STUDIES IN PRIMARY BILIARY CIRRHOSIS

B. G. TAAL

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PROEFSCHRIFT

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This thesis is a summary of our studies on primary biliary cirrhosis, which have been described in more detail in the following articles:

1. Primary biliary cirrhosis: a changing clinical presentation. Taal BG, Schalm SW. Neth J Med 1981;24:101-108.
2. Clinical diagnosis of primary biliary cirrhosis: a classification based on major and minor criteria. Taal BG, Schalm SW, ten Kate FWJ, Hermans JJ, Geertzen HGM, Feltkamp TEW. (submitted for publication).
3. Serum IgM in primary biliary cirrhosis. Taal BG, Schalm SW, de Bruyn AM, de Rooy FWM, Klein F. Clin Chim Acta 1980;108:457-463.
4. Monomeric (7S) IgM in primary biliary cirrhosis. Taal BG, Schalm SW, de Bruyn AM, Kornman-van de Bosch HJ. Scand J Gastroent 1981; in press.
5. Cryoglobulins in primary biliary cirrhosis: prevalence and modulation by immunosuppressive therapy. Taal BG, Schalm SW. Gastroenterology (submitted).
6. Low therapeutic value of D-penicillamine in primary biliary cirrhosis. Taal BG, Schalm SW, ten Kate FWJ, van Berge Henegouwen GP, Brandt (submitted for publication).
7. Treatment of primary biliary cirrhosis: a comparison of D-penicillamine, D-penicillamine plus prednisone, and a placebo. Taal BG, Schalm SW. (To be published in Zeitschrift für Gastroenterologie as part of a review article.)

Introduction

In 1892 Hanot described 'la cirrhose hypertrophique avec ictère chronique'. It was probably this condition that 58 years later Ahrens (1) called primary biliary cirrhosis: a syndrome seen predominantly in middle-aged women and characterized by longstanding pruritus, jaundice and massive enlargement of the liver, frequently accompanied by xanthelasma and increased serum lipids. In 1964 Foulk of the Mayo Clinics described a series of 49 patients with the primary biliary cirrhosis syndrome, of whom 50% had chronic cholangiolitic hepatitis without cirrhosis; the syndrome was associated with an increased serum alkaline phosphatase level (2). A few years later the diagnostic importance of antimitochondrial antibodies (3), as well as increased serum IgM levels (4), was discovered. From detailed histological studies by Rubin et al. (5) came the term of chronic non-suppurative destructive cholangitis, but primary biliary cirrhosis (PBC) is still the term most frequently used. In 1970-1971 the associations with the sicca syndrome (6) as well as with scleroderma and Raynaud's syndrome (7) were found. In addition to the usual complications of cirrhosis, hepatic osteodystrophy is prominent in this cholestatic syndrome (8), probably due to malabsorption of calcium and disturbances in vitamin D metabolism (9). As a result of the increasing availability of biochemical (alkaline phosphatase) and immunological (antimitochondrial antibodies, IgM) determinations, PBC is now frequently diagnosed in an earlier phase. The presenting clinical features of PBC therefore need redefining.

For the diagnosis of primary biliary cirrhosis the presence of antimitochondrial antibodies (AMA) is a sensitive marker (10). Since the first report on AMA by Doniach in 1966 (3), the prevalence of AMA in PBC has risen from 84 to 99%. Antimitochondrial antibodies, however, are also found in other diseases and the prevalence of AMA in chronic active hepatitis (CAH) is reported to be 11-28% (3,10). Differentiation of CAH and PBC can be very difficult in some cases where histological features are non-specific (11). The diagnosis as defined by an international committee in 1976 (14) is based on subjective evaluation of a combination of features. Because treatment and prognosis of PBC and CAH are different, there is a need for objective diagnostic guidelines.

The aetiology of PBC is unknown; abnormalities in both humoral and cellular immunity, however, have been connected with the pathogenesis of PBC. The antibodies to the inner membrane of mitochondria (AMA) are probably only of diagnostic importance (11), while antibodies to bile duct cells and impaired delayed hypersensitivity are thought to be secondary to bile duct damage and not related to the aetiology (15,16). Increased serum IgM concentration (17), and more recently circulating IgM containing immune complexes (18-22) have been described, but their significance in relation to pathogenesis remains uncertain. Equally unknown is the role of copper in primary biliary cirrhosis. Hepatic copper in PBC may reach concentrations comparable with those in Wilson's disease (28), but copper accumulation is probably secondary to cholestasis and not primarily related to PBC.

With regard to treatment, the immunosuppressive drug azathioprine (24,25) has been shown to have no significant effect, while corticosteroids (26,27) have never been properly evaluated. Recently, D-penicillamine has been tried because of its copper chelating properties. D-penicillamine, however, also has immunosuppressive properties and these may be more related to its possible beneficial effect in PBC (29-31). Its toxicity, however, appears to be the limiting factor for widespread use, but slow introduction and a small maintenance dose may retain the beneficial effects without the occurrence of severe side effects.

Aim of the study

The specific aims of the study were:

- To (re)define the clinical features of the primary biliary cirrhosis syndrome.
- To develop objective criteria which combine specificity with sensitivity for the diagnosis of PBC.
- To study the relation of IgM in various forms (pentameric, monomeric and cryoglobulin) to the activity of the non-suppurative destructive cholangitis.
- To test the therapeutic effects of 'go-slow-go-low' D-penicillamine therapy in a randomized controlled trial, and to determine whether any favourable effects were due to a reduction of hepatic copper or to a decrease of immunological disturbances.
- To perform a pilot study for evaluation of the effect of a combined therapy with small doses of D-penicillamine and prednisone.

Methods

SUBJECTS

The control group (Table I) consisted of 25 age- and sex-matched patients seen since 1978 in the out-patients clinic of the Department of Internal Medicine II of the University Hospital, Rotterdam, because of abdominal complaints; no organic disease was demonstrable in any of the patients of this group. In addition, a control group of 25 patients with various liver diseases was studied: 8 patients with HBsAg positive chronic active hepatitis, 8 with HBsAg-negative chronic active hepatitis, 6 with biopsy proven alcoholic cirrhosis, and 3 with cryptogenic cirrhosis.

The other subjects (Table I) were 25 consecutive patients with PBC referred to the University Hospital, Rotterdam, since 1978. The diagnostic criteria for PBC for this study comprised an increased serum alkaline phosphatase level, a positive antimitochondrial antibody test, no demonstrable abnormalities of the extrahepatic bile ducts at cholangiography and a liver biopsy showing lymphoplasmocellular infiltrates in enlarged portal tracts, paucity of interlobular bile ducts and/or granuloma around destroyed bile ducts. Subsequently, another group of 15 patients fulfilling these criteria were seen and some measurements in these patients were used for analysis.

Sixteen symptomatic patients from this group, with serum alkaline phosphatase levels at least twice the upper limit of normal, participated in the therapeutic studies. In addition, 8 patients from the Municipal Hospital, Arnhem, fulfilling the same entry criteria, also took part in the double-blind clinical trial with D-penicillamine.

In order to develop objective criteria for the diagnosis of PBC, 92 patients with a positive AMA test were studied from January 1979 until October 1980. They represent part of the 437 AMA-positive serum samples detected in 1976-1977 in the Department of Auto-immune Diseases of the Netherlands Red Cross Blood Transfusion Service in Amsterdam. This laboratory provides the AMA test for approximately 90% of the Netherlands. For each hospital with more than three AMA-positive patients we contacted the physicians, and with their help 92 patients were seen by the same investigator.

GENERAL METHODS

Each patient was examined by the same investigator. Histories were taken with the aid of a checklist with particular emphasis on symptoms such as fatigue, pain, itch and use of cholestyramine. At physical examination special attention was given to scratch marks, spider naevi, palmar erythema, xanthelasma or xanthoma, size of liver and spleen, ascites and/or oedema. In addition we asked about dry eyes, dry mouth and white fingers in cold weather, and looked for telangiectasia and diminished tear secretion (less than 10 mm in 5 minutes by means of the Schirmer test; SMP Division Cooper Laboratory, New York, USA).

Haematological studies were performed with EDTA blood (Coulter Counter). Clotting factors were assessed by the Normotest® method (Nyegaard, Oslo). Serum bilirubin and alkaline phosphatase were determined with the Technicon SMA 12-60; serum GPT (glutamic pyruvic transaminase), GOT (glutamic oxalacetic transaminase), and γ-glutamyltranspeptidase (γ-GT) levels were determined by an UV kinetic measurement (LKB reaction rate analyzer, Stockholm, Sweden); these biochemical measurements were expressed as a multiple of the upper limit of normal (defined as the

TABLE I: GENERAL FEATURES OF THE PATIENTS STUDIED

	Total number	*Sex*		*Age, years median(range)*	*Duration disease, yr median(range)*
		male	*female*		
Controls					
normal	25	4	21	54 (38-72)	4 (1-19)
chronic liver disease	25	15	10	51 (24-68)	not tested
Primary biliary cirrhosis					
Rotterdam I	25	4	21	54 (38-72)	3 (1-11)
Rotterdam II	15	1	14	49 (25-72)	2 (1-10)
Arnhem	8	0	8	50 (40-65)	5 (1-16)
AMA-positive patients	92	7	85	64 (33-82)	4 (1-28)

95th percentile of the normal population of the routine clinical chemistry laboratory); albumin and γ-globulin concentrations were measured by standard cellulose acetate electrophoresis. Fasting bile acids were measured by radioimmunoassay (32). An indirect immunofluorescent technique using fluorescein isothiocyanate conjugated sheep antihuman immunoglobulin (Netherlands Red Cross Blood Transfusion Service, Amsterdam) was used for demonstration of antibodies against nuclear antigens (ANF, on rat kidney) and mitochondria (AMA, on rat kidney) and antibodies against smooth muscle (SMA, on rat stomach) in serum diluted twenty-fold (33). When positive, the serum sample was titrated. As an assay for determination of immune complexes, the ^{125}I-Clq binding was measured according to Zubler (34).

Liver biopsies were performed using a Tru-Cut® needle (Travenol Laboratories, Deerfield, Illinois, USA) in patients under our care; liver biopsies from other centres were reviewed. Abnormalities including portal inflammation, bile duct lesions and granuloma, as well as the degree of fibrosis and piecemeal necrosis were scored by a pathologist who had no knowledge of the clinical features of the patients. A bile duct lesion was considered to be present when a mononuclear infiltration affected the bile duct epithelium with or without a granulomatous reaction in connection with a bile duct. In the case of atypical proliferation of duct epithelium, a bile duct lesion was suspected (Fig. 1). The degree of fibrosis was scored as none, mild (some periportal deposition), moderate (formation of some portoportal septa) or severe (extensive portoportal septa); the degree of piecemeal necrosis was similarly scored as absent, mild (in some portal tracts infiltration of lymphoplasmocellular elements into the liver parenchyma), moderate (infiltration around all portal tracts) or severe (around all portal tracts infiltration deep into the parenchyma forming bridging necrosis). For the clinical trial with D-penicillamine, a piece of 3 mm from each biopsy specimen of about 2 cm was transferred to a copper-free tube; liver copper was measured by neutron activation analysis (35).

Some aspects of bone metabolism were studied only in the first 25 patients with PBC and 25 normal controls. The bone mineral content per square centimetre was assessed by densitometric analysis of the radius of the right forearm (36) (Norland Instruments, Fort Atkinson, Wisconsin, USA). X-rays of the lumber spine and the pelvic bones were scored by a radiologist according to the degree of osteodystrophy (none, minimal, moderate or severe). Serum 25-hydroxycholecalciferol (vitamin D3) was determined according to Edelstein (37), while the serum calcium level was estimated with the Technicon SMA 12-60.

TREATMENT AND FOLLOW-UP

Each patient participating in a therapeutic trial was given detailed assessment before entry and at six-month intervals, including clinical, biochemical and immunological measurements as well as a liver biopsy when possible. For monitoring side effects, blood counts and urinalysis (for protein) were initially performed monthly and after six months at three months intervals. Symptoms were scored semi-quantitatively as follows: fatigue was scored as none, mild or severe (i.e. inability to perform normal daily tasks), while pruritus was scored as absent, intermittent or continuous with need of cholestyramine.

Sixteen consecutive symptomatic patients with PBC from two centres participated in a double-blind controlled trial. After randomization in each centre, patients received either the placebo or D-penicillamine in an increasing monthly dosage of 250-1000 mg daily for six months (slow introduction) and followed by 500 mg daily for another six months (low maintenance dose). Therapy was discontinued after one year and the patients were observed during the following 6 month-period without therapy. All patients received prophylactically 25 mg vitamine B6 daily.

Nine consecutive symptomatic patients from one centre (Rotterdam) took part in a pilot study and were treated with a combination of 250 mg D-penicillamine and 10 mg prednisone daily. This group of nine patients consisted of 3 patients from the D-penicillamine group and 4 from the placebo group, while 2 had not been treated before.

The therapeutic studies were approved by the local institutional Medical Ethics Committee on September 26th, 1977.

STATISTICS

For statistical analysis of the initial data on the patients with PBC and the age- and sex-matched controls, the (paired) Wilcoxon signed rank test was used because most data sets showed a clearly non-normal distribution. To test the significance of discrete variables, the McNemar test was used.

For comparison of the initial data on patients with PBC in various treatment groups the (unpaired) Wilcoxon two sample test was used. To compare data of the patients of one group during therapy, the (paired) Wilcoxon signed rank test was applied.

To test the correlation between two variables in a

group of patients the Spearman correlation coefficient was used.

Differences were considered to be statistically significant when the p-value was less than 0.05.

SPECIAL STUDIES

Classification procedures for diagnosis

Initially, internationally accepted, strict criteria for the diagnosis of PBC (4,14,38) or CAH (14,39-41) were used. PBC was established when the serum alkaline phosphatase concentration was at least twice the upper limit of normal, SGOT less than five times the upper limit of normal, serum IgM more than 2.8 g/l, the AMA titre equal to or larger than 1/40 and a duct lesion demonstrable in the liver biopsy specimen. CAH was diagnosed when the SGOT was at least five times the upper limit of normal, without a more than two-fold increase in serum alkaline phosphatase, and when in addition serum IgG was more than 18 g/l, the ANF or SMA test positive and moderate or severe piecemeal necrosis present in the liver. Patients were presumed to have no liver disease when both serum alkaline phosphatase and SGOT were normal, and neither duct lesions nor piecemeal necrosis were observed at liver biopsy. The diagnosis PBC, CAH or no liver disease was made only when all features of a single entity were found in one patient. Patients who did not fulfil the above criteria were considered to have undefined chronic liver disease.

Subsequently, cluster analysis was performed using 14 variables for the total group and 17 variables for those from whom a liver biopsy specimen was obtained. These variables consisted of: symptoms and signs (pruritus, abnormal tear secretion) as well as biochemical (bile acids, alkaline phosphatase, γ-GT, SGOT, SGPT), immunological (IgM, IgG, cryoglobulin M, ^{125}I-Clq binding, titre of AMA, SMA and ANF) and histological features (duct lesion, granuloma, piecemeal necrosis). This analysis leads to a subdivision of the group of patients into a few clusters (in our case three). The subdivision is made in such a way that intra-cluster variability is less than inter-cluster variability.

The difference from the previous classification procedure is that no pre-assigned weight is attached to the variables. An advantage of cluster analysis is that a possible bias caused by wrong weights to some variable is avoided; a disadvantage is that medical interpretation of the clusters created is often difficult. A computer programme was used according to Wishart (42). The importance of a variable to separate the patients into clusters was expressed in a rank number based on the F ratio (variance of the variable within the cluster divided by variance of the total group). When the F ratio is low, the rank number is low and consequently the variable is highly representative for a cluster.

Finally, a new diagnostic scheme (Table II) based on major and minor criteria was developed by analogy with diagnostic systems used for systemic lupus erythematosus (43) and other rheumatological conditions. Two levels of diagnostic accuracy

TABLE II: A NEW SYSTEM FOR THE DIAGNOSIS OF PRIMARY BILIARY CIRRHOSIS AND CHRONIC ACTIVE HEPATITIS

PBC*

Major criteria	*Minor criteria*
– AMA titre $\geq$ 1/20	– pruritus with need of medication
– liver biopsy: duct lesion**	– jaundice with normal clotting factors
	– alk. phosph. $\geq$ 2x the upper limit of normal
	– serum IgM > 2.8 g/l
	– Schirmer test < 10 mm tear secretion/5 min

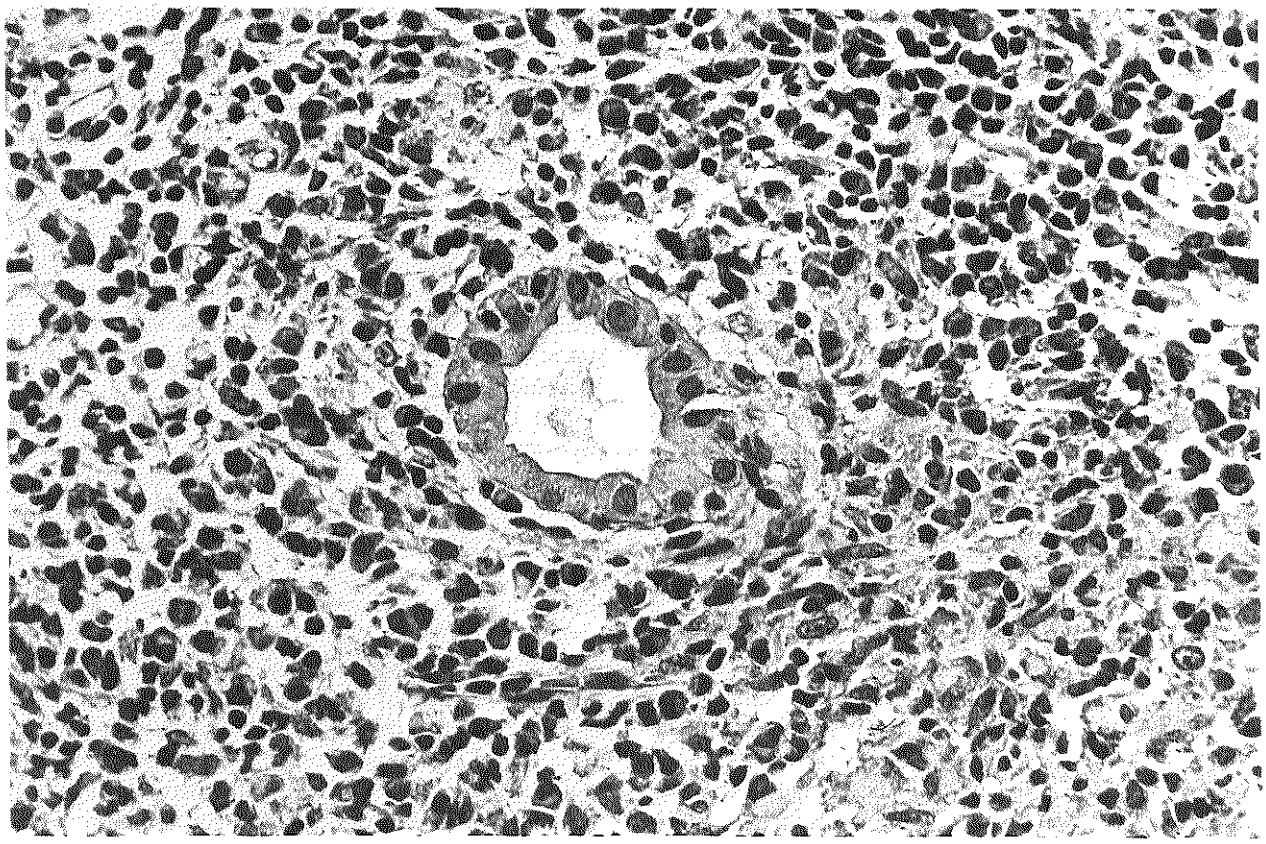
CAH*

Major criteria	*Minor criteria*
– ANF or SMA titre $\geq$ 1/20	– severe fatigue without pruritus
– liver biopsy: piecemeal necrosis***	– jaundice with abnormal clotting factors
	– SGOT $\geq$ 3x the upper limit of normal
	– γ-globulin > 20 g/l

*A definite diagnosis: 2 major and 2 minor, or 1 major and 4 minor criteria; a probable diagnosis: 2 major, or 1 major and 2 minor criteria.

