocytes, we performed the colocalization study. This analysis revealed that most of the hepatocytes (>80%) contained both proteins, hsp90 and ER, simultaneously in the same cell nuclei. Finally, hsp27 expression was also increased in hepatocytes of PBC patients (Table 2). This heat-shock protein appeared in the cytoplasm and at the periphery of the hepatocytes (Fig. 4). The immunostaining for hsp27 in the hepatocytes was observed only when the antibody against the hybrid hsp25/27 protein was applied; monoclonal antibody C11 against the human hsp27 yielded practically no staining on hepatocytes.

The predominant location of hsp90 and hsp70 in the

TABLE 2. Expression of steroid receptors and heat-shock proteins in hepatocytes

Patient no.	Age (yr)	Sex	Diagnosis	ER	PR	AR	hsp90	hsp70	hsp27
1	34	F	Normal	+ +(51)	+ +(10)	–	+ +(47)	+ +(76)	–
2	39	F	Normal	+ +(52)	–	+(10)	+ +(30)	+ +(81)	–
3	44	F	Normal	+ +(55)	–	–	+ +(22)	+ + +(82)	–
4	38	M	Normal	+ +(58)	–	–	+ +(26)	NA	–
5	55	M	Normal	+ +(57)	–	–	+(40)	+ + +(78)	+(<1)
6	64	M	Normal	+ +(58)	+(<5)	–	+ +(38)	+ + +(83)	+(<1)
7	43	F	PBC-1	+ +(86)	–	+(<10)	+ + +(84)	+ +(77)	+(8)
8	49	F	PBC-1	+ +(91)	–	+ +(<10)	+ + +(80)	+ +(83)	+(<5)
9	45	F	PBC-2	+ +(80)	+(<10)	+(<10)	+ +(76)	+(65)	+ +(29)
10	49	F	PBC-2	+ +(72)	+(<10)	–	+ +(79)	+ +(78)	+ +(56)
11	49	F	PBC-2	+ +(91)	+(<5)	+(<10)	+ +(88)	+ +(81)	+ +(20)
12	42	F	PBC-2	+ +(75)	–	–	+ +(78)	+ + +(88)	+ +(34)
13	58	F	PBC-3	+ +(79)	–	–	+ +(78)	+ +(82)	+ +(10)
14	38	F	PBC-3	+ +(86)	–	–	+ +(89)	+ +(88)	+ +(<5)
15	42	F	PBC-3	+ + +(87)	–	–	+ +(90)	+ +(80)	+(30)
16	57	F	PBC-4	+ +(77)	–	+(<5)	NA	NA	NA

NA, tissue not available for study.

Immunostaining was evaluated according to the intensity of staining and the proportion of stained cells (in parentheses). –, No staining; +, weak staining; + +, moderate staining; + + +, strong staining.

nuclei of hepatocytes was also seen in the rat livers, whereas little immunoreactivity was observed for hsp25, which appeared in the cytoplasm. The Western-blot study showed hsp70 in the cytosolic fraction but also in the pelleted fraction (nuclei and microsomes) (Fig. 5). It is important to mention the appearance in the Western blots of a band of 72,000 Da in the rat livers, which corresponds to hsc72. We confirmed this using the monoclonal antibody specific against the constitutive form of this hsp: hsc72. Although not shown here, hsc72 immunostaining was also predominant in the nuclei of rat and human hepatocytes. It did not appear in the nucleoli. (This location is seen in stressed cells).

