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Evidence of an Increased Nitric Oxide Production in Primary Biliary Cirrhosis

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OBJECTIVE: Although possible implications of nitric oxide in the pathophysiology of liver cirrhosis have been extensively studied, until now few articles have addressed the assessment of nitric oxide production in primary biliary cirrhosis. This study was directed to evaluate circulating nitrosyl-hemoglobin levels as well as neutrophil elastase and soluble adhesion molecule concentrations in this condition, by assuming these parameters as possible markers of either inflammatory response or neutrophil activation.

METHODS: Laboratory investigations were performed in 30 patients with primary biliary cirrhosis, in 13 patients with postviral and/or alcoholic cirrhosis, and in a group of eight subjects with chronic hepatitis.

RESULTS: Although no difference was detected with respect to chronic hepatitis subjects, higher levels of nitrosyl-hemoglobin adducts were found in primary biliary cirrhosis patients than in postviral or alcoholic cirrhotics and in normal subjects (3.55 ± 1.75 arbitrary units *vs* 1.95 ± 0.57 and 0.84 ± 0.34, $p = 0.0004$ and $p < 0.0001$, respectively). Similarly, more elevated concentrations of neutrophil elastase (213.7 ± 192.0 μg/L *vs* 51.1 ± 34.3 and 38.0 ± 11.5, $p < 0.0001$ and $p < 0.0001$, respectively) as well as of soluble forms of intercellular adhesion molecule 1 and endothelial-leukocyte adhesion molecule 1 were shown in primary biliary cirrhosis patients than in subjects with cirrhosis of other etiologies and in controls.

CONCLUSIONS: Highly enhanced nitric oxide production in primary biliary cirrhosis could be related to the development of strong inflammation and at least partially to neutrophil activation, thus suggesting a putative role of these cellular mediators in the development of liver damage owing to their ability to synthesize and release a wide variety of important factors, including elastase and nitric oxide. (Am J Gastroenterol 2001;96:869–875. © 2001 by Am. Coll. of Gastroenterology)

INTRODUCTION

Although the etiology of primary biliary cirrhosis (PBC) still remains to be clarified, both cellular and humoral mechanisms have been implicated in the progressive nonsuppurative destruction of small intrahepatic bile ducts as well as in the portal necroinflammation typically associated with this disorder (1). Indeed, cytokines and cell surface molecules may be involved in local adhesion and activation of leukocytes at the inflammatory sites (2). In particular, the sequence of changes affecting portal tracts and bile ducts appears to be regulated by the aberrant or increased expression of adhesion molecules (3). It has been demonstrated that intercellular adhesion molecule 1 (ICAM-1) is strongly and diffusely expressed during both acute and chronic liver inflammation including PBC (4–6). Accordingly, increased circulating levels of the soluble form of ICAM-1 (sICAM-1) have been shown in a number of inflammatory liver disorders (7, 8), suggesting their usefulness in monitoring disease progression as well as effects of therapies (9, 10). In addition to ICAM-1, endothelial leukocyte adhesion molecule 1 or E-selectin has also been found to be expressed in inflamed tissues predominantly in the early phase after induction with various cytokines (11, 12). However, the origin and possible implications of the soluble form of E-selectin (sE-selectin) are less well characterized. Both adhesion molecules may be involved in the migration and activation of polymorphonuclear neutrophils (PMNs) to sites of bile duct damage in PBC (3). Among leukocytes involved in changes affecting portal tracts and bile ducts in PBC, neutrophils may play a role by releasing highly reactive species and strong proteases. PMN-mediated responses are generally attributed to the coordinated effects of reactive oxidative species and proteolytic enzymes (13). Neutrophil elastase is the most potent among these latter and has been established as a highly specific and reliable index of *in vivo* neutrophil activation (14). Besides reactive oxygen intermediates, human neutrophils have been demonstrated to be capable of

Table 1. Laboratory Data of Primary Biliary Cirrhosis Patients (n = 30)