**A duct lesion is defined as a mononuclear infiltration affecting the bile duct epithelium with or without a granulomatous reaction in connection with a bile duct, or atypical proliferation (see methods).

***Piecemeal necrosis was considered to be when an infiltration into the liver parenchyma consisting of lymphoplasmocellular elements was found in all portal tracts (see methods).



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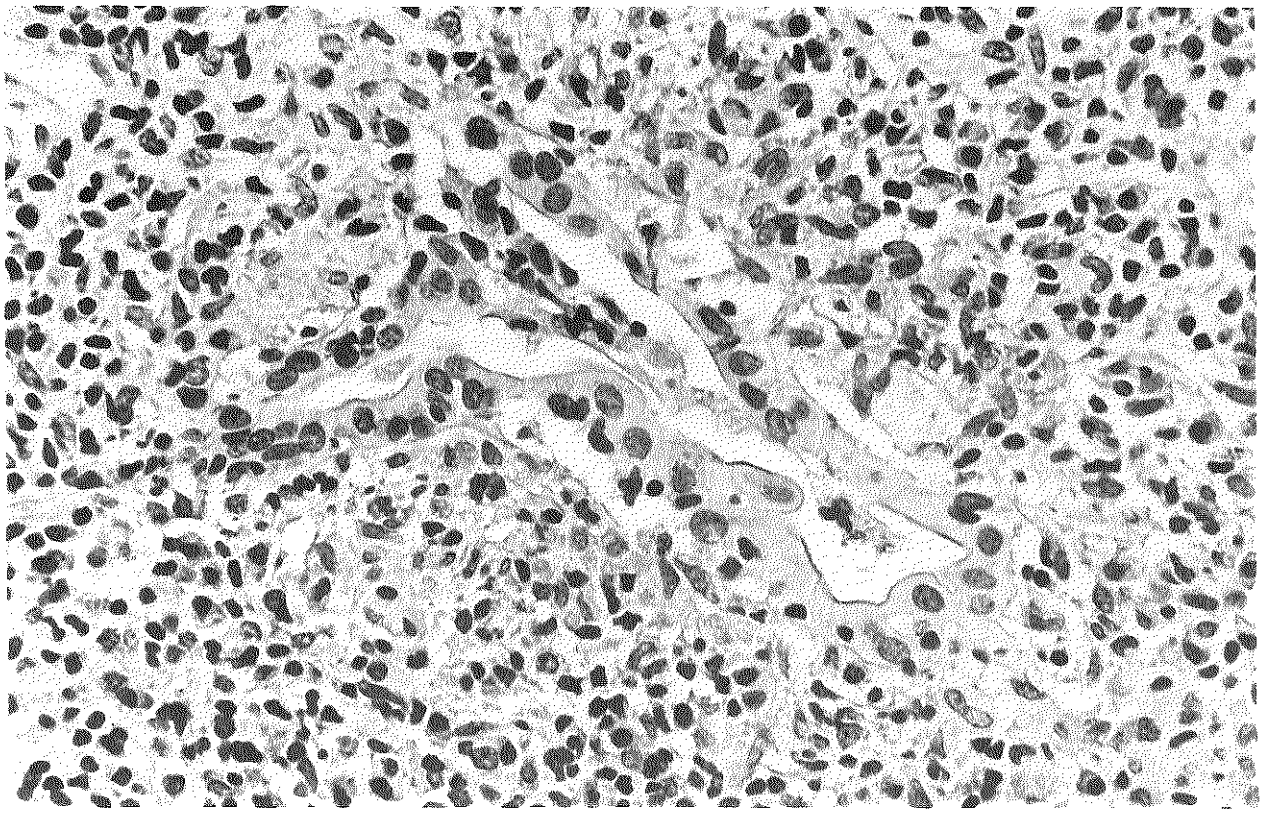

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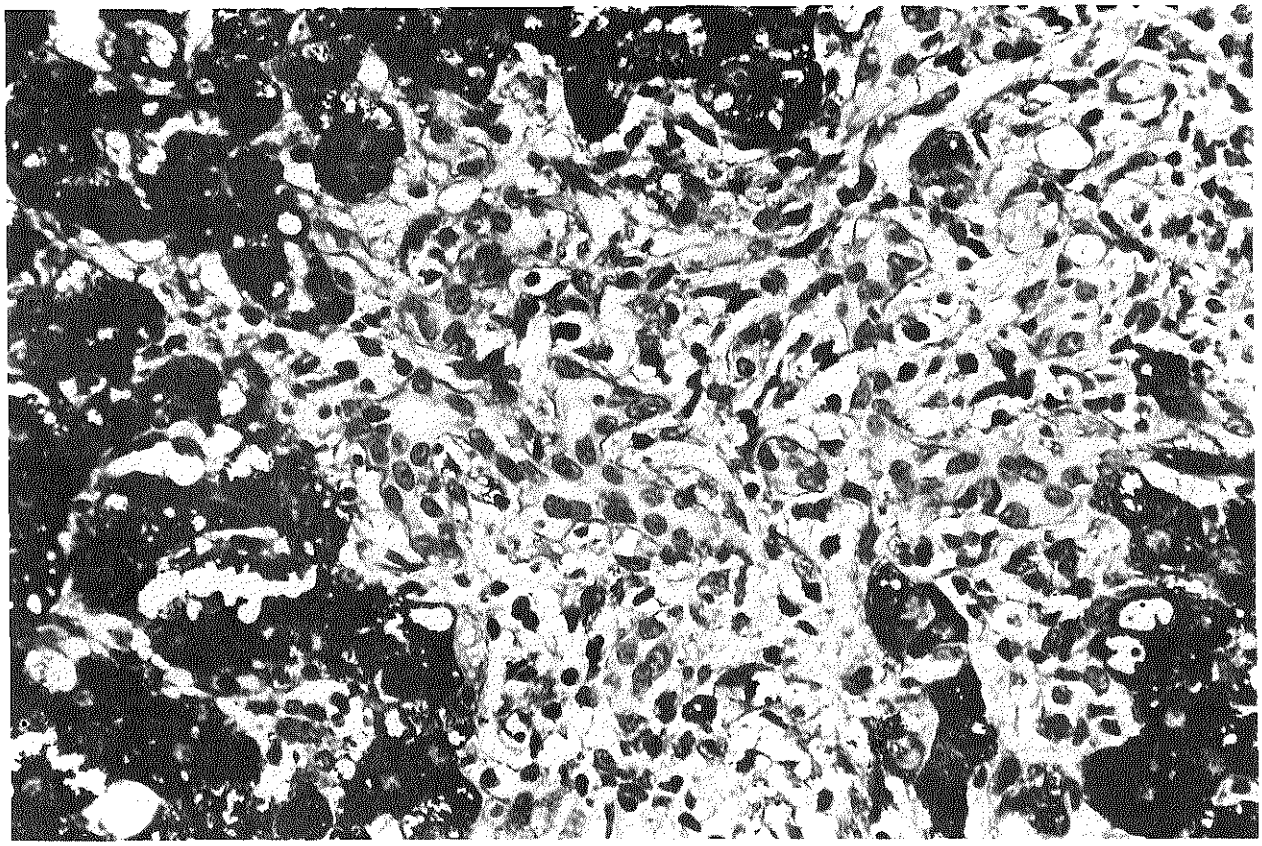


Fig. 1. A bile duct lesion characteristic for PBC, reveals a lymphoplasmocellular infiltrate affecting the bile duct epithelium (1A; haematoxylin-azaphloxin; 760x); frequently the infiltrate shows a granulomatous aspect with destruction of the bile duct (1B; haematoxylin-azaphloxin; 760x). In Figure 1C atypical proliferation of bile duct epithelium is present, which is suspicious for PBC (periodic acid Schiff; 760x).

were introduced. A diagnosis was considered to be definite when two major and two minor or one major and four minor criteria were fulfilled, and was regarded as probable when two major or one major and two minor criteria were fulfilled.

Immunoglobulin M measurements

Classical method – In serum samples processed at room temperature IgM was measured quantitatively by radial immunodiffusion according to Mancini (44). Partigen® immunoplates and standards (Behringwerke, Marburg, FRG) calibrated against WHO standards were used. The plates were incubated at 37°C for five days. The squared diameter of the precipitation ring was plotted against the concentration of the various standards to determine the calibration line.

Alternative method – Because the classical method may lead to erratic measurements in the presence of subgroups of IgM (45), IgM was also measured by the alternative method. The pentameric IgM (19S) in serum samples and standards was reduced to monomeric (7S) IgM with 1,4-dithioerythritol in phosphate-buffered saline (pH 7.4) to a final concentration of 0.01 M (30 min, 37°C). After appropriate dilution the samples were applied to the immunoplates and further processed as in the classical method. The standard used was a serum sample with a high monoclonal IgM concentration (absolute value 55 g/l) calibrated against purified IgM preparations using the radial immunodiffusion technique and checked in unprocessed serum by a non-immunological method (analytical ultracentrifugation).

Detection of 7S IgM

7S IgM was detected by double immunodiffusion in a polyacrylamide gel, prepared by dissolving 5g cyanogum 41 (Serva, Heidelberg, FRG), 0.4 ml tetramethylethylenediamine (Serva, Heidelberg, FRG) and 1 mg ammonium persulphate (Merck, Darmstadt, FRG) in 100 ml distilled water. The resulting solution was immediately poured into a glass

dish to form a layer 2.2 mm thick and then left to polymerize for 30 minutes. Sample wells containing 20 μl serum in two dilutions and antiserum wells were arranged in parallel rows rather than in the form of rosettes; all determinations were carried out in duplicate on two different plates. Rabbit anti-human IgM (Dako, Copenhagen, Denmark) was used as an antiserum. Reference 7S IgM, in a dilution ranging from 25 to 250 mg/l, and 19S IgM (dilution range: 5 to 10 g/l, comparable to the total IgM content in PBC sera) served as positive and negative controls. Reference 19S IgM was isolated from a serum sample containing a 19S IgM paraprotein by gel filtration through Sepharose 6B and dissolved in phosphate-buffered saline (PBS). Reference 7S IgM was obtained by reducing the 19S IgM solution with 1,4-dithioerythritol, blocking with ethyleneimine and removing excess reagent by dialysis against PBS. After incubation for two days at room temperature, the gel was washed with PBS, coloured with amido black and inspected for the presence of a precipitation line indicating the existence of 7S IgM.

Cryoglobulins

For isolation of cryoprecipitates, the method of Wands et al. (46) was used with only minor modifications. Venous blood drawn in warm polysterene tubes (Vacuplast®, Greiner, Nürtingen, FRG) was allowed to clot for 3 hours at 37°C and then centrifuged for 30 minutes at 200 g and 37°C. Ten ml of serum was incubated at 4°C for 10 days. The serum was then centrifuged for 1 hour at 8000 g and 4°C. The precipitate was resuspended in 10 ml phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA) pH 7.6 and 0.01% sodium azide, and stored overnight at 37°C. After centrifugation for 15 minutes at 2000 g and 37°C, the supernatant was stored at 4°C for 3 days. After centrifugation for 1 hour at 8000 g and 4°C, the precipitate was washed three times in PBS and finally redissolved in 500 μl 0.1 M sodium acetate pH 5.0 and 0.01% sodium azide. Using the Ouchterlony immunodiffusion technique, this cryoprecipitate was tested for the presence of albumin to check the wash procedure. In this cryoprecipitate preparation the protein content was measured by Lowry's technique (47) and immunoglobulins G, M and A were determined by a radial immunodiffusion technique (44) using special low-level immunoplates (Behringwerke, Marburg, FRG); the concentrations were expressed in milligrammes per litre original serum.

THE CLINICAL FEATURES OF PRIMARY BILIARY CIRRHOSIS

The clinical features of 25 PBC patients and their age- and sex-matched controls are summarized in Table III. Only two patients with PBC were asymptomatic and had been referred to us after an accidental finding of abnormal liver tests. Pruritus was present in 72% of the cases, but itching associated with scratch marks was a prominent feature in only a few cases; only four patients used cholestyramine. Fatigue was often present in both the PBC and the control group, but severe fatigue leading to incapacity to do normal daily work was encountered only in the PBC group. Pain in the liver region was often reported by PBC patients. Jaundice was found in 50% of the patients, and was transient in 25%. Advanced liver disease was rare; only two patients had complications of cirrhosis. Spider naevi, palmar erythema, xanthelasma and xanthoma were relatively rare in PBC patients, while enlargement of the liver and/or the spleen was present in 52%. Diminished tear secretion was noted in 48% of the PBC patients, but also in about 25% of the controls. In contrast, symptoms of dry eyes requiring methylcellulose eye-drop therapy were found in 20% of the PBC group, but in none of the controls. Raynaud's syndrome, as defined by white fingers in cold weather, was as frequent in the PBC as in the control group.

The results of the laboratory studies are summarized in Table IV. The haemoglobin concentration as well as the leucocyte and platelet counts were significantly ($p < 0.05$) lower in PBC patients than in controls, but overt anaemie, leucopenia and/or thrombopenia was rarely observed. Serum alkaline phosphatase, γ-glutamyltranspeptidase and serum transaminases were sensitive tests since the levels were abnormal in 96-100% of the PBC patients; serum bilirubin was increased in 60% of patients, and fasting bile acids in 84%. Serum albumin was significantly reduced in PBC patients, but in only two patients was this decrease of clinical importance in that it led to oedema. Serum cholesterol was not significantly higher in the PBC than in the control group.

Both serum IgG and IgM were significantly increased in PBC patients, and cryoglobulins, especially of the IgM class, were always present in various amounts. The median titre of antimitochondrial antibodies (AMA) was 1/320 with a range of 0-1/2560 (the presence of AMA was one of the criteria for inclusion in the studies; however, in one patient they were no longer demonstrable during the end-stage of the disease). Smooth muscle antibodies were present in 36% of the patients and in only 8% of controls ($p < 0.05$); antinuclear antibodies were found in 20% of the PBC and in 12% of the control population (no significant difference).

Of the findings relating to bone metabolism, neither serum calcium nor 25-hydroxycholecalciferol (vitamin D3) showed a significant difference between PBC patients and controls; six patients, however, were on vitamin D3 therapy. The bone mineral content per square centimetre was signifi-

TABLE III: SYMPTOMS AND SIGNS IN PBC PATIENTS AND AGE- AND SEX-MATCHED CONTROLS

		PBC $n=25$	*Controls* $n=25$
Asymptomatic		2	7
Symptomatic			
pruritus	intermittent	12*	0
	continuous	6*	0
fatigue	moderate	10	14
	severe	6*	0
pain	region of liver	10	4
	arthralgia	9	11
	low back	3	9*
jaundice	intermittent	6*	0
	continuous	6*	0
History of complications			
variceal haemorrhage		2	0
ascites/oedema		2	0
encephalopathy		1	0
Physical examination			
scratch marks		6*	0
xanthelasma		3	0
spider naevi		3	0
palmar erythema		3	1
hepatomegaly		9*	0
splenomegaly		4	0
Schirmer test < 10 mm		12*	5
Associated diseases			
Sjögren's syndrome**		5	0
Raynaud's syndrome***		7	7
scleroderma****		1	0

*Significantly increased according to the Mc Nemar test ($p < 0.05$).

**Defined as dry eyes requiring methylcellulose eye-drop therapy.

***Defined as a history of white fingers upon exposure to cold.

****Characterized by a mask-like face, thickened skin or diminished esophageal motility.

TABLE IV: INITIAL FEATURES IN PATIENTS WITH PBC AND AGE- AND SEX-MATCHED CONTROLS

		PBC n=25		Controls n=25	
		median	range	median	range
Haematology					
Hb	mmol/l	8.3	6.0-10.0	8.8*	7.6-10.0
MCV	fl	92	82-108	92	86-102
leucocytes	$x10^9/l$	6	4-11	7*	5-10
platelets	$x10^9/l$	199	95-468	253*	138-373
Normotest®	%	84	37-100	93	62-100
Blood chemistry					
alkaline phosphatase	xN**	4.3*	0.8-10.0	0.6	0.4-0.8
γ-GT	xN	8.8*	1.0-22.8	0.4	0.2-1.2
SGOT	xN	2.7*	1.1-6.6	0.5	0.3-1.1
SGPT	xN	2.7*	1.2-6.8	0.3	0.2-0.6
bile acids	μmol/l	11*	1-100	2	1-6
bilirubin	μmol/l	16*	6-497	6	2-22
albumin	g/l	44	28-64	50*	42-59
γ-globulin	g/l	18*	10-31	10	4-13
cholesterol	mmol/l	7.1	3.6-18.2	5.9	5.1-8.5
Immunology					
IgG	g/l	15*	10-29	11	8-17
IgM	g/l	5.1	2.5-12.4	1.4	0.7-2.1
cryoIgM	mg/l	11*	5-228	0	0-3
AMA titre		1/320	0-1/2560***	0	
Bone metabolism					
serum Calcium	mmol/l	2.5	2.1-2.6	2.4	2.3-2.6
vitamin D_3	μmol/l	56	15-100*****	46	19-98
BMC****	g/cm	0.61	0.43-0.81	0.66	0.48-0.77

*Significantly increased according to the (paired) Wilcoxon test ($p < 0.05$).
**N is defined as the 95th percentile of the normal population of the routine clinical chemistry laboratory.
***One patient with end-stage PBC and a positive AMA test at previous testings.
****BMC is the bone mineral content as measured by bone densitometry of the right forearm.
*****Six patients on vitamin D therapy.

cantly lower in the PBC than in the control group, but only a minority of the PBC patients showed signs of overt decalcification on the X-ray of the lumbar spine. Two patients showed severe bone disease as manifested by vertebral collapse.

Comment

In this series of 25 patients with the primary biliary cirrhosis syndrome we noted a change in clinical presentation. In contrast to the classical description of Ahrens (1), probably pertaining to the end-stage of the disease, the symptoms are much more variable. A small number of patients may even be asymptomatic; in others pruritus, non-specific symptoms such as fatigue and pain in the region of the liver, have become early and rather constant features.

The primary biliary cirrhosis syndrome is still encountered predominantly in middle-aged women, although we recently saw a 27-year-old man with PBC complicated by a variceal haemorrhage. The association of PBC with the sicca syndrome, as indicated by diminished tear production and the need for methylcellulose eye-drop therapy, was confirmed in our series. In the control group, however, quite a number of patients also had diminished tear production. The frequent occurrence in the control group of Raynaud's syndrome was unexpected and perhaps due to the fact that the question asked (white fingers in cold weather) was not sufficiently specific; this aspect requires further investigation.

The clinical features of PBC are obviously influenced by our definition of the disease, because selection criteria influence the population. Morphology often serves as the basis in defining diseases. When specific histological abnormalities of

the liver, such as portal inflammation, paucity of interlobular bile ducts and granuloma around destroyed bile ducts, are accompanied by biochemical and immunological abnormalities such as an increased serum alkaline phosphatase level and IgM, and the presence of antimitochondrial antibodies, we diagnose the primary biliary cirrhosis syndrome. In contrast, Geubel (44) differentiates patients with the abovementioned features according to their response to corticosteroid therapy. It is generally undesirable to use response to therapy as a criterion in the diagnosis of a disease, and especially of diseases with a chronic fluctuating course.

Some laboratory findings merit special comment. An increased serum IgM level (17) and cryoglobulins were present in all patients. Serum IgM is a sensitive and more specific measurement than the alkaline phosphatase level, and particularly useful when the antimitochondrial antibody test is not readily available. The increase in serum IgM level could be caused by continuous antigenic stimulation or by an abnormal change from IgM to IgG production (48). Determination of cryoglobulins is not a routine test and therefore of little diagnostic value, its uniform presence seems important, however, in view of the putative role in the pathogenesis of PBC (20). The other important finding pertains to the parameters of bone disease. For serum calcium and vitamin D3 there was no difference between the PBC and the control group, while the bone mineral content per square centimetre as measured by densitometry was only slightly lower in PBC patients than in controls. This technique is not widely available; however, X-rays of the lumbar spine and pelvic bones are probably adequate for detection of decalcification. In the present study overt osteodystrophy accompanied by vertebral collapse was rare, and associated exclusively with prolonged jaundice.