DISCUSSION

In this study we first defined the presence and distribution of ERs, PRs, ARs, hsp90, hsp70 and hsp27 in the normal human liver. However, the normal liver specimens studied here were obtained during cholecystectomies for cholelithiasis; this situation may have altered the expression of the proteins analyzed. With this proviso, in the normal human livers we found consistent expression of ERs in the hepatocytes and bile duct epithelial cells and little immunoreactivity in some Kupffer cells. We previously reported the expression of ERs in hepatocytes and bile duct cells (18). Positive nuclear ER immunostaining in hepatocytes has also been reported in mouse liver; however, these authors did not find ERs in Kupffer cells, smooth muscle of blood vessels or bile duct cells (38). Perhaps species differences account for these discrepancies in ER localization. In addition we used a higher ER antibody concentration (with two different antibodies) and a more sensitive immunohistochemical technique. The presence of ERs in the bile ducts is also supported by the finding of ERs in the gallbladder, as revealed by immunoenzymatic assay (39). In this study we also found that the expression of PRs (with two different PR antibodies) and ARs in the normal liver is very low or absent. In previous studies these receptor proteins have been detected by means of biochemical methods using ligand-receptor assays, which are more sensitive than immunohistochemical methods (13-17). When immunohistochemical study was used to detect PRs and ARs in the liver, the investigators found weak staining or no staining for PRs in rabbit and human hepatocytes (26, 40) and low AR expression in rodent livers (41). On the other hand, very little is known about the expression of heat-shock proteins in the normal human liver. Abundant expression of hsp65 has been found in hepatocytes and sinusoidal lining cells (42). This particular hsp has been mainly involved in the immune response (43). In a previous study, hsp70 was detected by means of immunocytochemical study in the cytoplasm of hepatocytes from patients with alcoholic liver disease (44). In this study we found high expression of hsp70, mainly in the nuclei of hepatocytes, in bile duct epithelial cells (cytoplasm and nuclei) and, expressed moderately, in Kupffer cells. The discrepancy in hsp70 localization in hepatocytes between our study and that of Omar et al. (44) may be due to the different liver diseases studied, but it may also be due to the different antibodies used. In their study of alcoholic liver disease, Omar et al. used a polyclonal antibody (44), whereas in this study we used two different monoclonal antibodies, one against the constitutive and inducible forms of hsp70 and the other against hsc72 (constitutive only). With the use of the latter antibody we were able to observe that, in the normal liver and in livers from PBC patients, most of the hsp70 form present in the nuclei of hepatocytes is the constitutive form. The elevated expression of this hsp70 in bile ductal cells should be useful to investigate this heat-shock protein as a marker of bile ducts in diseases like primary sclerosing cholangitis. On the other hand,

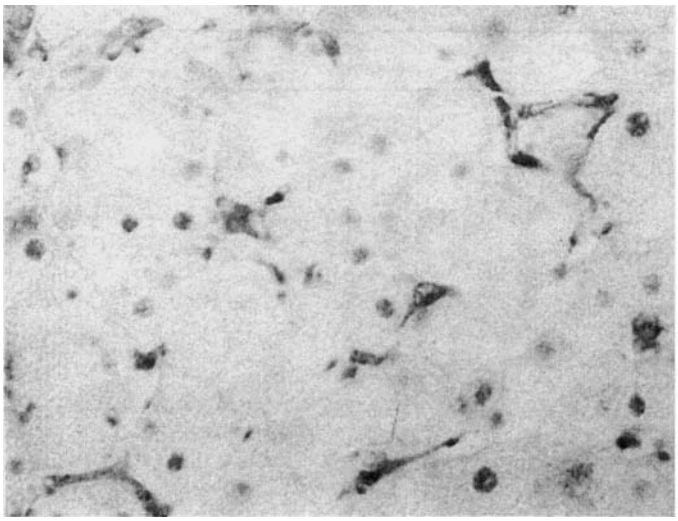

FIG. 3. Tissue section from a normal liver immunostained to reveal hsp90, which was predominantly seen in Kupffer cells, but also appeared in some nuclei of hepatocytes (original magnification ×280).

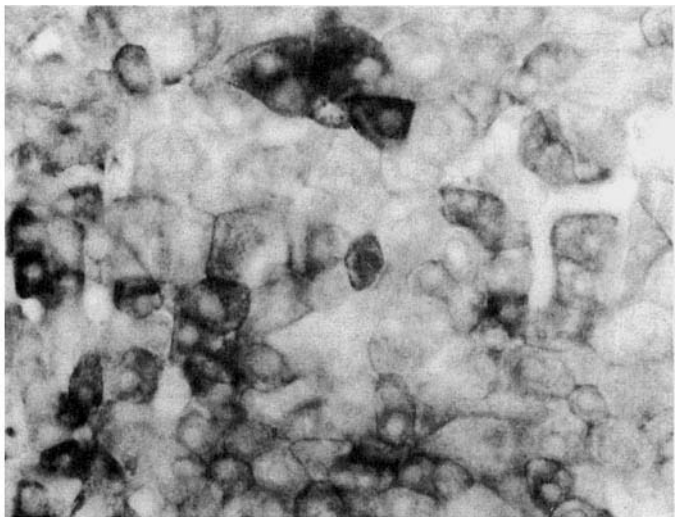

FIG. 4. Biopsy section from a patient with PBC (stage 2) immunostained with the antibody against hsp25/27 hybrid protein. Positive hsp27 staining can be seen in the cytoplasm and at the peripheries of several hepatocytes (original magnification ×280).