Variable	Mean Value ± SD	Minimum Value	Maximum Value	Normal Range
AST (IU/L)	75.6 ± 44.2	21	188	0–31 (women) 0–37 (men)
ALT (IU/L)	79.8 ± 56.2	14	279	0–31 (women) 0–37 (men)
ALP (IU/L)	513.2 ± 406.2	87	1679	40–145
GGT (IU/L)	275.3 ± 236.7	38	1118	8–35 (women) 10–50 (men)
TB (mg/dl)	1.3 ± 1.2	0.3	4.5	0.2–1.0
DB (mg/dl)	0.7 ± 0.9	0.1	3.6	0–0.2
ALB (g/dl)	4.0 ± 0.6	2.0	4.9	3.6–5.2
PT (INR)	0.96 ± 0.20	0.83	1.45	0.85–1.25
IgG (mg/dl)	1526.0 ± 516.2	200	2840	840–1660
IgA (mg/dl)	275.2 ± 95.0	93	530	90–395
IgM (mg/dl)	403.5 ± 225.0	113	919	48–220
Mayo score	4.49 ± 1.39	2.51	8.43	

AST = aspartate amino-transferase; ALT = alanine amino-transferase; ALP = alkaline phosphatase; GGT = γ-glutamyl transpeptidase; TB = total bilirubin; DB = direct bilirubin; ALB = albumin; PT = prothrombin time; INR = International Normalized Ratio; Ig = immunoglobulins.

synthesizing and releasing nitric oxide (NO) (15, 16), a versatile multifunctional molecule acting as both a protective and a toxic mediator (17, 18). Its production has been hypothesized to be altered in different pathophysiological conditions of the liver, such as cirrhosis and its complications (19, 20).

This study was undertaken to determine NO production, assessed by measuring circulating nitrosyl-hemoglobin adducts and serum neutrophil elastase, as an indirect marker of PMN leukocyte activation, in patients affected by PBC and in two groups of subjects with chronic hepatitis and postviral and/or alcoholic cirrhosis. We also looked at possible relationships of these parameters with soluble forms of ICAM-1 and E-selectin and with liver biochemical tests.

MATERIALS AND METHODS

Subjects

Thirty consecutive patients (mean age 53.3 yr, range 25–77), 26 female and four male, with PBC were studied. All had positive antimitochondrial antibody testing, and PBC was diagnosed an average of 39.9 months (range 1–146 months) before entering the study on the basis of both typical biochemical patterns and histopathological findings. At the time of diagnosis 13 patients were in stage I, four in stage II, 10 in stage III, and three in stage IV according to conventional criteria (21). Laboratory data as well as the prognostic Mayo score (22) at entry are shown in Table 1; viral markers were negative in all patients. Symptoms referrable to liver disease such as fatigue and pruritus were reported by 25 of the studied patients, whereas 19 presented with extrahepatic autoimmune disorders, including Sjögren's syndrome, arthropathies, Raynaud's phenomenon, and thyroid dysfunction. Esophageal varices with or without previous hemorrhage were documented in 10 cases. Substitutive bile salt therapy (12–15 mg/kg/day of ursodeoxycholic acid) was being administered in 15 patients at the time of the study, and only four were taking corticosteroids and/or immunosuppressives (5–15 mg/day of prednisone for three and 100 mg/day of azathioprine for one). A group of eight patients (mean age 44.7 yr, range 39–53), two female and six male, with biopsy-proven chronic hepatitis (CH) (B virus infection in five and C virus in the remaining three) was also enrolled. Thirteen patients (mean age 48.1 yr, range 26–62; two female and 11 male) with cirrhosis of diverse etiology (seven postviral hepatitis C, three postviral hepatitis B and C, and three alcoholic) and portal hypertension served as a reference group. An additional cohort including 10 patients with choledocholithiasis (mean age 62 yr, range 36–85; seven female and three male) was also studied. Informed written consent was obtained for each patient, and the study was performed according to the ethical guidelines of the 1975 Declaration of Helsinki.