OBJECTIVE DIAGNOSTIC CRITERIA FOR PRIMARY BILIARY CIRRHOSIS

In order to develop objective criteria for PBC we examined data on 92 AMA positive patients from 16 centres in The Netherlands. The diagnoses made by the patients own physicians were: PBC in 57 patients, chronic active hepatitis (CAH) in 10, undefined chronic liver disease in 9, and no liver disease in 16.

Using the system with strict diagnostic criteria a large group of patients (39/92 or 42%) remained without diagnosis (Table V). Another striking finding was that no instance of CAH was diagnosed with this system.

The results of cluster analysis in 92 patients with 14 variables and in 73 patients with 17 variables did not differ; only the results in the latter group with more complete data are therefore given. Initially we compared three clusters with the groups formed by the strict diagnostic criteria system. The percentage of patients with PBC was 84 in cluster I, 64 in cluster II and 27 in cluster III. Since cluster III was the largest, a similar number of patients with PBC was present in each cluster and consequently this method was useless for the purpose of diagnostic classification. Two, four or more clusters provided no additional information, because all subsequent clusters were obtained by adding or dividing the original three clusters. In cluster I the most important abnormalities of PBC such as AMA and duct lesions, were prominent as well as the immunological abnormalities, in cluster II the cholestatic features were marked, and in cluster III the AMA, duct lesions, immunological and cholestatic features were least pronounced. The formation of clusters therefore seemed to be based on the severity of the disease and yielded no specific nosological entity.

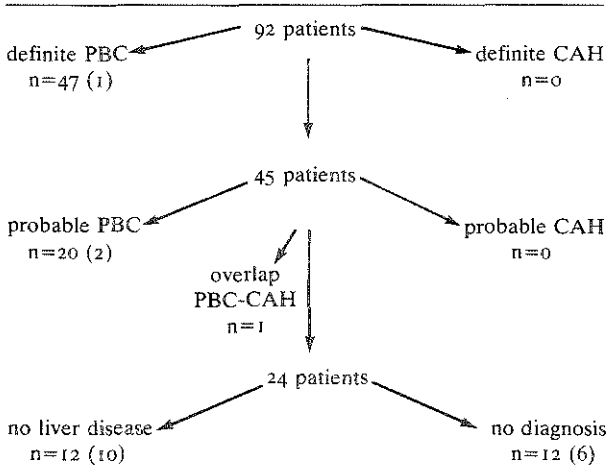
Our new diagnostic system based on major and minor criteria (Table II) distinguished two levels of

TABLE V: COMPARISON OF THE DIAGNOSIS BY STRICT DIAGNOSTIC CRITERIA WITH THAT MADE BY THE PERSONAL PHYSICIANS OF 92 AMA-POSITIVE PATIENTS

	*Diagnosis by strict criteria**				
Diagnosis by patients own physician	*PBC*	*CAH*	*undefined chronic liver disease*	*no liver disease*	*total number of patients*
PBC	29	0	27 (3)	1	57 (3)
CAH	6	0	4	0	10
Undefined chronic liver disease	5	0	3 (1)	1	9 (1)
No liver disease	1	0	5 (5)	10 (10)	16 (15)
Total number of patients	41	0	39 (9)	12 (10)	92 (19)

* Strict diagnostic criteria system: see methods.
In parentheses the number of patients of whom no liver biopsy specimen was available.

TABLE VI: DIAGNOSIS OF AMA POSITIVE PATIENTS USING A NEW DIAGNOSTIC SYSTEM WITH MAJOR AND MINOR CRITERIA



In parentheses the number of patients of whom no liver biopsy specimen was available.

accuracy: a definite and a probable diagnosis. The results are given in Table VI. Definite PBC was found in 47 patients (51%), and probable PBC in 20 (22%); PBC was therefore likely to be present in 67 out of 92 patients (73%). In 13% a diagnosis could not be established, but these patients had mild biochemical abnormalities. With this scheme, as with the method of strict criteria, none of the patients had definite or probable CAH. Seven patients showed some features of CAH, but had already been classified as having definite PBC. In one patient features of PBC and CAH were equally pronounced, so that the diagnosis remained uncertain.

Comment

The diagnostic criteria for PBC as proposed by the international group (14) are useful guidelines, but they lack specificity. We used strict criteria for the diagnosis PBC or CAH, but an unacceptably large group (42%) of patients with a positive AMA test could not be classified. Various attemps to improve our diagnostic methods by computer-aided cluster analysis were unsuccesful. The clusters seemed to be formed according to the severity of cholestasis and/or immunological abnormalities, and not according to a nosological entity.

A new diagnostic scheme was therefore designed by analogy with the systems used for rheumatological diseases (47). With our simple system Table II the majority of patients (86%) could be classified. Nearly all patients in whom no diagnosis could be made, had mild abnormalities without need for therapy.

Classical chronic active hepatitis was not found in this group of patients with a positive AMA test. Features of CAH, however, were present in 10% of the patients with PBC, but mostly in those with severe PBC. In only 1 out of 92 patients were criteria for both PBC and CAH present in equal number and strength.

Another advantage of the proposed system is that it obviates the need for a surgical liver biopsy. We found that the classical duct lesion (Fig. 1, p. 6 and 7) can also be detected in a needle biopsy specimen, especially when it comprises at least 10 portal tracts. Without histology a diagnosis could be made in 68% of the patients (Table VI), while classification was possible in 90% of 73 patients with a liver biopsy.

Our simple diagnostic system with major and minor criteria combines clinically acceptable specificity with sensitivity for the diagnosis of PBC. Such a diagnostic approach is particularly needed for systematic studies of the natural history of the disease and for evaluation of treatments.

SERUM IgM IN PRIMARY BILIARY CIRRHOSIS

Serum IgM was measured in 25 patients with PBC and 25 age- and sex-matched controls by two different immunodiffusion techniques.

With the classical method, the median serum IgM concentration for the controls was found to be 1.1 g/l (range 0.6 to 1.9 g/l). In PBC patients the median IgM concentration was 4.7 g/l (range 2.0-15.6 g/l). The 25 patients therefore all (100%) had a serum IgM level higher than their controls (Fig. 2). Using the alternative method, the median serum IgM concentration in controls was 0.6 g/l (range 0.3 to 1.0 g/l). For the PBC patients the median serum IgM concentration was 3.1 g/l (range 0.9 to 8.1 g/l). With this method, serum IgM levels were therefore found increased in 24 patients (96%). The values obtained by the alternative method differed significantly ($p < 0.05$; Wilcoxon signed rank test) from those obtained by the classical method. This applied to the PBC group as well as to the controls.

The two methods differed in two respects: the reduction step and the standard. Reduction of IgM to 7S monomers alone did not produce significant differences in average IgM content for each group, but the change to a different standard was responsible for the significant differences observed (Fig. 3).

Comment

Increased serum IgM levels have been reported in 73-85% of patients with PBC (49-51); in our study, serum IgM determined by the classical method was increased in all patients and was at least 1 g/l above the upper limit of normal controls in 92%. These

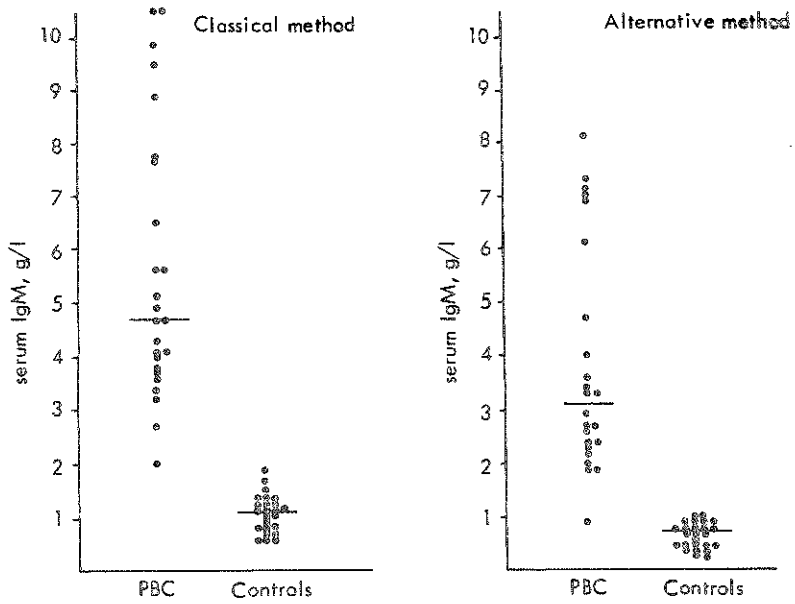


Fig. 2. Serum IgM levels in 25 PBC patients and age- and sex-matched controls, determined by the classical Mancini technique and by an alternative method which included reduction to 7S monomers and different standards. The horizontal lines denote the median values.

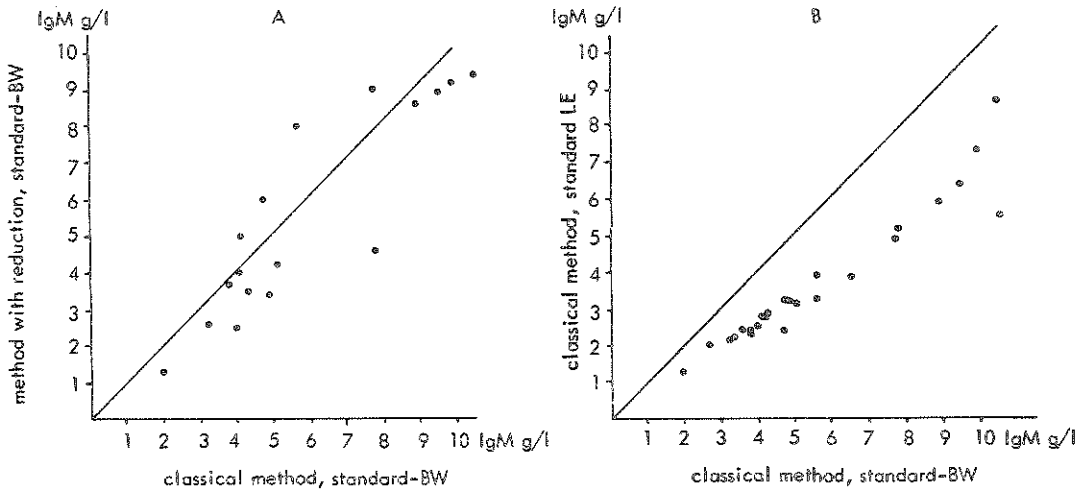


Fig. 3. Analysis of the differences in serum IgM in patients with PBC when measured by the classical Mancini technique and by the alternative method. Fig. 2A shows the effect of the reduction step: the line denotes identity. Data of 17 patients were available. In Fig. 2B the serum IgM of 25 patients measured by the classical method with a standard calibrated against the WHO standard (BW) is plotted against the serum IgM measured by the classical method and a standard calibrated against a serum with an absolute value of 55 g/l (determined by immunochemical as well as by non-immunological methods). The line again denotes identity.

results differ somewhat from data in the literature but this could be due to differences in methodology, the control group or the patient population. As compared with older series in the literature (4) the diagnosis of PBC in our patient group was probably established quite early, at a time when serum IgM may be higher than in the end-stages. The mean duration of the disease was 3 years from the first objectively found abnormalities (Table I).

The high frequency of increased serum IgM concentrations in PBC suggests that this test, which is readily available in most clinical chemistry laboratories, may be a useful screening test for patients with an increased alkaline phosphatase value with or without jaundice. If the serum IgM value is increased, then an antimitochondrial antibody test and a liver biopsy are probably more appropriate procedures than cholangiography.

We anticipated problems in the measurement of serum IgM, for large variations were reported when

various prominent laboratories assessed IgM by weight, using an international reference serum (52). Our classical method gave values for the control group which were comparable with those reported by other investigators, and significantly higher values for all patients with PBC. The very different values (in g/l) obtained with the alternative method demonstrate the importance of properly calibrated standards in the radial immunodiffusion technique. Although the classical method appears theoretically to be inferior to the alternative method, which includes reduction (53), the reduction step exerted no influence on the average IgM levels in either group; individual values, however, were sometimes quite different.

MONOMERIC (7S) IGM IN PRIMARY BILIARY CIRRHOSIS

A simple immunodiffusion technique was developed to detect 7S IgM in serum of 25 patients with PBC and 25 age- and sex-matched controls. The polyacrylamide double immunodiffusion technique yielded a precipitation line when the 7S IgM concentration exeeded 25 μg/ml. In 6 out of 25 patients with PBC a precipitation line was found, while none of the controls showed such a line. The amount of 7S IgM did not exceed 100 μg/ml in the positive sera. The immunological and biochemical features of both 7S IgM-positive and 7S IgM-negative PBC patients and controls are summarized in Table VII.

The immunological abnormalities (serum IgM, cryo-IgM, Clq binding) were considerably more prominent in 7S IgM-positive patients. In addition, serum alkaline phosphatase was significantly higher in 7S IgM-positive than in 7S IgM-negative patients; the results of the other liver tests were similar in the two groups of PBC patients.

Comment

We detected 7S IgM in 24% of the PBC patients using a double immunodiffusion technique in a polyacrylamide gel. Fakunle (54) recently found 7S IgM in one-third of this patients with PBC, using more complex methodology.

The presence of 7S IgM corresponded with high levels of total IgM and the presence of immune complexes as measured by cryoglobulins and ^{125}I-Clq binding. A possible explanation is that increased or accelerated immunoglobulin synthesis (56) is associated with production of IgM of abnormal size and structure, followed by formation of immune complexes. The finding of significantly higher serum alkaline phosphatase concentrations in 7S IgM-positive PBC supports the notion that bile duct damage, which is the key to the PBC syndrome, may be related to the presence of immune complexes.

CRYOGLOBULINS IN PRIMARY BILIARY CIRRHOSIS

As an assay for immune complex determination, cryoglobulins were measured in 25 patients with PBC, 25 controls without organic disease matched for age and sex, and 25 controls with various chronic liver diseases. In the normal controls, a precipitate was observed in only a few cases, and the protein concentration in the final solution was often hardly measurable. Cryoglobulins were detected in all patients with PBC and in 5 (20%) of the patients with various chronic liver diseases. In PBC the cryoglobulins mainly consisted of IgM with no or minimal IgA and IgG (Table VIII). The cryoglobulins in four out of the five patients with various chronic liver diseases, however, were a mixture of IgM and IgG. The distribution of cryoglobulins in PBC is depicted in Fig. 4; there is no normal distribution,

TABLE VII: IMMUNOLOGICAL AND BIOCHEMICAL FEATURES OF 7S IGM-NEGATIVE AND 7S IGM-POSITIVE PATIENTS WITH PBC, AND AGE- AND SEX-MATCHED CONTROLS

		Controls $n=25$ *median (range)*	*PBC 7S IgM-neg** $n=6$ *median (range)*	*PBC 7S IgM-pos** $n=19$ *median (range)*	*Wilcoxon-test p-value*
IgM classical	g/l	1.1 (0.6- 1.9)	4.1 (2.0- 7.8)	9.6** (7.7- 15.7)	0.0004
IgM alternative	g/l	0.7 (0.3- 1.0)	2.7 (0.9- 6.1)	7.1** (6.9- 9.2)	0.0003
Cryo IgM	mg/l	0 (0 - 2)	8 (5 - 34)	79 ** (66 -228)	0.0003
^{125}I-Clq binding	%	5 (3 -16)	12 (5 - 40)	23 ** (12 - 60)	0.024
Alkaline phosphatase	U/l	27 (18 -45)	135 (54 -329)	282 ** (167 -450)	0.044
Bilirubin	μmol/l	6 (3 -22)	16 (6 -497)	15 (7 - 65)	0.226
SGOT	U/l	15 (9 -30)	78 (33 -162)	96 (57 -156)	0.171

*All measurements in both PBC groups differed significantly from those in the control group.

**Significantly increased if compared to the 7S IgM-negative PBC patients, according to the (unpaired) Wilcoxon test ($p < 0.05$).

TABLE VIII: INITIAL CRYOGLOBULIN COMPOSITION IN PBC PATIENTS, AGE- AND SEX-MATCHED CONTROLS AND PATIENTS WITH VARIOUS CHRONIC LIVER DISEASE. THE VALUES ARE EXPRESSED IN MG PER LITRE ORIGINAL SERUM

Diagnosis	*Cryoglobulin analysis*				*Number of positive patients**
	Total protein mg/l median (range)	*IgM mg/l median (range)*	*IgG mg/l median (range)*	*IgA mg/l median (range)*	
Controls n = 25	3 (1- 12)	0 (0- 2)	0 (0- 1)	0 (0-1)	0
PBC n = 25	18 (6-233)	11 (5-228)	1 (0-4)	1 (0-2)	25
Various chronic liver disease n = 25	4 (0-220)	2 (0- 77)	0 (0-14)	not measured	5

*Cryoglobulins were considered positive when the cryoglobulin M content was at least 4 mg/l, which is twice the upper limit of normal.

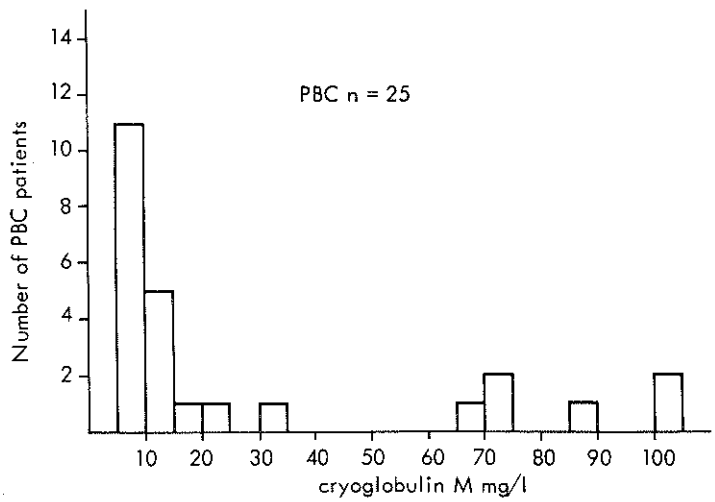


Fig. 4. The distribution of cryoglobulin M in 25 patients with PBC.

but possibly there are two sub-populations: 6 patients had a high concentration of cryoglobulin M (more than 35 mg/l), and 19 had a moderate one (less than 35 mg/l).

The relation of the initial cryglobulin M concentration with several other immunological (serum IgM, ^{125}I-Clq binding, AMA titre), biochemical (serum bilirubin, alkaline phosphatase, SGOT) and histological (fibrosis, piecemeal necrosis) features was tested in the group of patients with PBC. Cryoglobulin M was significantly related with serum IgM ($p < 0.003$) and with ^{125}I-Clq binding percentage ($p < 0.001$) (Fig. 5). The correlation of cryoglobulin M with serum alkaline phosphatase ($p = 0.8$) and with the degree of liver fibrosis ($p = 0.10$) was not significant in the group of 25 patients with PBC, but highly significant in a larger group of 40 patients with PBC (serum alkaline phosphatase $p = 0.002$, and liver fibrosis $p = 0.01$) (Fig. 6).

Comment

Cryoglobulins were detected in our first 25 patients with PBC by the method of Wands who reported the presence of cryoglobulins in 90% of patients with PBC (20). In terms of cryoglobulin levels, patients with PBC did not seem to be a homogeneous group. The group with a high cryoglobulin level differed in biochemistry (alkaline phosphatase) and immunology (^{125}I-Clq binding, serum IgM) from the group with mild cryoglobulinaemia. Berg (57) also postulated the existence of subgroups in PBC: a classical form and a form with features of chronic active hepatitis. He based his subdivision on two different types of antimitochondrial antibodies. It would be of interest to establish whether our subgroups are similar to those described by Berg.