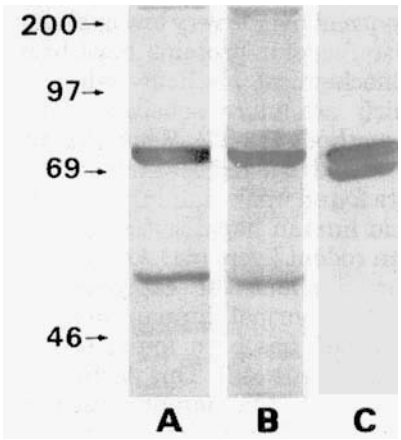

FIG. 5. Western blot of normal rat liver tissue incubated with the monoclonal antibody N27 F3-4, which recognized two hsp70 family members: the constitutive hsc72 (72,000 Da) and the inducible hsp70 (70,000 Da). Lane A, cytosol fraction; lane B, pellet fraction; lane C, cytosol from a human endometrial carcinoma used as positive control. Molecular weight standards (in kilodaltons) are shown on the left. Note that the positive control shows two close bands; one is hsp70 and the other is hsc72, whereas in the livers only hsc72 is seen. The results show that in the liver hsc72 has two fractions, one extractable (cytosolic) and one nonextractable (it remains in the pellet fraction). We do not know the significance of the lower molecular weight band seen in the livers only (at about 56 kD). This band was not caused by degradation; it remained after cytosol preparation with protease inhibitors.

our study revealed increased expression of hsp90 in Kupffer cells. This protein was seen in moderate quantities in normal hepatocytes and bile duct cells. Finally, in a previous study we showed absence of hsp27 in human normal hepatocytes (18). In this study we confirmed this observation and added the finding that this heat-shock protein is moderately to highly expressed in the cytoplasm of bile ductal cells. A recent study has shown very low amounts of two different hsp27 isoforms in human normal liver; levels of this protein were increased after heat shock (45).

In the patients with PBC we observed increased expression of ER and hsp90 in the bile ducts and in the hepatocytes. There is no explanation for this; estrogens themselves regulate the expression of ER. In cells with high ER content estradiol caused down-regulation of ERs (46), but the hormone caused up-regulation of ERs in a cell line with low ER content (47). Higher estrogen levels have been found in patients with PBC (10, 48) but this was seen in patients with late-stage disease (liver dysfunction, portalsystemic shunts of cirrhotic patients), making it difficult to explain up-regulation of ERs by elevated estrogens. In a recent study, Becker et al. (49) reported reduced ER concentrations in patients with chronic liver dysfunction. In our study only one case had stage 4 PBC; this patient also showed increased ER expression. Therefore it seems that increased ER expression is a specific event in PBC.

On the other hand, we found a correlation between the increased expression of ER and that of hsp90 in PBC patients. The hsp90 was present mainly in the nuclei of the cells; it is involved in steroid hormone receptor assembly and activation (19). The hsp70 is another of the heat-shock proteins that have been described as chaperones of steroid receptors. We found it already highly expressed in the normal hepatocytes and bile ducts; it remaining increased in hepatocytes of PBC patients. In rat hepatocytes hsp70 expression has been associated with growth rate (50) and with thermotolerance (51, 52). The hsp27 was also increased in the hepatocytes of PBC patients; in the hepatocytes this protein was detected with one of the antibodies used, suggesting that the two antibodies against hsp27 used here detect different

hsp27 epitopes. Increased hsp27 expression has been recently reported in neoplastic liver tissues (45). Other heat-shock proteins found in the liver include hsp65 and hgp105 (53, 54). The hsp65 was reported to be increased in cholestatic liver disorders (53) and in CAH (42), suggesting a role in immune-mediated liver disease. In this study we show that hsp70 (more specifically, hsc72), hsp27 and hsp90 are highly expressed in the livers of patients with PBC, but given the complexity of functions attributed to heat-shock proteins it is difficult to know the specific role of these heat-shock proteins in patients with PBC. They may be chaperoning ERs and other proteins, including those up-regulated by estrogens. Estrogens are basically cholestatic, and PBC is a cholestatic disease; therefore, the finding of a high expression of ERs in PBC patients may have therapeutic implications. It will be of interest to know whether these patients benefit from antiestrogen therapy.

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