NO Determination

NO production was assessed directly as nitrosyl-hemoglobin complexes. The binding of NO to the heme iron of deoxyhemoglobin, mainly on the venous side of the circulation, leads to the formation of stable nitrosyl-hemoglobin adducts (23, 24). These NO-containing compounds function as either carrier or store systems for NO released into the bloodstream by several cellular sources (25). Due to the unpaired electron residing on NO, nitrosyl-heme adducts may interact with a magnetic field, *i.e.*, they are paramagnetic. With this property, the technique of electron paramagnetic resonance (EPR) spectroscopy has been used to detect circulating NO-hemoglobin derivatives as means to study the *in vivo* generation of NO (26). EPR spectra were recorded with heparinized whole blood samples. The quantitative analysis of characteristic EPR signals was carried out by double integration of typical triplet patterns centered at a spectroscopic splitting factor of 2.015 with 15.6 Gauss line widths. In each case the magnitude of the EPR peaks was considered to be proportional to the amounts of nitrosyl-hemoglobin adducts. Because of the unavailability of a reliable standard for these nitrosyl-hemoglobin com-

plexes, results were expressed in arbitrary units (AUs) as calculated by the instrument software from the area under the three-line EPR spectra. Intra- and extra-assay variations, calculated on seven replicates in a 1-day experiment and on 10 replicates over a 30-day period, were 7.3% and 14.7%, respectively. Nitrosyl-hemoglobin spectra from specimens stored at −80°C appeared stable for at least 2 months.

Neutrophil Elastase Measurement

The content of neutrophil elastase in serum samples was determined by a homogeneous immunoenzymatic assay based on the agglutination of latex particles in the presence of elastase–α_1-protease inhibitor complexes. This method (Ecoline, Merck, Darmstadt, Germany) has been applied to an automated analyzer (Merck Mega), yielding 2.6% and 6.7% variations in intra- and extra-assay replicates, respectively. Reference values (29–86 mg/L) indicated by the purchasing company did not significantly differ from those observed in a group of healthy volunteers in our laboratory.

Soluble ICAM-1 and E-Selectin Concentrations

Two photometric one-step "sandwich" enzyme immunoassays were used for the quantification of human sICAM-1 and E-selectin (Boehringer Mannheim, Mannheim, Germany) in serum samples. Intra- and interassay variances were 6.6% and 13.4% for sICAM-1 and 8% and 15% for sE-selectin, according to manufacturer indications. No cross-reaction with serum components other than the soluble forms of the adhesion molecules has been found for each assay kit. Normal values as indicated by the manufacturer did not differ with respect to findings in our experience with healthy volunteers and were assumed to be reliable controls.

Morphological Evaluations

Needle liver biopsies performed close to the time of blood withdrawal for the biochemical measurements included in this study were available from 17 PBC patients. The degree of neutrophil infiltration of liver biopsies was evaluated on 5-μm-thick sections from tissue fixed in neutral buffered 4% formaldehyde solution, dehydrated in graded ethanol and then embedded in paraffin. The sections were stained with the naphtol AS-D chloroacetate esterase technique as described by Moloney *et al*. (27) with slight modifications, where the substrate was dissolved in dimethyl sulphoxide/Triton X-100 (9:1, vol/vol). Fast garnet GBC, a diazonium salt, was used as a pair for the naphtol released by the action of the esterase specifically present in the neutrophils. Finally, the sections were counterstained with Harris hematoxylin, thereby providing a greenish background against which the neutrophils appeared red.

Statistical Analysis

Differences between PBC patients and the other studied groups as well as within the PBC group were evaluated by Student's *t* test. Spearman rank analysis was performed to assess correlations among considered parameters. In each case a p value of <0.05 was assumed to be statistically significant.