The cryoprecipitate was almost exclusively composed of IgM. In contrast, IgG was present in the precipitate found in 4 out of 5 patients with other

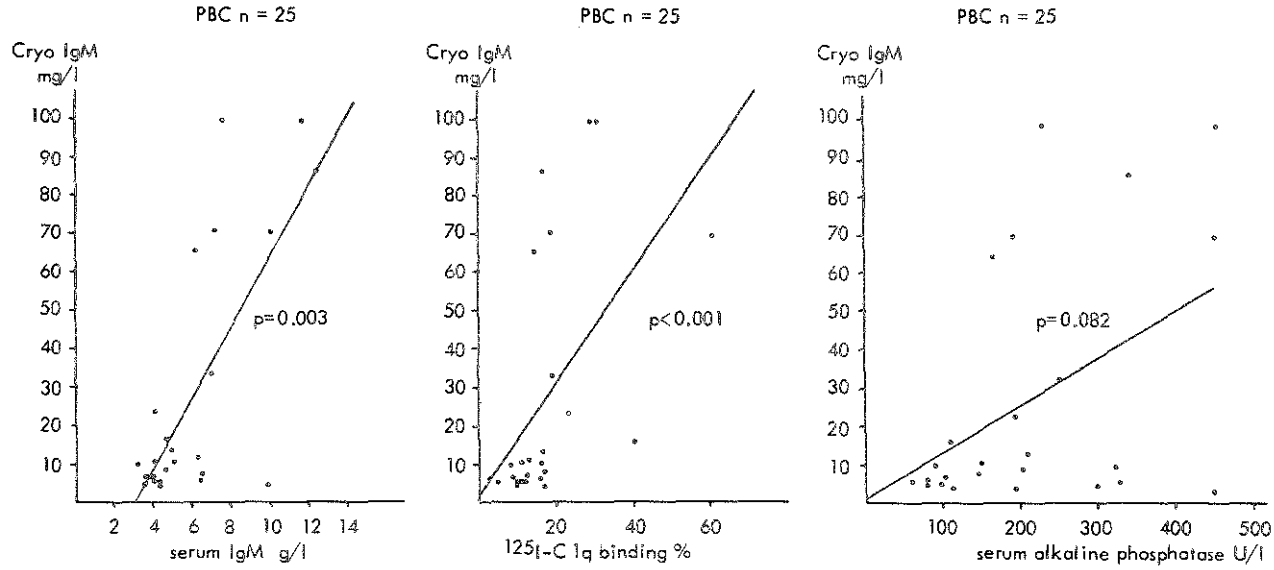


Fig. 5. The relationship between levels of cryoglobulin M and serum IgM (Spearman correlation coefficient 0.57, $p < 0.001$); cryo IgM and ^{125}I-C1q binding percentage (Spearman correlation coefficient 0.68, $p = 0.003$) as wel as cryo IgM and alkaline phosphatase (Spearman correlation coefficient 0.35, $p = 0.08$) in sera of 25 patients with PBC.

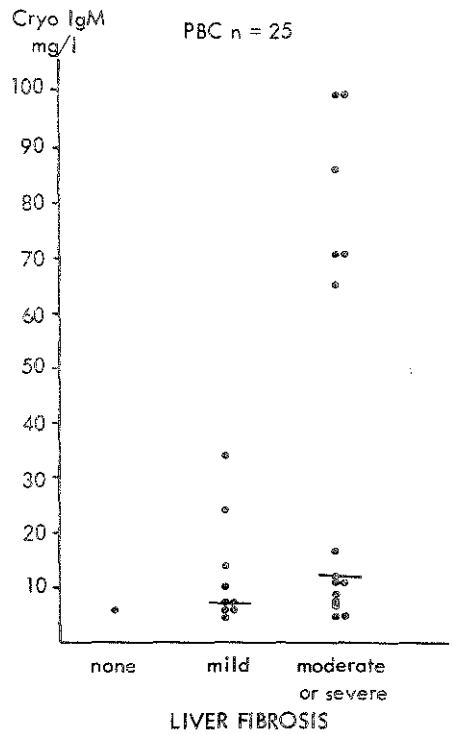


Fig. 6. The relationship between levels of cryoglobulin M and the degree of fibrosis in the liver biopsy specimen (Spearman correlation coefficient 0.13, $p = 0.10$) in 25 patients with PBC.

types of chronic liver disease. The hypothetical role of cryoglobulins in the pathogenesis of liver damage is based on the concept that cryoglobulins are circulating immune complexes. Evidence that cryoglobulins represent immune complexes was provided by Wands (20); but this will not be convincing until it is demonstrated that they consist of antigens, antibodies and complement components. The size and composition of the immune complexes (in particular the role of complement) are probably related to the type of immune-complex disease (58). It has been suggested that the presence of IgG in cryoglobulins is associated with inflammation, while IgM-containing cryoglobulins could be harmless.

We observed IgG-containing cryoglobulins in patients with chronic parenchymal liver disease, and found no correlation between piecemeal necrosis and cryoglobulin M in PBC. We did, however, find a positive correlation with liver fibrosis, which is probably the result of persistent destruction of the bile ducts. The large-size cryoglobulins in PBC may therefore be harmful for bile ducts.

The role of cryoglobulins in the pathogenesis of PBC may also appear from the effects of treatment. Recently, Epstein et al. (30) (and our therapeutic studies) reported a reduction of immune complexes simultaneous with improvement in liver tests.

D-PENICILLAMINE THERAPY IN PRIMARY BILIARY CIRRHOSIS

Advanced PBC is a disease with an average survival of about 5 years (4). Preliminary results indicate that D-penicillamine may have a beneficial effect on survival (59).

TABLE IX: FREQUENCY OF SIDE EFFECTS IN PATIENTS WITH PBC DURING TREATMENT WITH D-PENICILLAMINE OR A PLACEBO

	D-penicillamine n = 11				*Total number side effects*	*Placebo n = 13*				*Total number side effects*
Month	3	6	12	18		3	6	12	18	
Dosage, mg daily	750	1000	500	0		750	1000	500	0	
Side-effects										
exanthema	1	2	0	0	2	2	1	0	0	2
gastro-intestinal	5	2 (1)	0	0	5 (1)	3 (1)	3	0	0	3 (1)
renal or hemopoietic	0	0	0	0	0	0	0	0	0	0
other	0	1 (1)	0	0	1 (1)	2 (2)	1 (1)	0	0	3 (3)

In parentheses the number of patients whose therapy was discontinued.

TABLE X: BIOCHEMICAL FEATURES (MEDIAN VALUES) IN PATIENTS WITH PBC, BY TREATMENT GROUP

	D-penicillamine n = 11				*Placebo n = 13*			
Month	0	6	12	18	0	6	12	18
Dosage, mg daily	0	1000	500	0	0	1000	500	0
Bilirubin, μmol/l	20	16	14	23	21	23	23	23
Alkaline phosphatase, xN*	4.4	4.2	5.0	5.3	4.7	4.6	4.4	4.3
SGOT, xN	2.9	2.4	2.9	3.2	3.4	3.1	3.2	3.5
Albumin, g/l	45	46	44	43	43	44	43	44

*N is the upper limit of normal defined as the 95th percentile of the normal population of the clinical chemistry laboratory.

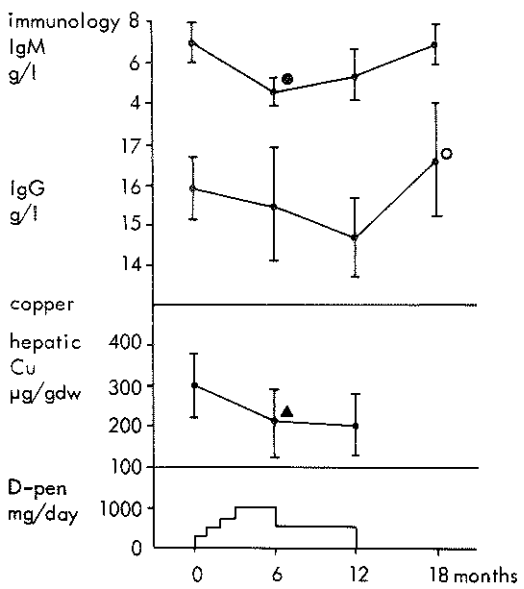


Fig. 7. The immunological features and hepatic copper in the patients with PBC during treatment with D-penicillamine. Serum IgM ● decreased significantly within 6 months, but rose again during maintenance dosage while the lowering of hepatic copper ▲ concentration persisted. Insufficient data were available at the 18th month. After therapy an increase of serum IgG ○ was found. Data are presented as the mean and the standard error of the mean.

From March 1978 until October 1979, 24 patients entered our study; 11 received D-penicillamine and 13 a placebo. The initial features of both groups (age and sex, symptoms, signs, histology, biochemistry and immunology) were comparable. The patients were predominantly middle-aged women suffering from pruritus. Only a minority of patients (17%) was jaundiced. Frank cirrhosis was present in a minority (9-23%), but marked or severe fibrosis was seen in 50-70% of the patients.

The subjective effect of drug therapy was impressive: 50% of the patients treated with either D-penicillamine or placebo felt better with regard to fatigue and/or pruritus after six months. After discontinuation of treatment at 1 year, this beneficial effect disappeared in both groups. Frequency and nature of side effects encountered during the treatment period of one year are given in Table IX. Most side effects, especially nausea and vomiting, were seen in both groups during introduction of the drug; loss of taste occured only in the D-penicillamine group. Otherwise the high incidence of side effects in the placebo group with subsequent withdrawal of four patients is to be noted. The reasons for discontinuation of drug therapy were persistent vomiting and pulmonary tuberculosis in the D-penicillamine group (2 out of 11), and vomiting (two

cases), pneumonitis and arthralgia for the placebo group (4 out of 13).

None of the patients developed liver failure or died during the 18 months of observation. Some biochemical measurements (Table X) such as alkaline phosphatase and SGOT showed improvement during the first six-month period, when the dose of D-penicillamine was relatively large; these changes, however, failed to attain statistical significance and disappeared in the following treatment period with the reduced dosage. Serum IgM showed a statistically significant fall in the first six-month period of D-penicillamine therapy. After one year of treatment the median value was still lower, but not significant, than at the start. Serum IgG concentration was low during D-penicillamine therapy, and increased significantly after discontinuation of the drug. The hepatic copper concentration showed a significant decrease within six months and remained at a relatively low level during further follow-up (Fig. 7).

Biochemical and immunological measurements in the patients treated with the placebo were rather stable, with the exception of the small but significant increase in serum IgG.

Two or more liver biopsy specimens were obtained from 20 patients (10 in each group). No significant histological improvement was observed with D-penicillamine. Most patients showed little variation in the histological features and stage of the disease. One patient progressed from stage III to stage IV, and another patient had regression of stage I to non-specific hepatitis (Fig. 8 and 9).

Comment

Preliminary results of D-penicillamine therapy in PBC have recently been reported (29, 31, 59, 60). The incidence of side effects has been particularly disturbing. Withdrawal of D-penicillamine was necessary in 7 out of 50 patients (14%), when the dose was increased to 1000 mg daily within 6 weeks; and withdrawal was 30% when this dosage was reached within three weeks. On the basis of experience with the drug in rheumatoid arthritis (61), D-penicillamine was introduced more slowly in our study (go-slow), reaching 1000 mg daily in 12 weeks. In addition, the maintenance dose was reduced to 500 mg after six months (go-low) in order to avoid serious side effects that often occur upon prolonged administration of the full dose (61). Side effects were not prevented by slow introduction, but they were often of a mild nature; withdrawal was necessary in 2 out of 11 patients (18%). The incidence of withdrawal of D-penicillamine therapy in this study should be assessed against the high incidence of discontinuation of treatment in patients treated with placebo (31%). Fear for severe D-penicillamine toxicity by the patients as well as by the physicians and nurses involved in the trial, seemed to be responsible.

The effect of D-penicillamine on symptoms has never been mentioned in previous reports (29, 31, 59, 60). In our study, improvement was found in both treatment groups with an equal incidence. The slight improvement in fatigue or pruritus only was observed during the period of medication and not in the last six-month period; it is therefore probably due to a 'placebo' effect in both groups.

TABLE XI: INFLUENCE OF THREE TREATMENT SCHEDULES ON SYMPTOMS AS WELL AS BIOCHEMICAL AND IMMUNOLOGICAL DATA IN PBC

Treatment	*Group*	*Fatigue number of patients*	*Pruritus number of patients*	*Bilirubin N* < 12 umol/l median (range)*	*Alk. phosph. N < 45 U/l median (range)*	*SGOT N < 30 U/l median (range)*
Placebo (n = 7)	before**	6	5	24 (14- 70)	212 (125-543)	102 (50-199)
	after 6 months	2	6	18 (11- 68)	209 (120-530)	126 (63-277)
D-penicillamine (n = 9)	before**	6	8	18 (6-140)	203 (96-365)	83 (46-256)
	after 6 months	3	6	13 (6-142)	199 (95-475)	67 (45-256)
D-penicillamine and prednisone (n = 9)	before**	8	6	16 (9-558)	240 (95-558)	93 (61-162)
	after 6 months	1	4	9***(3-268)	139***(46-581)	100 (29-136)

*N is defined as the 95th percentile of the normal population of the routine clinical chemistry laboratory.
**No significant differences were found for initial features between the groups.
***Significantly decreased after 6 months, according to the (paired) Wilcoxon test ($p < 0.05$).

The two earliest studies showed a beneficial effect on liver tests associated with a decrease in hepatic copper (28, 29). Later authors (31) found only a decrease in serum IgM and hepatic copper, comparable with the findings in the present study. Our study suggests that the effect of D-penicillamine is probably dose-related. A dose of 1000 mg daily was associated with improvement in biochemical measurements within six months, but the abnormalities (with the exception of those in hepatic copper) returned during maintenance with a smaller dose.

Improvement in liver tests may be a reflection of decreased levels of hepatic copper or reduction of immunological abnormalities. Our observations support the hypothesis that liver damage is more closely correlated with immunological abnormalities than with hepatic copper accumulation, for the fall in hepatic copper was sustained during a period when the liver disease became more active.

COMBINED THERAPY WITH D-PENICILLAMINE AND PREDNISONE IN PRIMARY BILIARY CIRRHOSIS

A pilot study was made of combined therapy with D-penicillamine and prednisone and the results were compared with those in D-penicillamine or placebo treated patients (the Rotterdam patients from our double-blind controlled trial).

The initial features of these three groups at the start of the treatment were comparable (Table XI). The initial serum IgM concentration appeared higher in the group receiving combined therapy, but the difference form the other groups was not statistically significant. Histological evaluation showed that all stages of PBC were distributed equally among the three treatment groups.

IgM $N < 2.8$ *g/l* *median (range)*	*Cryo IgM* $N < 2$ *mg/l* *median (range)*
4.8 (2.6-15.7)	9 (4- 71)
4.9 (3.1-16.0)	15 (3-170)
6.4 (3.2-13.2)	11 (6-228)
4.0***(2.3- 8.3)	10 (1- 47)
8.9 (3.2-12.8)	15 (2-116)
5.1***(1.6- 8.0)	4***(0- 12)

The results indicate that the combination of small doses of D-penicillamine with prednisone is superior to either D-penicillamine alone or a placebo.

The symptomatic improvement with the combined therapy was marked; symptoms decreased in 8 out of the 9 patients and even disappeared in 5 patients. In the D-penicillamine group, 2 patients showed no symptoms after treatment. No side effects of the combined therapy were observed; in contrast, D-penicillamine alone was associated with side effects of varying severity in 4 out of 9 patients. D-penicillamine alone led to a significant decrease in serum IgM, but no difference in either serum bilirubin, alkaline phosphatase or cryo-IgM. During treatment with D-penicillamine plus prednisone a significant decrease was observed in serum bilirubin, alkaline phosphatase and IgM.

Comment

In this pilot study, the beneficial effect of a combination of D-penicillamine and prednisone, both in small doses, on symptoms was sometimes remarkable: severe fatigue could diminish rapidly and the patients were often able to resume normal daily tasks. Pruritus also decreased. The symptomatic relief was accompanied by statistically significant biochemical improvements. During treatment with D-penicillamine alone (up to 1000 mg daily) the only significant change was a decrease in serum IgM, but there was only minor improvement in either symptoms or biochemical measurements.

Administration of D-penicillamine plus prednisone was the only therapy associated with a fall in cryoglobulins and improvement in tests of cholestasis. These observations may be regarded as evidence in support of the pathogenetic role of cryoglobulins in PBC. It will be very difficult to obtain direct proof of the role of cryoglobulins.

Although none of the patients was in an advanced cirrhotic stage of the disease, many of the liver biopsy specimens showed extensive fibrosis; these results, therefore, do not exclusively apply to the early stage of PBC. We did not attempt to evaluate the effect of treatment on liver histology, since the treatment period was considered too short to influence this.

Aggravation of osteodystrophy is probably the major contraindication to long-term corticosteroid therapy for this disease (62, 63); recent advances in diagnosis and treatment of osteodystrophy (64) may eventually allow long-term low-dosage prednisone therapy for selected patients with PBC. Further controlled studies of the combination of prednisone with D-penicillamine are needed.

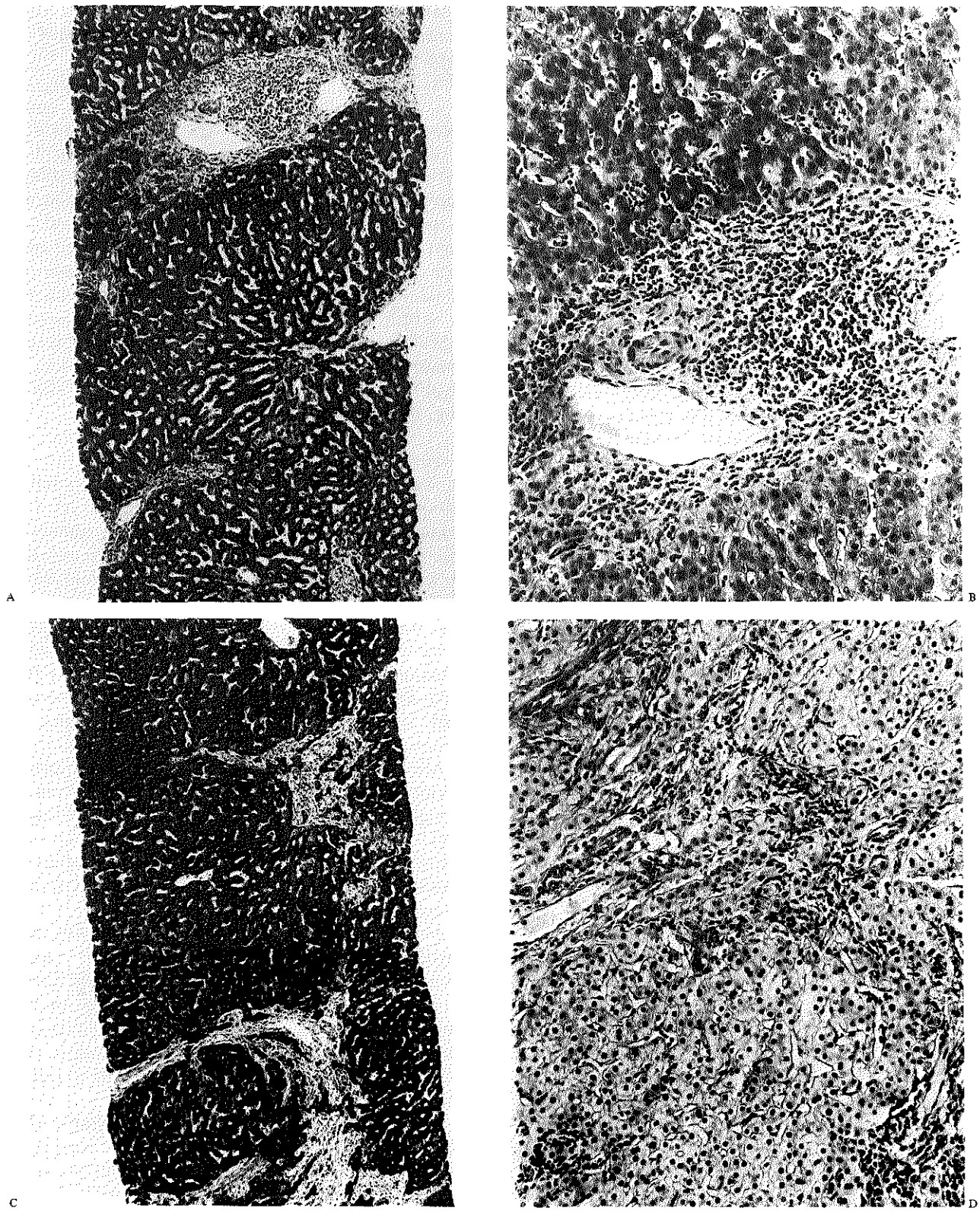


Fig. 8. The liver biopsies from a patient with progressive liver disease during D-penicillamine therapy. The degree of fibrosis increased from mild (before treatment, A; periodic acid Schiff; 60x) to severe (after one year of D-penicillamine therapy) with the formation of portoportal septa (C). The degree of piecemeal necrosis around the portal tracts as shown in detail (haematoxylin-azaphloxin; 150x) was also progressive (from mild in B to mild-moderate in D).

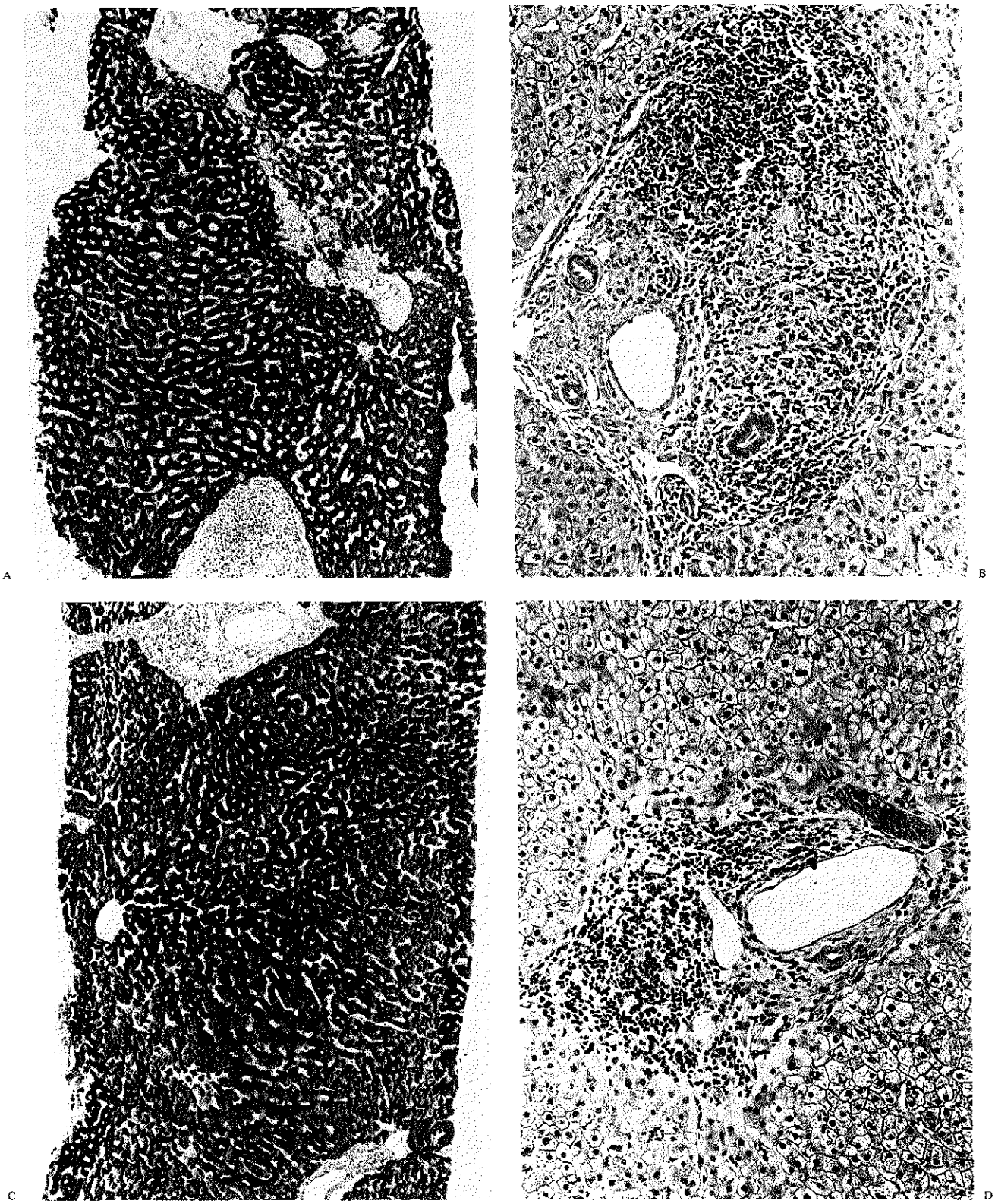


Fig. 9. The liver biopsies from a patient with some regression of the liver disease during D-penicillamine therapy. The degree of fibrosis was already mild to moderate (before treatment, A; periodic acid Schiff; 60x) and decreased (after one year of D-penicillamine therapy, C). The degree of piecemeal necrosis around the portal tracts (in detail; haematoxylin-azaphloxin; 150x) was already mild (B) and disappeared almost entirely (D).

General discussion

The classical syndrome of primary biliary cirrhosis (a middle-aged woman with pruritus, hepatosplenomegaly, increased serum cholesterol and a characteristic bile duct lesion on liver histology) used to be regarded as rare. Today, due to the widespread availability of serological tests for alkaline phosphatase, immunoglobulin M and antimitochondrial antibodies, the syndrome appears not to be rare, and is frequently diagnosed in the early stage of chronic cholangiolytic hepatitis. The natural history of the disease is ill-defined, and the median survival of 5 years (4) probably applies to the advanced stage of the disease. Evidence is accumulating that asymptomatic patients with PBC have an excellent prognosis (65), and some of our patients with antimitochondrial antibodies and a characteristic bile duct lesion on liver histology had normal biochemistry and serology two years later, suggesting the existence of a transient form of the disease. Further studies of the natural history will disclose whether there are subgroups without progression (comparable to chronic persistent hepatitis) and subgroups with rapidly progressive disease (comparable to chronic active hepatitis).

It is not surprising that the presenting features of PBC have changed. In the past, symptoms and signs were predominantly the result of chronic cholestasis. But we found that the characteristic abnormalities in immunology and biochemistry (AMA, IgM, alkaline phosphatase) are often accompanied by non-specific symptoms such as fatigue and pain in the region of the liver, and dry eyes as a sign of the frequent association with the sicca syndrome. The disease is still found predominantly in women.

For the study of the natural history of the disease and for defining subgroups in need of therapy, it is important to recognize the disease in an early stage. Since there are variations in the early expression of PBC, the nosological entity of PBC may be as difficult to define as several rheumatological diseases. The diagnostic criteria for PBC as proposed by the International Association for Study of the Liver (IASL) lack specificity. The development of a diagnostic system with major and minor criteria is a new approach in the field of hepatology. Encouraging initial results have been obtained with this diagnostic system, which distinguishes two levels of accuracy: definite and probable PBC. This simple system led to a classification in 86% of the patients studied.

The aetiology of primary biliary cirrhosis is unknown. Early studies focused on a possible derangement of lipid metabolism (1). Somewhat surprising, the role of virus, bacteria or exogenous agents such as drug and food-components has not been studied. In the last decade two major theories on pathogenesis have been advanced. The discovery of the frequent occurrence of antimitochondrial antibodies and raised serum IgM levels prompted the hypothesis that PBC is an auto-immune disease, and the predominance of the disease in women – albeit unexplained – would fit this hypothesis. Other workers (28) have focused on the role of copper as an important factor in the pathogenesis of liver fibrosis, without explaining the bile duct lesions and other characteristic immunological features of primary biliary cirrhosis. Our study concentrated on the raised immunoglobulin M levels, which result either from continuous stimulation of variable antigens (e.g. trypanosomiasis) or from a defect in the normal transition of IgM to IgG production after stimulation with a single antigen. Our findings of 7S IgM in 24% of patients and of abnormal amounts of cryoglobulins in nearly all patients with PBC provide evidence to support the hypothesis that, in PBC, increased or accelerated immunoglobulin synthesis is associated with production of IgM of abnormal size and structure. The association between liver fibrosis with cholestasis and the presence of 7S IgM and/or high concentration of cryoglobulins adds to the postulate that large circulating immune complexes play a role in the destruction of bile ducts. Also our studies with D-penicillamine and prednisone suggest that large circulating immune complexes rather than copper are causally related to the cholestatic disease process.

The studies on serum IgM were initially hampered by uncertainty about the accuracy of the classical immunodiffusion assay according to Mancini. Comparison of this method with an alternative method including reduction to monomers and the use of a standard checked by a non-immunological calibration method, demonstrated the importance of properly calibrated standards with international approval. Newer methods of assaying IgM (e.g. a non-diffusion method such as nephelometry) may facilitate the solution of this methodological problem.

For the patient, effective therapy is the most important and ultimate goal. In the past 5 years, progress has been made in the treatment of PBC. In addition to improved supportive care, D-penicillamine therapy has been reported to prolong survival. This drug was proposed as a probable

therapeutic agent in view of its copper-binding properties, but recent studies in PBC and rheumatological diseases have demonstrated an anti-inflammatory (or immunosuppressive) action of this drug. In this respect, the failure of azathioprine to change the course of the disease in two large controlled trials is to be noted. Corticosteroids have never been tested in a controlled study, for fear of aggravation of osteodystrophy. D-penicillamine therapy, however, is less effective in PBC than prednisone in chronic active hepatitis. In our small controlled trial D-penicillamine produced no significant subjective improvement, and the biochemical and immunological changes were only minor in spite of a relatively large dose. The combination of small doses of D-penicillamine and prednisone, however, led to both subjective and biochemical improvement in a therapeutic pilot study. Since the osteodystrophy of PBC may respond to therapy with new vitamine D metabolites, benefits of corticosteroids may now outweigh the risk of such therapy, and controlled evaluation of the combined therapy is the logical consequence to the results of our pilot study. Further advances in the eludication of the pathogenesis of PBC and its treatment are to be expected in the near future, especially when large groups of patients are studied in a few centres and when the study of the clinical aspects of these patients can be linked to basic research in immunology, microbiology and pharmacology.

Summary

1. The clinical features of PBC have changed since the disease is more frequently diagnosed in the non-cirrhotic stage. The female predisposition is still unmistakable, but non-specific symptoms such as fatigue and pain in the region of the liver have replaced pruritus and jaundice as major symptoms.
2. The internationally accepted diagnostic criteria for PBC lack specificity or sensitivity; the early as well as the end-stage of the disease is thus not readily recognizable. The newly proposed system, based on major and minor criteria, distinguishes two levels of accuracy: definite and probable PBC. This simple system proved to combine clinically acceptable specificity with sensitivity; its ultimate value can only be determined in other populations of patients with PBC and chronic active hepatitis.
3. Serum IgM is a sensitive and simple screening test for PBC in patients with cholestasis. Methods to quantitate serum IgM urgently require standardization.
4. The presence of 7S IgM in PBC is probably an expression of an accelerated immune response related to the activity of the disease.
5. Cryoglobulins are nearly always present in PBC and consist mainly of IgM. Cryoglobulin levels correlate with some biochemical (alkaline phosphatase) and histological (liver fibrosis) markers of the disease.
6. The finding of significantly higher serum alkaline phosphatase concentrations in 7S IgM-positive and strongly cryoglobulin positive patients with PBC supports the postulate that bile duct damage, which is the key to the PBC syndrome, may be related to the presence of immune complexes.
7. D-penicillamine can be administered to patients with PBC with a low incidence of side effects if the 'go-slow-go-low' dose schedule is used. A relatively high dosage is required for a beneficial effect.
8. Combined therapy with small doses of D-penicillamine and prednisone produced marked subjective and significant objective improvement. The combination was superior to D-penicillamine alone.

Samenvatting

1. De klinische presentatie van PBC is veranderd nu de diagnose vaker wordt gesteld in een stadium waarin nog geen cirrose is ontstaan. Het frequent voorkomen van deze ziekte bij vrouwen is nog steeds opvallend; niet-specifieke symptomen echter, zoals vermoeidheid en pijn in de leverstreek, staan tegenwoordig meer op de voorgrond dan jeuk en icterus.
2. De internationaal aanvaarde diagnostische criteria voor PBC zijn niet specifiek of gevoelig; in het begin- maar ook in het eindstadium van de ziekte is deze soms moeilijk te herkennen. De nu voorgestelde methode, gebaseerd op 'major' en 'minor' criteria, onderscheidt twee niveau's van nauwkeurigheid: zeker en waarschijnlijk PBC. Deze methode is eenvoudig in gebruik en leidt tot klinisch acceptabele specificiteit en sensibiliteit; de betekenis van deze methode zal blijken uit de toepassing in andere groepen patiënten met chronische leverziekten.
3. Serum IgM is een gevoelige en eenvoudige screening-test voor PBC bij patiënten met cholestase. Bij de verschillende methoden om IgM te kwantificeren blijkt dringende behoefte te bestaan aan standaardisatie.
4. De aanwezigheid van 7S IgM bij PBC is waarschijnlijk een uiting van een versnelde immuun respons in samenhang met de activiteit van de ziekte.
5. Cryoglobulinen komen vrijwel altijd voor bij PBC en bestaan voornamelijk uit IgM. De hoeveelheid cryoglobulinen correleert met enkele belangrijke biochemische (alkalische fosfatase) en histologische (lever fibrose) kenmerken van de ziekte.
6. Het voorkomen van een significant hogere serum alkalische fosfatase concentratie bij patiënten met 7S IgM en duidelijk aanwezige cryoglobulinen, pleit voor de veronderstelling dat de galgang aantasting, die de kenmerkende lesie is bij PBC, samenhangt met de aanwezigheid van immuuncomplexen.
7. D-penicillamine kan worden toegepast bij PBC, met weinig kans op bijwerkingen wanneer het toegediend wordt volgens het 'go-slow-go-low' principe. Een betrekkelijk hoge dosis is echter nodig voor een gunstig effect.
8. Tijdens een combinatie therapie bestaande uit een lage dosering D-penicillamine plus prednison trad een opmerkelijke subjectieve, maar ook een significante objectieve verbetering op. De combinatie therapie had een gunstiger effect dan D-penicillamine alleen bij patiënten met PBC.

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Verantwoording

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De interesse voor klinisch onderzoek werd gestimuleerd door mijn opleider Prof. Dr. M. Frenkel. De aanzet tot het onderzoek van patiënten met primair biliaire cirrose en met name de behandeling, gaf Solko Schalm. Onder zijn leiding leerde ik systematisch onderzoek. Door verwijzing door vele internisten uit de omgeving steeg het aantal patiënten met PBC snel.

Om de vele gegevens op te slaan werd een computer programma opgesteld met de zeer gewaardeerde steun van Eugene Clermont, die ook later statistische technieken in een computerprogramma verwerkte. Prof. R. van Strik gaf daarbij waardevolle adviezen.

Op de afdeling Pathologische Anatomie leerde ik van Fibo ten Kate steeds meer te zien in een leverbiopt. Corrie van Pelt was daarbij onmisbaar door de vele biopten op te sporen. Johan van Lier en Dirk Nonnekes hielpen bij de immunofluorescentie. Paula Delfos zorgde snel voor prachtige foto's.

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De samenwerking met Ton de Bruijn, Dr. F. Klein en Hilda Kornman-van de Bosch resulteerde in beter inzicht in immunologische technieken en hun toepassing. Ook waren de gesprekken met Bert Feltkamp, Riekie Geertzen en Erik Hak uit Amsterdam boeiend en stimulerend. Jo Hermans uit Leiden liet me kennis maken met nieuwe statistische methoden zoals cluster en discriminant analyse.

Met Dr. Gerard P. van Berge Henegouwen en Dr. Karl-H. Brandt heb ik plezierig samen gewerkt en ik waardeer het vele werk dat zij deden voor ons gezamenlijke onderzoek.

De hulp op de afdeling 4 Noord van Paula St. Maartensdijk-Schuiff en Pauline van Horst-in 't Veld en vele anderen bij de organisatie en opvang van de patiënten was onontbeerlijk. Tevens dank ik Mark van Blankenstein voor de gastvrijheid op 4 Noord, waar de patiënten met PBC werden opgenomen. Ook het polikliniek team bestaande uit Elleke Schalekamp, Jeanine Boender, Heleen Versteeg-Leenheer en Annette van Zelm was behulpzaam bij het verzamelen van gegevens.

Het manuscript van dit proefschrift werd met enthousiasme getypt door Anke de Graaf; Marieke Horsch-Blekking en Ellis van der Waarde-Masthoff hebben met zorg de artikelen getypt; Marianne Kuipers-Leuvekamp zorgde voor de vele administratie bij het in kaart brengen van het serum archief. De medewerkers van de grafische studio (Audiovisuele Dienst, Erasmus Universiteit) verzorgden de figuren. Mevr. G. Bieger-Smith was onmisbaar voor de juiste toepassing van de engelse taal in de verschillende artikelen.

Tenslotte zou dit proefschrift niet tot stand zijn gekomen zonder het aanstekelijke enthousiasme van Solko Schalm en de onmisbare steun van mijn ouders.

Curriculum vitae

De schrijfster van dit proefschrift werd op 3 april 1949 geboren te 's Gravenhage. Het diploma gymnasium β werd verkregen in 1967 aan het Alexander Hegius Gymnasium te Deventer. Daarna studeerde zij aan de Medische Faculteit te Groningen; na de co-schappen in het Ziekenhuis 'Ziekenzorg' te Enschede werd het arts-examen afgelegd in 1974. Daarna volgde een assistentschap gedurende enkele maanden op de afdeling Heelkunde (hoofd: Prof. Dr. P. J. Kuijer) van het Academisch Ziekenhuis te Groningen.

In september 1974 begon zij de opleiding tot internist op de afdeling Inwendige Geneeskunde II (opleider Prof. Dr. M. Frenkel) van het Academisch Ziekenhuis Dijkzigt te Rotterdam. Op 1 september 1979 werd zij ingeschreven in het Specialisten Register. Tijdens de opleiding werd het onderzoek naar verschillende aspecten van primair biliaire cirrose begonnen en voortgezet in de functie van wetenschappelijk medewerkster aan de Erasmus Universiteit te Rotterdam.

STUDIES IN PRIMARY BILIARY CIRRHOSIS

(papers)

B.G.Taal

november 1981

Supplement of the thesis of B.G.Taal

CONTENTS

Primary biliary cirrhosis: a changing clinical presentation

B. G. VAAL and S. W. SCHALM

SUMMARY

Primary biliary cirrhosis is now being diagnosed more frequently in the non-cirrhotic stage due to the increased availability of such laboratory determinations as serum alkaline phosphatase and IgM levels and the presence of antimitochondrial antibodies. The classical signs of the disease, jaundice, hepatomegaly and xanthelasma, have been replaced by non-specific symptoms such as fatigue, abdominal pain and pruritus, or the dry eyes and mouth associated with the sicca syndrome. The disease can no longer be regarded as rare.

There are no specific physical signs; a minority of patients are jaundiced. Serum alkaline phosphatase and IgM are nearly always increased. The presence of antimitochondrial antibodies is highly specific, but the titres may be low in the end-stage of the disease. Osteodystrophy is not common, but can develop as a complication in the advanced stage in the event of prolonged jaundice. *Neth J Med 24,* 101.

INTRODUCTION

In 1892 HANNOT described 'la cirrhose hypertrophique avec ictère chronique'. It was probably this condition that AHRENS et al.[1] 58 years later called primary biliary cirrhosis: a syndrome seen predominantly in middle-aged women and characterized by longstanding pruritus, jaundice and massive enlargement of the liver, frequently accompanied by xanthelasma and increased serum lipids. In 1964 49 patients with the primary biliary cirrhosis syndrome were described, of whom 50 per cent had chronic cholangiolitic hepatitis without cirrhosis; the syndrome was associated with an increased serum alkaline phosphatase level[2]. A few years later the diagnostic importance of antimitochondrial antibodies[3] as well as increased serum IgM levels[4], was discovered. From detailed histological studies by RUBIN et al.[5] came the term chronic non-suppurative destructive cholangitis, but primary biliary cirrhosis is still the term most frequently used. In 1970-1971 the association with the sicca syndrome[6] as well as scleroderma and Raynaud's syndrome[7] was found. In addition to the usual complications of cirrhosis, hepatic osteodystrophy is prominent in this cholestatic syndrome[8], probably due to malabsorption of calcium and disturbances in vitamin D metabolism[9].