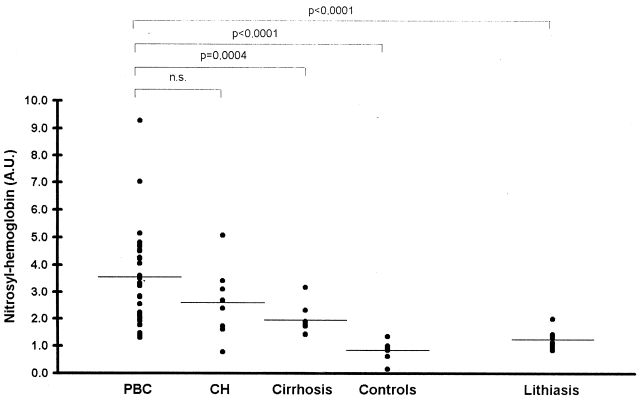

Figure 1. Nitric oxide levels measured in 30 patients with PBC, as compared to those detected in eight subjects with CH, 13 with postviral and/or alcoholic cirrhosis, eight normal healthy volunteers, and 10 patients with choledocholithiasis. Student's *t* test *p* values for pairwise comparisons were CH *vs* cirrhosis, not significant; CH *vs* controls, $p = 0.006$; CH *vs* lithiasis, $p = 0.02$; cirrhosis *vs* controls, $p = 0.0005$; cirrhosis *vs* lithiasis, $p = 0.008$; controls *vs* lithiasis, $p = 0.04$. ● = single values; lines = mean values for each of the five groups of subjects.

RESULTS

Increased amounts of EPR-detectable circulating nitrosyl-hemoglobin adducts were found in PBC with respect to cirrhotic patients and controls (3.55 ± 1.75 AUs *vs* 1.95 ± 0.57 and 0.84 ± 0.34, $p = 0.0004$ and $p < 0.0001$, respectively), whereas no significant difference was shown between PBC and CH subjects (Fig. 1). It is noteworthy that higher nitrosyl-hemoglobin levels were detected in PBC patients than in lithiasis subjects (1.24 ± 0.38, $p < 0.0001$). Furthermore, a superimposable behavior was observed for soluble adhesion molecules: both were elevated in PBC

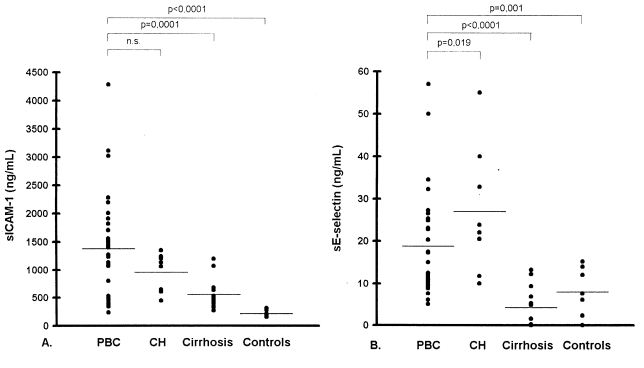

Figure 2. Individual and mean values of sICAM-1 (*A*) and sE-selectin (*B*): findings in 30 PBC patients, eight CH subjects, 13 postviral and/or alcoholic cirrhotics, and eight healthy controls. Pairwise comparisons by Student's *t* test yielded the following *p* values: CH *vs* cirrhosis, $p = 0.015$ and $p = 0.003$ for sICAM-1 and sE-selectin, respectively; CH *vs* controls, $p = 0.0004$ and $p = 0.008$; cirrhosis *vs* controls, $p = 0.0008$ and not significant.

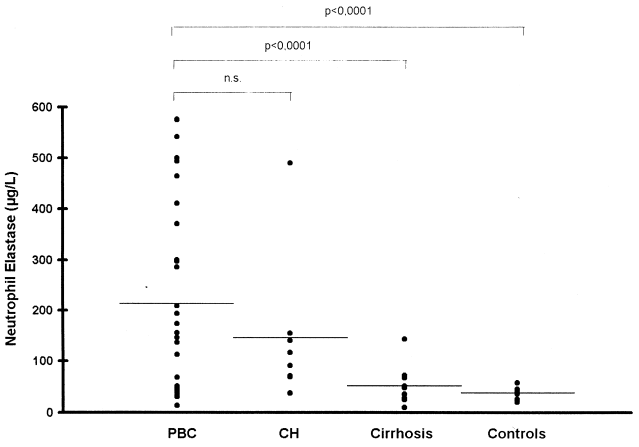

Figure 3. Neutrophil elastase results from 30 PBC patients compared to those detected in eight CH and 13 cirrhotic patients, as well as eight healthy controls. Individual and mean values are indicated. Student's *t* test *p* values for pairwise comparisons were CH *vs* cirrhosis, not significant; CH *vs* controls, not significant; cirrhosis *vs* controls, not significant.