As a result of the increasing availability of biochemical determinations such as serum alkaline phosphatase and IgM levels and the presence of antimitochondrial antibodies, the primary biliary cirrhosis syndrome is now frequently detected in an earlier phase. We noted that the presenting features of this syndrome have changed markedly: a fairly non-specific clinical syndrome characterized by fatigue, pain in the region of the liver, pruritus and arthralgia has emerged.

In order to define the presenting symptoms and signs of the primary biliary cirrhosis syndrome (PBC) more precisely, we compared a group of such patients with an age- and sex-matched control group.

PATIENTS AND METHODS

Patients and controls

We studied 25 consecutive patients with PBC referred to the University Hospital, Rotterdam, since 1978. The diagnostic criteria for PBC in this study consisted of an increased serum alkaline phosphatase level, a positive antimitochondrial antibody test, no demonstrable abnormalities of the extrahepatic bile ducts at cholangiography, and a liver biopsy showing lymphoplasmocellular infiltrates in enlarged portal tracts, paucity of interlobular bile ducts and/or granuloma around destroyed bile ducts.

The control group consisted of 25 age- and sex-matched patients seen since 1978 at the out-patient clinic of the Department of Internal Medicine II of the University Hospital, Rotterdam, because of abdominal complaints; no organic gastrointestinal disease was demonstrable in any of the patients of this group.

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Methods

Each patient and his control were studied in an identical procedure. Histories were taken with the aid of a checklist with particular emphasis on such symptoms as fatigue, pain, itch, use of cholestyramine and previous liver disease. Previous use of corticosteroids and/or D-penicillamine was recorded also.

At physical examination, special attention was given to scratch marks, spider naevi, palmar erythema, xanthelasma or xanthoma, size of liver and spleen, ascites and/or oedema. In addition we asked about dry eyes, dry mouth and white fingers in cold weather and looked for telangiectases, diminished tear secretion (by means of the Schirmer test; SMP Division Cooper Laboratory, New York) and decreased oesophageal motility (by means of fluoroscopy) to detect the so-called associated diseases.

Haematological studies were performed with EDTA blood (Coulter Counter). Clotting factors were assessed by the Normotest method (Nyegaard, Oslo). The serum bilirubin, GPT (glutamic pyruvic transaminase), GOT (glutamic oxalacetic transaminase), alkaline phosphatase and γ-glutamyltranspeptidase levels were determined with the Technicon SMA 12; albumin and γ-globulin concentrations were measured by standard electrophoresis. Fasting bile acids were measured by means of a radio-immunoassay[10]. Serum immunoglobulins were assayed by the radial immunodiffusion method of Mancini (Partigen plates, Behringwerke, Marburg). An indirect immunofluorescent technique using fluorescein isothiocyanate-conjugated anti-γ-globulin (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam) demonstrated antibodies against nuclear antigens and mitochondria (on rat kidney) and antibodies against smooth muscles (on rat stomach) in serum diluted ten-fold[11]. Cryoglobulins were isolated from 10 ml serum harvested at 37°C and stored for ten days at 4°C[12]; after appropriate washing, the immunoglobulin content of the cryoprecipitate was determined by radial immunodiffusion.

The bone mineral content per square centimetre was assessed by densitometric analysis of the radius of the right forearm[13] (Norland Instruments, Fort Atkinson, Wisconsin). X-rays of the lumbar spine and the pelvic bones were classified by an independent radiologist according to the degree of osteodystrophy (none, minimal, moderate or severe). 25-Hydroxycholecalciferol (vitamin D3) was determined according to EDELSTEIN[14], while the serum calcium level was estimated with the Technicon SMA 12.

For statistical comparison the Wilcoxon test for paired samples was used for data sets with a skewed distribution; Student's t-test was applied when the distribution was normal. To test the significance of discrete variables the X^2 test was used, including the Yates correction for small numbers. Statistical significance was assumed whenever the p-value was less than 0.05.

TABLE I: SYMPTOMS AND SIGNS IN PBC PATIENTS AND CONTROLS

		PBC (n=25)	*Controls (n=25)*
Asymptomatic		2	7
Symptomatic			
pruritus	intermittent	12*	0
	continuous	6*	0
fatigue	moderate	10	14
	severe	6*	0
pain	region of liver	10	4
	arthralgia	9	11
	low back	3	9*
jaundice	intermittent	6*	0
	continuous	6*	0
History of complications			
variceal haemorrhage		2	0
ascites/oedema		2	0
encephalopathy		1	0
Physical examination			
scratch marks		6*	0
xanthelasma		3	0
spider naevi		3	0
palmar erythema		3	1
hepatomegaly		9*	0
splenomegaly		4	0
Schirmer test < 10 mm		12*	5
Associated diseases			
Sjögren's syndrome**		5	0
Raynaud's syndrome***		7	7
scleroderma§		1	0

*significantly increased according to the X^2 test ($p < 0.05$)
**defined as dry eyes requiring methylcellulose eye-drop therapy
***defined as a history of white fingers upon exposure to cold § characterized by a mask-like face, thickened skin or diminished oesophageal motility.

RESULTS

The group of patients with PBC comprised 21 women and 4 men with a median age of 54 years (range 38-72); the control group was of exactly the same sex and age distribution.

Data on history and physical examination are summarized in Table I. Only two patients with PBC had no symptoms at all, and were referred to us after the accidental finding of biochemical abnor-

TABLE II: INITIAL BIOCHEMICAL FEATURES (HAEMATOLOGY, BLOOD CHEMISTRY AND IMMUNOLOGY) IN PBC PATIENTS AND CONTROLS

	PBC (n=25)		Controls (n=25)	
	median	range	median	range
Haematology				
Hb (mmol/l)	8.3	6.0–10.0	8.8*	7.6–10.0
MCV (fl)	92	82-108	92	86–102
leucocytes ($\times 10^9$/l)	6	4–11	7*	5–10
platelets ($\times 10^9$l)	199	95–468	253*	138–373
normotest (%)	84	37–100	93	62–100
Blood chemistry				
alk. phosphatase (xN**)	4.3*	0.8–10.0	0.6	0.4–0.8
γ-GT (xN)	8.8*	1.0–22.8	0.4	0.2–1.2
SGOT (xN)	2.7*	1.1–6.6	0.5	0.3–1.1
SGPT (xN)	2.7*	1.2–6.8	0.3	0.2–0.6
bile acids (μmol/l)	11*	1–100	2	1-6
bilirubin (μmol/l)	16*	6–497	6	2–22
albumin (g/l)	44	28–64	50*	42–59
γ-globulin (g/l)	18*	10–31	10	4–13
cholesterol (mmol/l)	7.1	3.6–18.2	5.9	5.1–8.5
Immunolgy				
IgG (g/l)	15*	10–29	11	8-17
IgM (g/l)	5.1*	2.5–12.4	1.4	0.7–2.1
cryo IgM (g/l)	0.011*	0.005–0.228	0.0	0.0–0.003
AMA titre	1/320*	0***–1/2560	0	

*significantly increased according to the paired Wilcoxon test ($p < 0.05$)
**N is defined as the 95 percentile of the normal population of the routine clinical chemistry laboratory
***one patient with end-stage PBC and positive AMA tests at previous testing

malities related to the liver. Pruritus was present in 72 per cent of the cases, but itching associated with scratch marks was a prominent feature in only a few cases; only four patients used cholestyramine. Fatigue was often present in both the PBC and the control group, but severe fatigue preventing normal daily work was encountered only in the PBC group. Pain in the region of the liver was often reported by PBC patients. Jaundice was found in 50 per cent of the patients, and was transient in 25 per cent. Advanced liver disease was rare; only two patients had complications of cirrhosis. Spider naevi, palmar erythema, xanthelasma and xanthoma were relatively rare in PBC patients, while enlargement of the liver and/or spleen was present in 52 per cent. Diminished secretion of tear fluid (less than 10 mm in five minutes according to the Schirmer test) was noted in 48 per cent of the PBC patients but often also in the controls (5/22; 3 controls on amitriptyline medication were excluded). In contrast, dry eyes requiring methylcellulose eye-drop therapy were found in 20 per cent of the PBC group but in none of the controls. Raynaud's syndrome, as defined by us, was as frequent in the PBC as in the control group.

The results of the haematological and blood chemistry studies are summarized in Table II. The concentration of haemoglobin as well as leucocyte and platelet counts were significantly ($p < 0.05$) lower in PBC patients than in controls; overt anaemia, leucocytopenia and/or thrombocytopenia, however, was rare. Serum alkaline phosphatase, γ-glutamyltranspeptidase and serum transaminases were sensitive tests because the levels were abnormal in 96-100 per cent of the PBC patients (Fig. 1); serum bilirubin was increased in 60 per cent of patients and fasting bile acids in 84 per cent. Serum albumin was significantly reduced in PBC patients, but in only two patients was this decrease of clinical importance in that it led to oedema. Serum cholesterol was not significantly higher in the PBC than in the control group.

The results of the immunological measurements are shown in Fig. 2. Both serum IgG and IgM were significantly increased in PBC patients and cryoglobulins, especially of the IgM class, were always present in various amounts. The median titre of the antimitochondrial antibodies (AMA) was 1/320 with a range of 0-1/2560 (the presence of AMA was one of the criteria for inclusion in the studies; however, in one patient they were no longer demonstrable during the end-stage of the disease). Smooth muscle antibodies were present in 36 per cent of the patients and in only 8 per cent of controls ($p <$

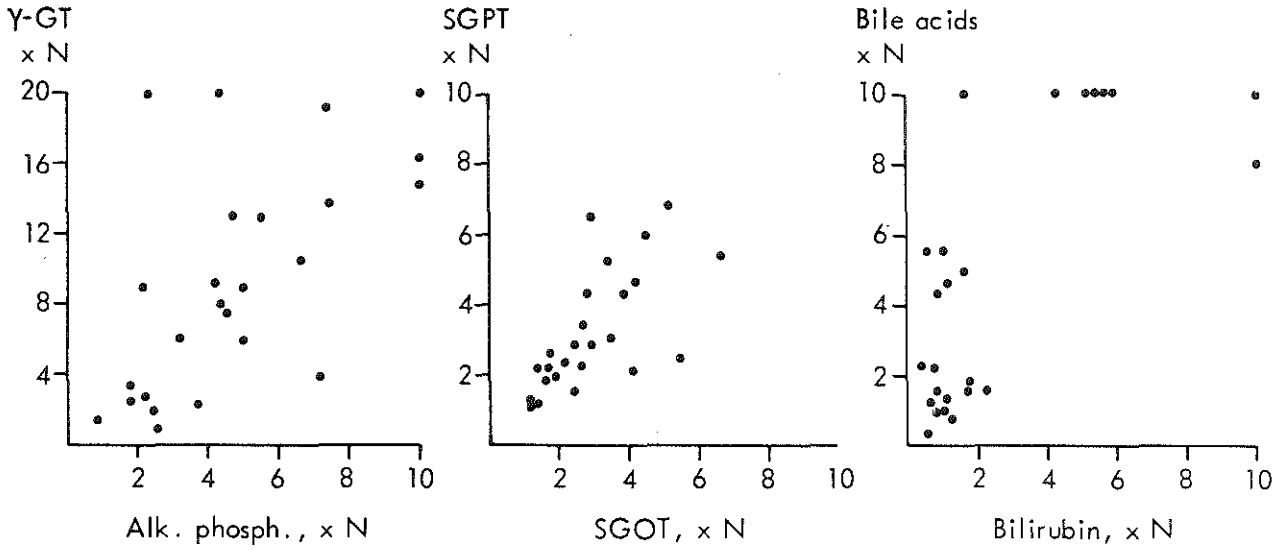

Fig. 1. Biochemical findings in PBC patients expressed as a factor of the upper limit of normal (N), which is defined as the 95 percentile of the normal population of the clinical laboratory. Alkaline phosphatase and γ-glutamyltranspeptidase are as sensitive as cholestatic enzymes; serum GPT and GOT also show an almost identical increase in all PBC patients. Fasting bile acids are increased more often (84%) than serum bilirubin (60%).

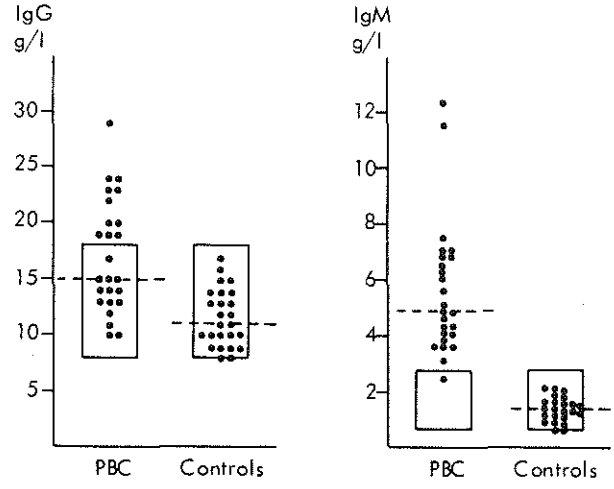

Fig. 2. Serum immunoglobulins in PBC patients and controls. The horizontal lines denote the median value for each group (patients or controls), the lined area represents the range of normal values used in our laboratory. Although the differences between PBC patients and controls are statistically significant ($p < 0.05$) for serum immunoglobulin G, the overlap is rather large; in contrast, serum immunoglobulin M shows a complete separation between PBC patients and controls.

0.05); antinuclear antibodies were found in 20 per cent of the PBC and in 12 per cent of the control population (no significant difference).

The findings relating to bone metabolism are listed in Table III. Neither serum calcium nor 25-hydroxycholecalciferol (vitamin D3) showed a statistical difference between PBC patients and controls (Fig. 3); six patients, however, were on vitamin D3 therapy. The bone mineral content per square centimetre was significantly lower in the PBC than in the control group but only a minority of the PBC patients showed signs of overt decalcification on the X-ray of the lumbar spine. Severe bone disease as manifested by vertebral collapse was present in two patients.

DISCUSSION

In this series of 25 patients with the PBC syndrome we noted a change in clinical presentation. Contrary to the classical description of AHRENS et al.[1], probably pertaining to the end-stage of the disease, the symptoms may now vary. In addition to pruritus, non-specific symptoms such as fatigue and pain in the region of the liver, have become prominent features; this finding appears to be true for each of the four histological stages of PBC[5] in our small group of patients.

Because of the apparently non-specific clinical features of PBC, a control group is essential for evaluation of the significance of such symptoms and signs. Although fatigue was often evident in the histories of the control group, the severity differed markedly from that in the PBC group.

Due to the easy availability of laboratory tests, PBC is now diagnosed even in asymptomatic patients[15,16]. The percentage of asymptomatic patients

TABLE III: INITIAL FEATURES OF BONE METABOLISM IN PBC PATIENTS AND CONTROLS

	PBC (n=25)		Controls(n=25)	
	median	*range*	*median*	*range*
Serum calcium (mmol/l)	2.5	2.1–2.6	2.4	2.3–2.6
Serum 25-OH-vitamin D (μmol/l)	56	15–100*	46	19–98
Bone mineral content /g/cm^2)	0.61	0.43–0.81	0.66**	0.48–0.77
X-ray lumbar spine				
no abnormalities (%)	64		not performed	
slight decalcification (%)	28			
severe decalcification (%)	8			

*6 patients on vitamin D therapy
**significant difference according to the paired Wilcoxon test ($p < 0.05$)

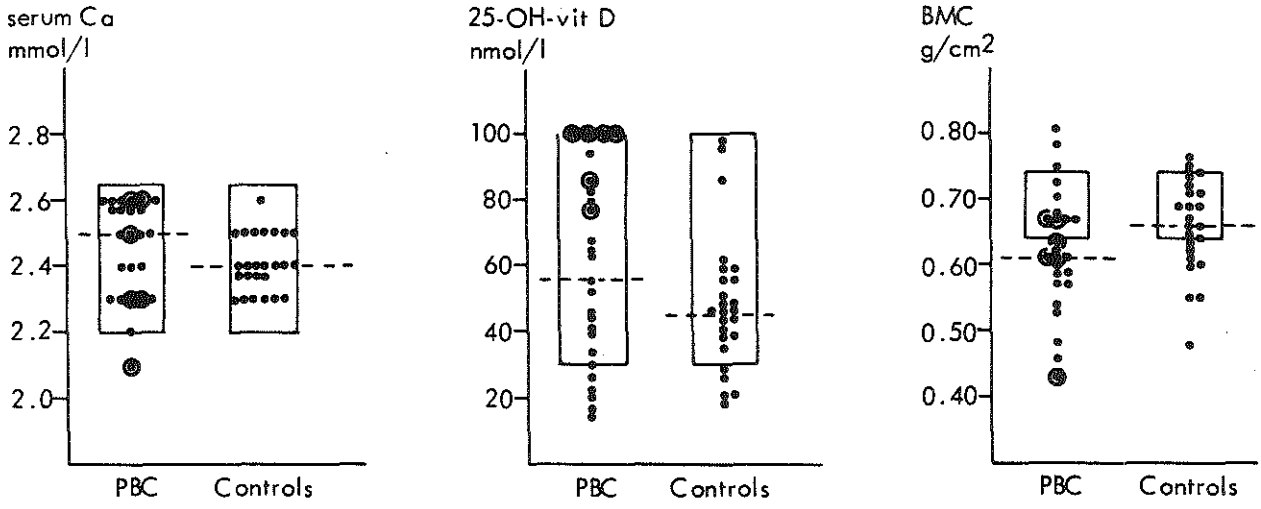

Fig. 3. Indices of bone metabolism for patients with PBC and controls; serum calcium and 25-hydroxycholecalciferol (vitamin D3) show no statistically significant differences, in contrast to the bone mineral content, which was significantly lower in the PBC group. Encircled dots represent patients receiving vitamin D therapy.

may be as large as 20[16]; this percentage, however, depends on the definition of 'asymptomatic'. In our group with PBC, 7 patients, referred because of accidental finding of abnormal liver tests, had symptoms which could be detected only by systematic questioning; if these patients are excluded we have a small number of asymptomatic patients (8 per cent).

Prior to 1978 the diagnosis PBC was made only occasionally in our hospital. Yet our impression is that the disease is not rare: since 1978 we have seen 25 PBC patients. Others have had similar experience (HAMLYN, unpublished observations).

The PBC syndrome is still encountered predominantly in middle-aged women, although we recently saw a 27-year-old man with PBC complicated by a variceal haemorrhage.

The association of PBC with the sicca syndrome, as indicated by diminished tear production and the need for methylcellulose eye-drop therapy, was confirmed in our series. In the control group, however, several patients also had diminished tear production, even without the use of drugs such as amitriptyline. The frequent occurrence of Raynaud's syndrome in the control group was unexpected and perhaps due to the fact that the question asked (white fingers in cold weather) was not sufficiently specific; this aspect requires further investigation.

The definition of PBC is obviously an important factor in our study, because selection criteria influence the population. Morphology often serves as the basis for defining diseases. When specific histological abnormalities of the liver, such as portal inflammation, paucity of interlobular ducts and

granuloma around destroyed bile ducts, are accompanied by biochemical and immunological abnormalities such as an increased alkaline phosphatase level and the presence of antimitochondrial antibodies, the PBC syndrome is diagnosed. In contrast, GEUBEL et al.[17] differentiate patients with the abovementioned features according to their response to corticosteroid therapy. Reaction to therapy, however, should not be a factor in the diagnosis of a disease.

Three sets of laboratory findings merit special comment. A slight but significant ($p < 0.05$) decrease in haemoglobin content and in the leucocyte and platelet counts was seen in PBC; it could be due to mild splenomegaly but this was only occasionally confirmed by physical examination or echography. The second striking laboratory finding was an increased IgM level[18] and the presence of cryoglobulins in all patients. The serum IgM is a sensitive and more specific finding than the alkaline phosphatase level, and particularly useful when the antimitochondrial antibody test is not easily available. The increased serum IgM level could be caused by continuous antigenic stimulation or by an abnormal change from IgM to IgG production[19]. The cryoglobulins found in PBC serum mainly consisted of IgM; their role in the pathogenesis[12] is not fully understood. The third important finding pertains to the parameters of bone disease. In serum calcium and vitamin D3 there was no difference between the PBC and the control group, but the bone mineral content per square centimetre as measured by densitometry was significantly lower in PBC patients than in controls. This technique is not easily available; however, X-rays of the lumbar spine and pelvic bones are probably adequate for detection of decalcification. In the present study overt osteodystrophy accompanied by vertebral collapse was rare and exclusively associated with prolonged jaundice or corticosteroid therapy.