patients in comparison to cirrhotics and controls [1375.7 ± 939.7 ng/ml *vs* 560 ± 282.3 and 213.3 ± 55.3, $p = 0.0001$ and $p < 0.0001$, respectively, for sICAM-1 (Fig. 2A); 18.9 ± 12.7 ng/ml *vs* 4.1 ± 4.9 and 7.9 ± 5.5, $p < 0.0001$ and $p = 0.0011$, respectively, for sE-selectin (Fig. 2B)] but similar to levels detected in CH subjects. Nevertheless, sICAM-1 and sE-selectin findings were not correlated in our study population. On the other hand, the concentrations of neutrophil elastase were significantly increased in PBC patients only (213.7 ± 192.0 mg/l *vs* 51.1 ± 34.3 and 38.0 ± 11.5 in controls and in cirrhotic patients, respectively; $p <$ 0.0001 for both comparisons), whereas they did not differ among CH subjects, cirrhotics, and controls (Fig. 3). Neither gender nor age differences were found for all considered parameters.

sICAM-1 and sE-selectin levels were unaffected by hydrophylic bile salt administration (1368.7 ± 777.5 ng/ml in patients who were given this therapy *vs* 1382.7 ± 1106.5 in those who were not undertaking any treatment for at least 6 months preceding the study, $p = 0.97$, and 18.7 ± 14.4 ng/ml *vs* 19.0 ± 10.7, $p = 0.94$, respectively). Similarly, substitutive therapy did not influence NO production (3.53 ± 1.94 AUs *vs* 3.34 ± 1.62, $p = 0.76$) or neutrophil elastase release (197.0 ± 183.0 µg/L *vs* 230.3 ± 205.6, $p =$ 0.64).

Interestingly, 10 patients who had portal hypertension and a history of esophageal varices with or without previous hemorrhage had lower nitrosyl-hemoglobin levels and neutrophil elastase concentrations as compared to the other 20 patients (2.65 ± 1.23 AUs *vs* 3.81 ± 1.87, $p < 0.05$, and 115.5 ± 112.0 mg/L *vs* 262.7 ± 206.7, $p = 0.0174$, respectively).

In Figure 4 results of correlations reaching statistical significance among studied parameters and biochemical features in the group of all PBC patients are reported.