In the past, research into pathogenesis and treatment was severely limited by the small number of PBC patients. Since a large number of patients with various clinical presentations of the PBC syndrome can now be detected, progress in the study of aetiology and therapy can be expected in the next five years.

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CLINICAL DIAGNOSIS OF PRIMARY BILIARY CIRRHOSIS: A CLASSIFICATION BASED ON MAJOR AND MINOR CRITERIA.

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Running title: Diagnosis of primary biliary cirrhosis.

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SUMMARY

The presence of antimitochondrial antibodies (AMA) is a sensitive marker for the diagnosis of primary biliary cirrhosis (PBC). Since these antibodies are not specific for PBC and differentiation of PBC from chronic active hepatitis (CAH) has important therapeutic and prognostic implications, additional diagnostic criteria were investigated in 92 patients with AMA. Patients were classified as PBC, CAH, undefined chronic liver disease, or no liver disease by means of three objective methods and these diagnoses were compared with that of the patients's own physician. Using internationally accepted strict diagnostic criteria, 42% of 92 AMA-positive patients could be classified. An unbiased computer cluster analysis with 17 variables yielded groups which varied in the severity of the disease, but did not separate clinically different nosological entities.

With a diagnostic scheme which uses major and minor criteria for both PBC and CAH, the large majority of patients (86%) could be classified : definite PBC (n=47), probable PBC (n=20) and no liver disease (n=12). Seven patients with definite PBC also showed some features of CAH, but there were no patients with only classical CAH. Patients who could not be classified frequently had very mild liver disease not requiring treatment (6/12) or no liver biopsy (6/12).

This new diagnostic scheme is promising as it seems to combine sensitivity with specificity for the diagnosis of PBC. If validated in another group of patients with liver diseases, it may be helpful for studies on the natural history of the disease and for evaluation of treatments.

Keywords: antimitochondrial antibodies, primary biliary cirrhosis, chronic active hepatitis, diagnosis, cluster analysis.

INTRODUCTION

The presence of antimitochondrial antibodies (AMA) is a sensitive marker for primary biliary cirrhosis (1), since the prevalence of AMA in PBC has risen from a reported 84% in 1966 (2-4) to 99% recently (5). The detection of AMA, however, is not specific for PBC, since the prevalence of AMA in CAH was found to be 11-28% (1-4). It is not clear whether these patients indeed had CAH due to the lack of objective criteria for this diagnosis. The diagnosis of PBC and CAH as defined by the International Association for the Study of the Liver (IASL) is based on the subjective evaluation of a combination of features (6). Because PBC and CAH differ as far as treatment and prognosis are concerned, it is important to establish the appropriate diagnosis early in the course of the disease.

Our various attempts to find a diagnostic scheme which combines sensitivity with specificity for the diagnosis of PBC are reported here.

MATERIALS AND METHODS

In 1976-1977 437 serum samples out of 12500 samples submitted for the detection of AMA to the Department of Autoimmune Diseases of the Netherlands Red Cross Blood Transfusion Service in Amsterdam, contained antimitochondrial antibodies. This laboratory performs the majority of such serological tests in the Netherlands. After inquiring at other diagnostic laboratories we estimate that these samples represent approximately 90% of

the AMA-positive sera in the Netherlands. In 1978 we contacted the local physicians in each hospital with more than three positive AMA patients; with their cooperation we were able to study 92 patients between January 1979 and October 1980.

Each patient was seen by the same investigator. The history was taken with the aid of a check list, with particular emphasis on symptoms such as fatigue and pruritus as well as previous diseases and the use of alcohol and drugs. At physical examination special attention was given to scratch marks, spider nevi, palmar erythema, xanthelasma, size of the liver and spleen, ascites and/or edema. In addition, tear secretion was measured using the Schirmer test (SMP Division Cooper Laboratory, New York, USA).

Blood was drawn at room temperature for hematological studies and biochemical measurements. Hematological studies were performed by Coulter Counter and clotting factors were assessed by the Normotest (Nyegaard, Oslo, Norway). Serum bilirubin and alkaline phosphatase were determined with the Technicon SMA 12-60; serum glutamic oxaloacetic transaminase (SGOT or aspartate aminotransferase), glutamic pyruvic transaminase (SGPT or alanine aminotransferase) and γ-glutamyltranspeptidase (γ-GT) concentrations were measured by an UV kinetic method (LKB reaction Rate Analyzer, Stockholm, Sweden); albumin and γ-globulin levels were ascertained by standard cellulose acetate electrophoresis. Fasting bile acids were determined by means of a radioimmunoassay (7). Serum immunoglobulins were assayed by the radial immunodiffusion technique of Mancini (8) using Partigen ^{R} immunoplates (Behringwerke, Marburg, F.R.G.). An indirect immunofluorescent technique using fluo-

rescein isothiocyanate conjugated sheep antihuman immunoglobulin demonstrated antibodies against nuclear antigens (ANF) and mitochondria (on rat kidney) and antibodies against smooth muscle (SMA, on rat stomach) in serum diluted twenty-fold. When the test was positive, the antibody titer was measured after dilution of the serum sample. The ^{125}I-C1q binding was performed as an assay for immune complexes according to Zubler (9).

Cryoglobulins were isolated from 10 ml of serum harvested at 37°C and stored for ten days at 4°C (10); after appropriate washing, the immunoglobulin content of the cryoprecipitate was determined by radial immunodiffusion using low-level Partigen ^{R} immunoplates (Behringwerke, Marburg, F.R.G.).

When a liver biopsy had been taken within the past 5 years, the slides were reviewed by a pathologist who had no knowledge of the clinical features and laboratory results. A bile duct lesion was considered to be present when mononuclear infiltration affected the bile duct epithelium with or without granulomatous reaction around a bile duct. In the case of atypical proliferation of duct epithelium a bile duct lesion was suspected The degree of fibrosis was scored as none, mild (some periportal deposition), moderate (formation of some portoportal septa) or severe (extensive portoportal septa); the degree of piecemeal necrosis was similarly scored as absent, mild (infiltration in some portal tracts of lymphoplasmocellular elements into the liver parenchyma), moderate (infiltration around all portal tracts)

or severe (around all portal tracts; infiltration deep parenchyma forming bridges of necrosis).

CLASSIFICATION PROCEDURES

Initially, internationally accepted strict criteria for the diagnosis of PBC (6, 11-14) and CAH (6, 15-17) were used. PBC was established when the serum alkaline phosphatase concentration was at least twice the upper limit of normal, SGOT less than five times the upper limit of normal, serum IgM exceeded 2.8 g/l, the AMA titer was equal to or larger than 1/40 and a duct lesion was demonstrated during histological examination of the liver. The diagnosis of CAH was made when the SGOT was at least five times the upper limit of normal without a more than twofold elevation of the serum alkaline phosphatase and when, in addition, serum IgG was more than 18 g/l, the ANF or SMA test was positive, and moderate or severe piecemeal necrosis was present in the liver. Patients were presumed to have no liver disease when both serum alkaline phosphatase and SGOT were normal and neither duct lesions nor piecemeal necrosis were observed in the liver biopsy. The diagnosis of PBC, CAH or no liver disease was only made when all features of a single entity were found in one patient. Patients who did not fulfill the above criteria were considered to have undefined chronic liver disease.

Subsequently, cluster analysis was performed using 14 variables for the total group and 17 variables for those from whom a liver biopsy was obtained. These variables consisted of:

symptoms and signs (pruritus and tear secretion) as well as biochemical (bile acids, alkaline phosphatase, γ-GT, SGOT and SGPT), immunclogical (IgM, IgG, cryoglobulin M, ^{125}I-Clq binding and AMA, SMA and ANF titers) and histological features (duct lesion, granuloma and piecemeal necrosis). This analysis leads to a subdivision of the group of patients into several clusters (in our case three). The subdivision is made in such a way that within the subgroup or cluster the patients show less variability than between the subgroups. The difference with the previous classification procedure is that no pre-assigned weight is attached to the variables. An advantage of cluster analysis is that a possible bias caused by the incorrect loading of some variable is avoided; a disadvantage is that a medical interpretation of the clusters that are created is often not obvious. A computer program was used according to the method of Wishart (18) and Nie (19). The ability of a variable to separate the patients into clusters was expressed as a rank-number based on the F-ratio (variance of the variable within the cluster divided by the variance of the total group). When the F-ratio is low, the rank number is low and the variable is highly representative of a cluster.

Finally, similar to diagnostic systems used for systemic lupus erythematosus and other rheumatological conditions (20), a new diagnostic scheme based on major and minor criteria (table I) was developed. Two levels of probability of the diagnosis were introduced. The diagnosis was considered definite when 2 major plus 2 minor criteria were identified, or 1 major plus 4 minor a probable diagnosis was made when 2 major

or 1 major plus 2 minor criteria were present.

RESULTS

The group of AMA-positive patients consisted of 92 subjects (7 men and 85 women) with a median age of 64 years (range 33-82). In 73 cases a liver biopsy was obtained. The diagnosis of the patient's own physician yielded 57 with PBC, 10 with CAH, 9 with undefined chronic liver disease and 16 without liver disease.

According to the strict diagnostic criteria, a large group of patients (39/92 or 42%) had undefined liver disease (table II). No cases of chronic active hepatitis were diagnosed, and in comparison with the diagnosis provided by the patients' physicians fewer patients were identified as having PBC (29 versus 57). Ten out of 11 patients classified by us as having no liver disease had not had a liver biopsy; so this group may appear deceptively large.

Cluster analysis was performed using 14 variables and 92 patients as well as 17 variables and 73 patients (see classification procedures). Because the results did not differ, only the results of the more complete latter group are reported in table III. Initially, two clusters of 33 and 40 patients were formed; further separation yielded three clusters by division of the first cluster in 14 and 19 patients. Since the patients had been separated into three diagnostic groups with the strict criteria (no patients with CAH), we stopped

cluster analysis at three clusters and compared the groups formed by cluster analysis with those obtained with the strict criteria system (table III). The percentage of patients with PBC was 84% in cluster I, 64% in cluster II and 27% in cluster III. Since cluster III was the largest, a similar number of patients with PBC was present in each cluster and consequently this method was useless for the purpose of diagnostic classification. Two, 4 or more clusters provided no additional information, since all subsequent clusters are obtained by adding to or dividing the original three clusters. The list of variables in table IV includes the three variables of each cluster which, on the basis of the F-ratio, represented the most characteristic features of that cluster. In cluster I the major abnormalities of PBC, such as AMA and a lesion of the bile ducts, as well as several immunological abnormalities are definite; in cluster II the cholestasis is the determining feature, while in cluster III AMA titer, a lesion of the bile ducts and immunological and cholestatic features are the least common properties. Therefore, the clusters apparently have been formed according to the severity of the disease. There is, however, a poor agreement between the cluster classification and the classification based on the strict diagnostic criteria (table III).

Our new diagnostic scheme, which utilizes the major and minor criteria for PBC and CAH (table I), encompasses two levels of diagnostic accuracy for these two conditions. The results are shown in table V. For 47 patients (51%) the diagnosis was definite PBC, and for

20 patients (22%) probable PBC, thus in total PBC was diagnosed in 67 of 92 patients (73%). For the group of 73 patients with a liver biopsy, the percentage of PBC even increased to 90%. With this scheme, as with the method of strict criteria, none of the patients had definite or probable CAH. Seven patients had some features of CAH, but had already been classified as having definite PBC. In one patient the features of both probable PBC and probable CAH were present; thus, this patient remained unclassified. For 12 patients (13%) the abnormalities were mild and there were not sufficient features of either PBC or CAH to reach a diagnosis. In six cases of this group and in 10 of 12 subjects without liver disease, however, a liver biopsy had not been obtained.

The results of the new diagnostic scheme are compared with those of the strict diagnostic criteria method in table VI. The large group (39 patients) with undefined chronic liver disease, was reduced with the new scheme to 12 patients, as 27 patients could be classified as PBC. Thus, with the new scheme a group of only 13 patients (1 undefined liver disease and 12 no diagnosis) or 14% remained unclassified.

Seven patients had definite PBC with some features of CAH as shown in table VII. All cases had high antimitochondrial antibody titers and a characteristic duct lesion, and most of them (6 out of 7) also had an elevated serum IgM concentration. Signs of cholestasis were prominent. These 7 patients had severe

PBC, associated with piecemeal necrosis, ANF or SMA and elevated SGOT.

A liver biopsy was available for 8 out of the 13 patients without diagnosis; the features of these 8 patients (1 with PBC or CAH, 6 with neither PBC nor CAH) are listed in table VIII. As far as the major criteria for PBC are concerned, either AMA or a bile duct lesion was present in all cases, but the alkaline phosphatase level was only slightly elevated. Of the major criteria for CAH SMA and ANF were found in 6 of the 7 patients, but SGOT was normal in 6 of them. In three of these 7 patients the antimitochondrial antibodies previously found in 1976 or 1977 could not been demonstrated again in 1979. In all 3 cases a liver biopsy, taken at the time of the positive AMA test, showed a lesion of the bile duct. According to the new diagnostic scheme these patients had probable PBC at the time of the liver biopsy, but no firm diagnosis could be made at the time of our investigation.

DISCUSSION

The diagnostic criteria for primary biliary cirrhosis (PBC) proposed by the international group (6) are useful guidelines but they are still susceptible to various interpretations. Therefore, initially we used the strict criteria for diagnosis of PBC or CAH. However, an unacceptably large group of patients (42%) with a positive AMA test could not be classified with these criteria. Various attempts to improve our diagnostic methods using computer-aided cluster analysis were equally unsuccess-

ful. Cluster formation appeared to be based on the severity of cholestasis and/or immunological abnormalities; we were unable to find a program which would yield a nosological entity. Using another advanced statistical method (discriminant analysis) Schaffner et al reported similar results for 167 patients with biopsy-verified chronic active hepatitis (21). Further separation within the AMA-positive group was not possible, although his method was correct in 81% of the cases in distinguishing viral, autoimmune and AMA-positive chronic hepatitis.

Therefore, we searched for new diagnostic systems and the new scheme which we propose was designed more or less along the lines of the systems used for SLE and other rheumatological diseases (20). With this simple method the majority of patients (86%) could be classified. Most of those for whom no diagnosis could be made had very mild abnormalities with no need for therapy. In only one out of 92 patients were the criteria for both PBC and CAH present in equal strength.

In this study patients with repeatedly positive antimitochondrial antibodies and biopsy-proven liver disease frequently had PBC (88%). Classical chronic active hepatitis was not found. Features of CAH were present in 8% of the patients with PBC, especially those with severe PBC. Transient antimitochondrial antibodies were seen in a minority of patients (9%); in several of these cases the liver biopsy taken at the time of the positive AMA test showed bile duct lesions. Because no follow-up biopsy was available, one cannot speculate about the possibility of "transient" PBC in these patients.

Needle aspiration biopsy of the liver proved to be of great diagnostic importance. Without histology no diagnosis could be made in 32% of the cases, whereas 88% of the patients with a liver biopsy could be classified. In our experience, a laparotomy is almost never required for the diagnosis and classification of this group of patients. Two conditions, however, need to be fulfilled. Firstly, the liver biopsy must contain enough portal tracts, preferably at least 10. Secondly, the pathologist should carefully inspect every portal tract to detect infiltration of the bile duct epithelium. In addition, an intralobular bile duct is missing in more than 5 of the 10 portal tracts (3) , this finding may support the diagnosis of PBC.

Our simple diagnostic scheme with major and minor criteria appears to combine sensitivity and specificity for the diagnosis of PBC. Applying this system to the patient population of the University Hospital Dijkzigt in Rotterdam our 57 patients with "clinically" PBC 52 cases (91%) were classified as definite PBC and 5 patients (9%) were classified as having probable PBC. For 12 subjects with "clinically" dubious PBC the diagnosis was probable PBC in 4 patients (33%), while 8 patients (67%) could not be classified; this group of patients had either a positive AMA test or a lesion of the bile ducts, and the biochemical abnormalities were mild. The validity of our new diagnostic scheme with major and minor criteria can only be proved by testing it in variuos groups of patients with liver disease in other centers, and prospective follow-up.

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Table I. A new scheme for diagnosis of primary biliary cirrhosis and chronic active hepatitis.

PBC *

major criteria:	minor criteria:
- AMA titer $\geqslant$ 1/20	- pruritus with need for medication
- liver biopsy : duct lesion +	- jaundice with normal clotting factors II,VII,IX,X.
	- alk. phosph. $\geqslant$ twice the upper limit of normal
	- serum IgM $>$ 2.8 g/l
	- Schirmer test $<$ 10 mm tear secretion in 5 minutes.

CAH*

major criteria:	minor criteria:
- ANF or SMA titer $\geqslant$ 1/20	- severe fatigue without pruritus
- liver biopsy : piecemeal necrosis ‡	- jaundice with abnormal clotting
	- SGOT $\geqslant$ three times the upper limit of normal
	- γ-globulin $>$ 20 g/l

* A definite diagnosis: 2 major plus 2 minor or 1 major plus 4 minor criteria; A probable diagnosis: 2 major or 1 major plus 2 minor criteria

+ A duct lesion is defined as infiltration or destruction of an interlobular bile duct with or without granuloma; absence of bile ducts in more than half of the portal tracts in a liver biopsy containing at least 10 portal triads

‡ Piecemeal necrosis is defined as moderate or severe parenchymal necrosis.

Table II. Comparison of the diagnosis obtained with the strict diagnostic criteria and that of the local physician for 92 AMA-positive patients.

diagnosis own physician	strict diagnostic criteria*				
	PBC	CAH	undefined chronic liver disease	no liver disease	total number of patients
PBC	29	0	27 (3)	1	57 (3)
CAH	6	0	4	0	10
undefined chronic liver disease	5	0	3 (1)	1	9 (1)
no liver disease	1	0	5 (5)	10 (10)	16 (15)
total number of patients	41	0	39 (9)	12 (10)	92 (19)

In parentheses : no liver biopsy available

* See classification procedures

Table III. Cluster analysis using 17 clinical, biochemical and histological variables and 73 AMA-positive patients; comparison of 3 clusters and the 3 groups formed by the strict diagnostic criteria.