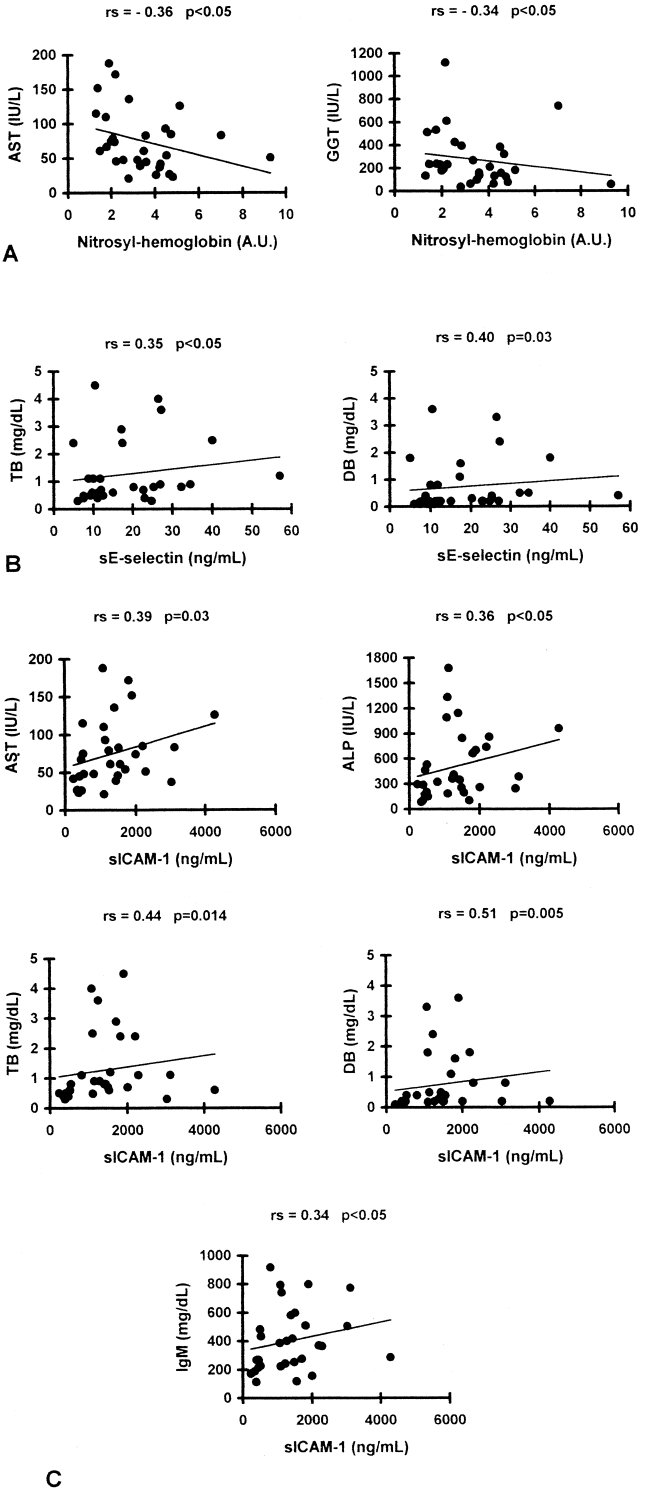

Figure 4. Statistically significant Spearman rank correlations among studied parameters in patients with primary biliary cirrhosis. (*A*) Correlations between nitrosyl-hemoglobin levels and AST as well as γ-glutamyl transpeptidase (GGT) values. (*B*) Correlations between sE-selectin levels and total bilirubin (TB) as well as direct bilirubin (DB) values. (*C*) Correlations between sICAM-1 and AST, alkaline phosphatase (ALP), TB, DB, and IgM.

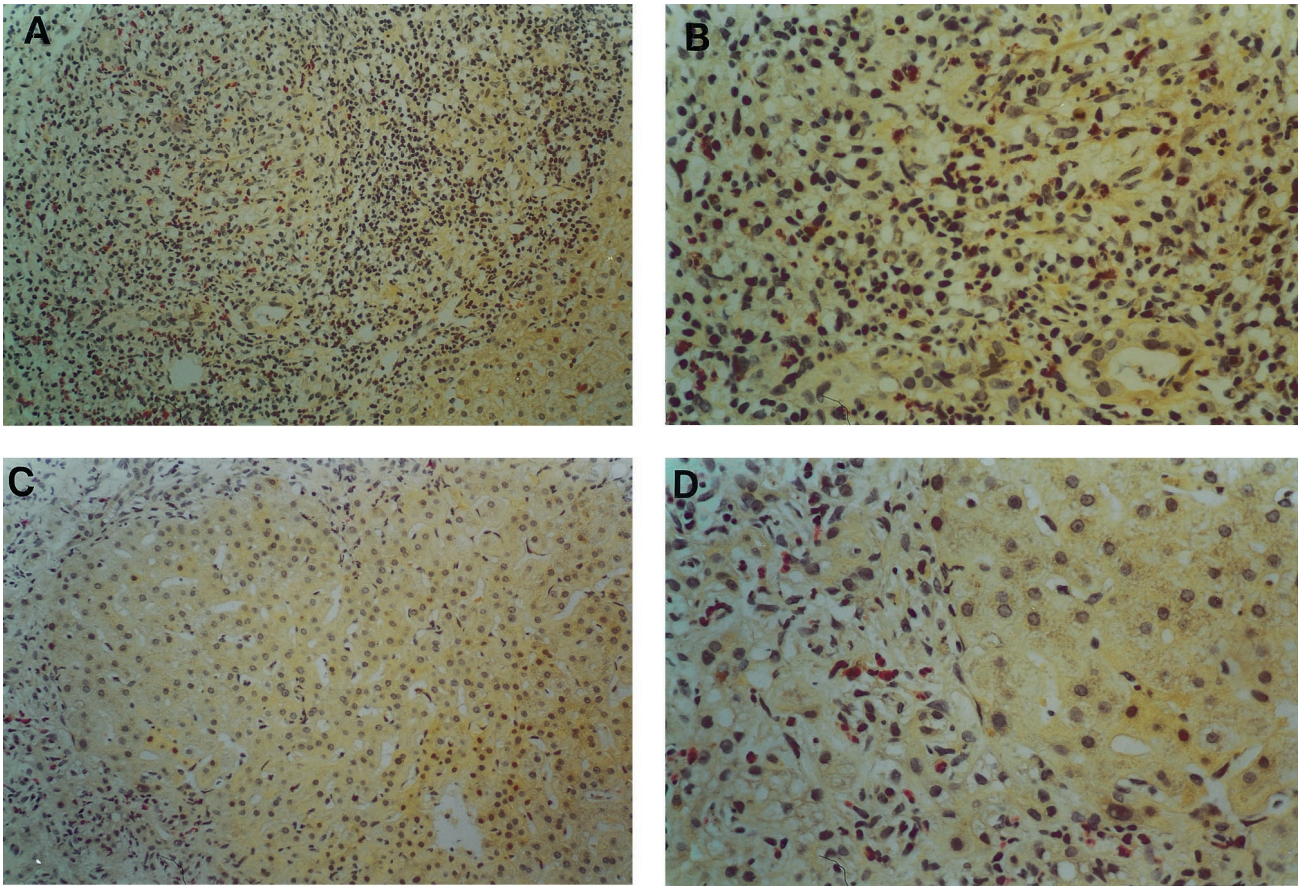

Figure 5. (*A*, *B*) PBC stages I–II. Portal tract expanded by granulomatous inflammation in which numerous red-stained neutrophils are visible. *A*, ×200; *B*, ×400. (*C*, *D*) PBC stages III–IV. Bridging fibrosis between portal tracts accompanied by a moderate infiltrate of lymphocytes, macrophages, and occasional plasma cells. Neutrophils appear as red-stained cells diffusely distributed. *C*, ×200; *D*, ×400. AS-D chloroacetate counterstained with Harris hematoxylin.

The morphological assessment revealed the presence of a variable number of neutrophils infiltrating the portal tracts in all observed specimens but one (Fig. 5). Neutrophil infiltrates ranged from scarce and isolated to some cases where they appeared as the most represented cell types, with a reproducible correlation between the degree of neutrophil accumulation, though qualitatively assessed, and both nitrosyl-hemoglobin and neutrophil elastase levels.

DISCUSSION

In agreement with previous reports (28, 29), we have found that serum concentrations of soluble forms of ICAM-1 and E-selectin are significantly elevated in PBC patients relative to normal subjects. Although similar to levels measured in CH subjects, in PBC they are highly increased as compared to those detected in cirrhotic patients. This may reflect upregulated expression of adhesion molecules on bile ducts, hepatocytes, vascular endothelial cells, and infiltrating leukocytes, a critical step in the pathogenesis of immune-mediated destruction of interlobular bile ducts and hepatocytes (3, 6). Although not specific to PBC, increased serum levels of circulating forms of adhesion molecules have been correlated with the stage of progression of the disease (9, 10, 29). Indeed, this is supported by relationships between soluble adhesion molecules and biochemical markers of cholestasis in our study. Both adhesion molecules included in this work are capable of enhancing PMN recruitment to sites of inflammatory damage (30, 31). sICAM-1 has been shown to induce elastase release by PMNs and to participate as a priming stimulus in the production of reactive oxygen species in response to a second activating effector substance (32). On the other hand, E-selectin is particularly important in the early adhesion and rolling of neutrophils (33, 34). From these observations, it could be hypothesized that extremely high sICAM-1 and sE-selectin amounts measured though nonspecific tests of neutrophil responses may reflect an enhanced PMN activation in PBC, as demonstrated by our findings of elevated neutrophil elastase concentrations mainly in this group of patients. Among reactive molecules, human neutrophils are capable also of releasing NO. The greatly augmented levels of circulating nitrosyl-hemoglobin adducts detected in our study suggest that an enhanced NO production by these as well as by other cell types may occur in PBC. Till now, few articles have addressed the determination of circulating NO in PBC patients; yet hyperproduc-

tion of NO, considered by many authors a feature of cirrhosis and chronic liver disease *per se*, seems to be highly upregulated in PBC. EPR-detectable nitrosyl-hemoglobin complexes are useful in assessing NO synthesis, and their increased levels may be directly related to NO biological effects (24). Although an ubiquitously enhanced NO generation is likely to occur in PBC from a variety of cellular sources including Kupffer cells, endothelial cells, and leukocytes, from our results one can suppose that activated neutrophils in this condition release relatively large amounts of NO. Several mechanisms may account for the enhanced NO production by neutrophils, including cytokine-mediated stimulation of inducible NO synthase (15) and adherence-dependent activation as demonstrated for hydrogen peroxide (35). Cholestasis itself may induce the upregulation of NO synthetic pathways and/or the release of the mediator (36, 37). It should be considered that, even if large duct obstruction could be associated with cholangiolitis and neutrophil infiltration, the duration of the disease in our lithiasis controls, always longer than 1 yr, may favor the hypothesis that in all of them cholestasis was acutely precipitated by stones, a condition presumably not accompanied by neutrophil activation and NO production. The systemic activation of neutrophils is not the entire explanation, as demonstrated by the lack of a significant correlation between nitrosyl-hemoglobin and neutrophil elastase levels, thus suggesting different sources and/or different modulation. Nevertheless, a complex series of NO/neutrophil interactions may condition liver cell injury in the pathogenesis of PBC. On one hand, NO participates in the generation of strong oxidants from neutrophils through its reactions with reactive oxygen species and myeloperoxidase and contributes to the reduction in antiproteinase activity (38). On the other hand, it exerts protective and anti-inflammatory effects at least in part by preventing neutrophil adhesion and modulating neutrophil responses (39). This may be supported by our findings of an inverse correlation between nitrosyl-hemoglobin levels and the Mayo score in a population of 45 PBC patients including 15 subjects in addition to those enrolled in this study ($r_s = 0.31$, $p = 0.04$ by Spearman rank test) (40) as well as of the occurrence of lower nitrosyl-hemoglobin levels in patients with portal hypertension.

Due to the cross-sectional nature of this study, effects of substitutive treatment cannot be substantiated even if no differences were found between the group of patients who were undertaking this kind of therapy and the nontreatment group. However, our data seem to indicate that substitutive bile salts have no influence on biochemical markers of either neutrophil activation or cellular adhesion.

Whether the expression of adhesion molecules and the release of soluble mediators are an early event in the pathogenesis of bile duct damage or a secondary response to inflammation is not readily deducible from our results. Changes affecting vascular and infiltrating cells in the portal tract could be related to the inflammatory process itself, and abnormalities of neutrophil functions have been described in cirrhosis and cholestasis as a consequence rather than a cause of the liver disease (41). Despite limitations of the measurement of systemic markers, however, it should be noted that neutrophil infiltrates are present in the liver in the majority of PBC biopsies in our study population, with a good correlation between the degree of neutrophil accumulation and circulating levels of assessed mediators. From our findings it can be suggested that, once recruited and primed due to the intervention of cell adhesion molecules, neutrophils may mediate a wide variety of functions by releasing a number of important effector substances, including NO and elastase. In addition to neutrophils, several different cells are likely to be involved in the generation of NO as well as other soluble factors, thereby triggering and sustaining the chronic inflammatory process of PBC.

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