	Cluster I	Cluster II	Cluster III	Total number of patients
strict diagnostic criteria				
PBC	16	10	13	39
undefined chronic liver disease	3	4	26	33
no liver disease	0	0	1	1
total number of patients	19	14	40	73

Table IV. Characteristics of the groups formed by cluster analysis.

variables:	Cluster I n=19			Cluster II n=14			Cluster III n=40		
	rank * number	mean	st.dev.	rank * number	mean	st.dev	rank * number	mean	st.dev
AMA titer (1-5)+	1	4.7	0.6	6	4.1	0.9	17	3.8	1.4
γ-GT xN‡	2	7.8	4.9	17	15.0	11.5	5	4.5	3.7
duct lesion (1-3)§	3	2.7	0.6	9	2.4	0.8	16	2.3	0.9
cryo IgM mg/l	17	73	82	1	14	20	1	7	10
^{125}I-C1q %	7	58	25	2	18	11	8	18	19
IgG g/l	11	26	7	3	17	4	10	17	6
bile acids umol/l	12	32	34	16	51	44	2	8	11
SGPT xN‡	13	2.7	1.4	13	2.8	1.5	3	1.1	0.5

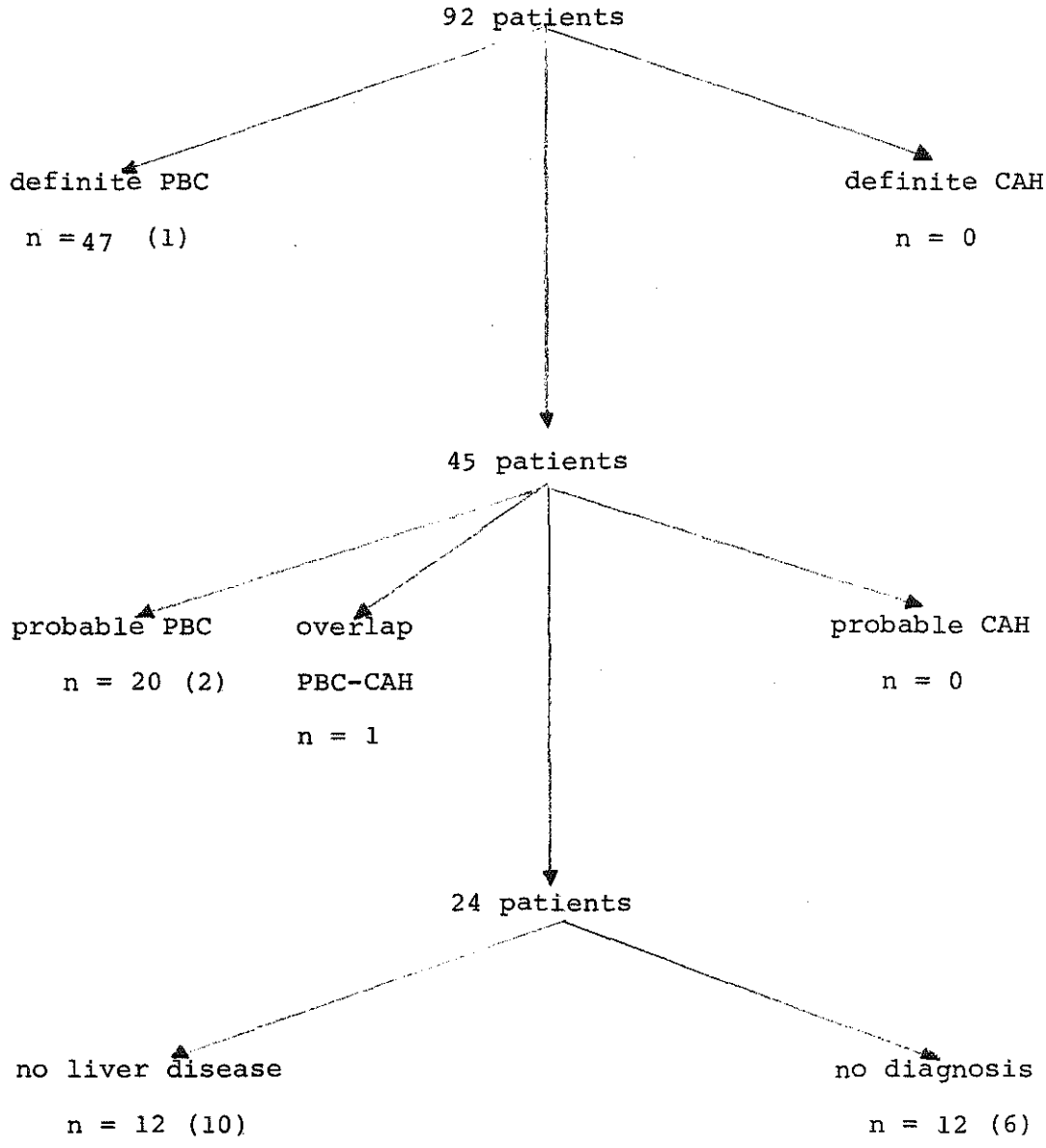
* The rank number of a variable is based on the F-ratio; the members of a cluster exhibit the closest resemblance when the rank number is low and the greatest difference when the rank number is high (see methods).

+ AMA titer : 1 = negative; 2=1/20 ; 3=1/40 , 1/80 ; 4=1/160 , 1/320 ; 5=1/640 or more.

‡ N is the upper limit of normal, defined as the 95 percentile of the normal population of the routine clinical chemistry laboratory.

§ Duct lesion is coded : 1= no lesion; 2= atypical proliferation or a doubtful lesion ; 3=destruction of the duct.

Table V. Diagnosis of AMA-positive patients using the new diagnostic scheme.



In parentheses : no liver biopsy available

Table VI. Comparison of the diagnosis obtained with the new diagnostic scheme and that provided by the strict diagnostic criteria in 92 AMA-positive patients.

strict diagnostic criteria	diagnosis, new scheme				
	PBC	CAH	no diagnosis	no liver disease	total number of patients
PBC	36(5)	0	1	0	41
CAH	0	0	0	0	0
undefined liver disease	27(1)	0	11	1	39
no liver disease	0	0	1	11	12
total number of patients	68 (20)	0	12	12	92

In parentheses: the number of patients with probable PBC.

Table VII. Features of those patients already classified as having definite PBC with the new diagnostic scheme, but who also appear to have some features of CAH.

Case number	PBC-criteria: MAJOR AMA 1-5*	duct lesion	MINOR itch 1-4+	bili/NT umol/%‡	alk.ph. xN§	IgM g/l	Schirmer test mm	CAH-criteria: MAJOR ANF/SMA	piecemeal‖ 1-4	MINOR fatigue 1-3¶	bili/NT ‡	SGOT xN§	γgl g/l	Number of criteria: PBC major	PBC minor	CAH major	CAH minor
4	5	+	3	30/99	6.6	10.6	7	SMA	3	2	30/99	2.8	19	2	4	2	0
9	3	+	1	16/66	3.9	4.5	0	ANF	4	2	16/66	1.8	31	2	3	2	1
21	5	+	1	8/89	3.2	10.1	19	ANF	3	1	8/89	1.3	24	2	2	2	1
32	4	+	4	500/70	2.8	2.8	17	-	3	3	500/70	3.3	21	2	3	1	2
45	4	+	2	23/81	4.8	6.8	0	ANF	2	2	23/81	4.0	31	2	3	1	2
54	5	+	4	72/49	7.7	13.3	1	-	3	2	72/49	5.2	22	2	4	1	3
48	4	+	1	21/97	4.3	5.4	5	SMA	3	2	21/97	4.9	17	2	3	2	1

* The AMA titer is coded: 1=negative; 2=1/20; 3=1/40; 4=1/160, 1/320; 5=1/640 or more.

+ Itch is coded: 1=none; 2=intermittent; 3=continuous; 4=continuous with the need for medication.

‡ Jaundice is defined as a bilirubin level at least twice the upper limit of normal (24umol/l); the Normo-test is abnormal when below 65%.

§ N is the upper limit of normal, defined as the 95th percentile of the normal population of the routine clinical chemistry laboratory.

‖ Piecemeal necrosis is coded: 1=none; 2= mild; 3=moderate; 4=severe.

¶ Fatigue is scored: 1=none; 2=moderate; 3=severe, leading to the inability to do normal daily tasks.

Table VIII. Features of the 7 patients for whom a liver biopsy was available but no diagnosis could be made with the new diagnostic scheme: 1 patient with PBC and CAH; 6 patients with neither PBC nor CAH. Symbols as in table VII.

Case number	PBC							CAH						Number of criteria			
	MAJOR		MINOR					MAJOR		MINOR				PBC		CAH	
	AMA 1-5*	duct lesion	itch 1-4†	bili/NT umol/%‡	alk.ph. xN§	IgM g/l	Schirmer test mm	ANF/ SMA	piece meal 1-4¶	fatigue 1-3¶	bili/NT ‡	SGOT xN§	γ-glob g/l	major	minor	major	minor
72	5	-	1	10/99	3.5	4.8	21	SMA	3	2	10/99	2.9	9	1	2	2	0
8	4	-	2	5/	1.4	2.7	11	-	1	2	5/	0.6	30	1	0	0	1
17	1	+	1	4/	1.0	0.7	30	ANF ± SMA	2	2	4/	0.7	9	1	0	1	0
31	5	-	1	8/88	1.4	4.0	20	ANF	2	2	8/88	0.7	30	1	1	1	1
37	3	-	1	6/99	1.4	2.4	12	ANF	1	1	6/99	1.0	7	1	0	1	0
55	1	+	2	19/99	1.0	3.7	14	ANF ± SMA	2	2	19:99	0.8	15	1	1	1	0
68	1	+	2	4/	1.3	3.1	7	ANF ± SMA	1	3	4/	0.7	10	1	2	1	0

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SERUM IgM IN PRIMARY BILIARY CIRRHOSIS

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Summary

Serum IgM concentrations were measured in 25 patients with primary biliary cirrhosis (PBC) and 25 age- and sex-matched controls by the classical Mancini technique and by a modified method, which included reduction to 7 S monomers. Standards calibrated against the WHO standard, as well as a serum standard with an absolute value calibrated by immunological and non-immunological techniques, were used. All patients had an elevated IgM level; measurement of serum IgM is therefore a simple and sensitive screening test for patients with cholestasis. When measured with the standard calibrated against the WHO standard, the average IgM levels for PBC patients and controls agreed with results previously reported; the average IgM levels were much lower when a serum standard with an absolute IgM value was used; further standardization is needed.

The differences in the ratio of the IgM measured by the classical method (pentameric IgM) to that measured by the alternative method (monomeric IgM) support the existence of different IgM subgroups or the in vivo presence of monomeric IgM in some patients with PBC.

Introduction

Primary biliary cirrhosis (PBC) is a disease of unknown etiology, characterized by continuous portal inflammation with destruction of the interlobular bile ducts, leading to fibrosis and subsequently to cirrhosis. Although the primary cause of the destructive inflammation of intrahepatic bile ducts is unknown, an abnormal host response has been suggested as a pathogenetic factor. Anti-mitochondrial antibodies (AMA) have been found in 80—95% of these

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patients [1,2]; the serum concentration of immunoglobulin M is raised in 73—85% of the cases [3—6]; the IgM response after antigenic stimulation [7] is prolonged, and recently immune complexes [6,8,9] were demonstrated in the serum of nearly all patients.

In view of our interest in the above-mentioned immunological measurements for the evaluation of therapy in PBC, our attention was drawn to the large differences in the immunoglobulin M concentration as estimated by means of radial immunodiffusion in various outstanding laboratories, as well as the need for standardization of calibrating proteins and antisera [10]. De Bruyn and Klein [11] suggested that adequate absolute measurement of IgM by the radial immunodiffusion technique is only possible after reduction to 7 S monomers. Therefore, we compared the classical Mancini technique [12] with their alternative method for the estimation of serum IgM in patients with PBC and in controls.

Materials and methods

Patients

A group of 25 consecutive patients with PBC, admitted since 1978 to the Rotterdam University Hospital, was studied. The diagnostic criteria for PBC comprise a positive AMA-test *, elevation of serum alkaline phosphatase, no demonstrable abnormalities of the extrahepatic bile ducts on the cholangiogram and a liver biopsy showing mononuclear portal inflammation and bile duct lesions, with or without fibrosis and bile duct proliferation.

The control group consisted of 25 age- and sex-matched individuals admitted to the Rotterdam University Hospital since 1978 for abdominal complaints, but without demonstrable somatic abnormalities.

Immunoglobulin M measurement

Classical method. In serum samples processed at room temperature immunoglobulin M was measured quantitatively by radial immunodiffusion according to the method of Mancini [12]. Partigen ® immunoplates and standards (Behringwerke, Marburg, F.R.G.) calibrated against WHO standards were used. The plates were incubated at 37°C for 5 days. The square diameter of the precipitation ring was plotted against the concentration of the various standards to determine the calibration line.

Alternative method [11]. The pentameric IgM (19 S) in serum samples and standards was reduced to monomeric IgM (7 S) with 1,4-dithioerythritol in phosphate-buffered saline (pH 7.4) to a final concentration of 0.01 mol/l (30 min, 37°C). After appropriate dilution the samples were applied to the immunoplates and further processed as in the classical method. The standard used by De Bruyn and Klein [11] was a serum sample with a high monoclonal IgM concentration (absolute value 55 g/l) calibrated against purified IgM preparations using the radial immunodiffusion technique and checked in un-

* Immunofluorescence on rat kidney (Prof. T.E.W. Feltkamp, Centraal Laboratorium voor de Bloedtransfusie Dienst, Amsterdam).

processed serum by a non-immunological calibration method (analytical ultracentrifugation).

Calculations

Ratio. The concentration of serum IgM determined according to the classical method was divided by the concentration of IgM found with the alternative method to obtain the IgM-ratio.

Statistics. For statistical analysis of the data the unpaired Wilcoxon or Student's *t* test was used to compare the results for PBC patients and controls; the classical method was compared with the alternative method by means of paired tests. Differences were considered significant when the *p*-value was less than 0.05.

Results

The patient and control groups were adequately matched as far as age and sex are concerned. Twenty-one patients out of 25 (84%) were women; the median age was 54 years (range 38—72 years).

Using the classical method the median serum IgM concentration for controls was found to be 1.1 g/l with a range of 0.6 to 1.9 g/l. For PBC patients the median IgM concentration was 4.7 g/l with a range of 2.0 to 15.7 g/l. Thus all of the 25 patients (100%) had an elevated serum IgM level; in 23 patients (92%) it was at least 1 g/l above the upper limit of normal (Fig. 1). Using the alternative method the median serum IgM concentration for controls was 0.6 g/l with a range of 0.3 to 1.0 g/l. For the PBC patients the median serum IgM concentration was 3.1 g/l with a range of 0.9 to 8.1 g/l. Thus the serum IgM levels

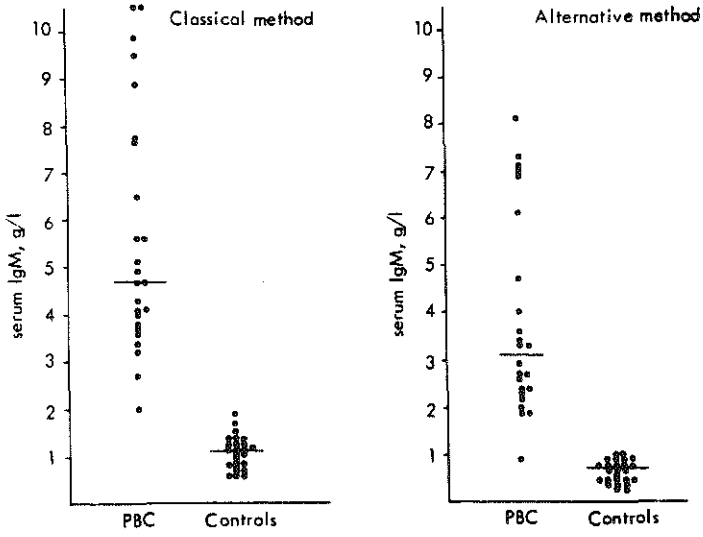

Fig. 1. Serum IgM levels in PBC patients and controls, determined by the classical Mancini technique and by an alternative method which included reduction to 7 S monomers and different standards. The horizontal lines denote the median values.

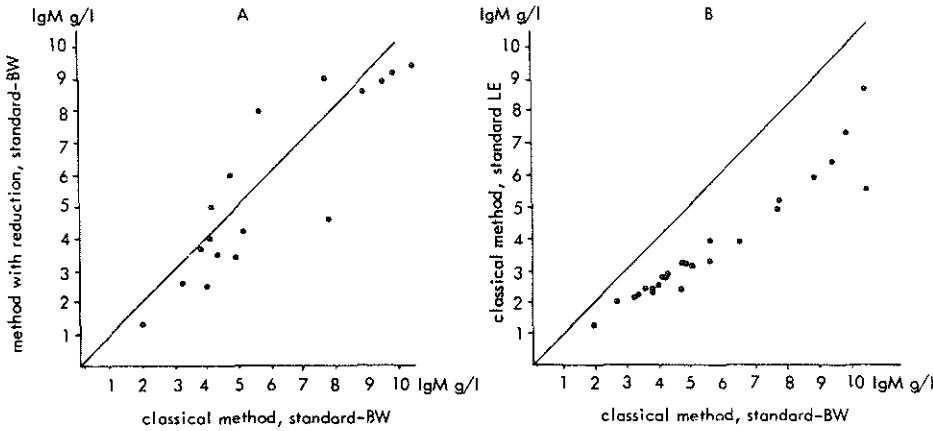

Fig. 2. Analysis of the differences in serum IgM when measured by the classical Mancini technique and by the alternative method. Fig. 2A shows the effect of the reduction step: the line denotes identity. In Fig. 2B the serum IgM measured by the classical method with a standard calibrated against the WHO standard (BW) is plotted against the serum IgM measured by the classical method and a standard calibrated against a serum with an absolute value of 55 g/l (determined by immunochemical as well as non-immunochemical methods). The line again denotes identity.

were elevated in 24 patients (96%); in 21 (84%) the level exceeded the upper limit of normal by at least 1 g/l (Fig. 1). The values obtained by the alternative method differed significantly ($p < 0.05$) from those obtained by the classical method. This was true for the PBC group as well as the controls.

Since the two methods differed in two respects (reduction step and standard) we assessed their separate effects on IgM measurement. Reduction of IgM to 7 S monomers did not produce significant differences in the average IgM content for each group, whereas the effect of the standard was statistically significant (Fig. 2). Although the average IgM levels for the groups were not changed by the reduction step, Fig. 2A demonstrates that variations in the individual values can be appreciable.

The IgM-ratio (pentameric IgM/monomeric IgM) for each patient and control is shown in Fig. 3. For controls the ratio was frequently between 1.5 and 1.9; for PBC patients it ranged mainly between 1.3 and 1.7. This difference in IgM ratio was statistically significant ($p < 0.05$).

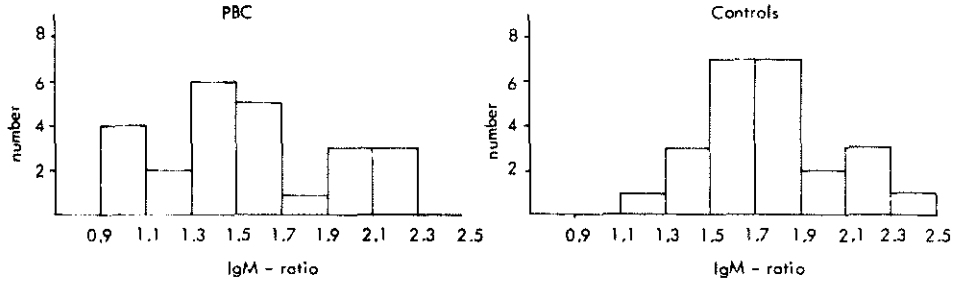

Fig. 3. The IgM ratio (serum IgM classical method/serum IgM alternative method) for PBC patients and controls.

Discussion

Elevated serum IgM levels have been reported in 73—85% of the patients with PBC; in our study serum IgM determined by the classical method was elevated in all patients and was at least 1 g/l above the upper limit of normal in 92%. These results differ from data in the literature but this could be due to variations in methodology, the control group or the patient population. The values for our control group did not differ from those reported by other investigators (Table I); therefore neither the methodology of serum IgM measurement nor the composition of the control group explains the observed difference. In our patient group the diagnosis of PBC was probably established quite early due to the use of the anti-mitochondrial antibody test for women with clinical or biochemical signs of cholestasis. The mean duration of the disease was 3 years, 9 out of 25 patients were asymptomatic and only 3 patients had severe complications of cirrhosis such as gastrointestinal bleeding, ascites or encephalopathy. It is conceivable that the serum IgM concentration is particularly high in the early phase of the disease.

The high frequency of elevated serum IgM concentrations in PBC patients suggests that this test, which is readily available in most clinical chemistry laboratories, might be a useful screening method for patients with a raised alkaline phosphatase with or without jaundice. If the serum IgM is elevated, an anti-mitochondrial antibody test and a liver biopsy are probably more appropriate diagnostic procedures than cholangiography.

We anticipated problems in the precise and accurate measurement of serum IgM, since large variations were reported when various outstanding laboratories assessed IgM by weight, using an international reference serum [10]. The heterogeneity of the estimates appeared to be due partly to differences in antisera, but could also be explained by the use of calibrating proteins from different subgroups [13]. The existence of subgroups was proposed by Klein et al.: they showed that human serum produces different calibration lines with the same antiserum, even when no qualitative differences could be detected by other techniques. The differences between the groups are eliminated by reduction of the IgM paraprotein to 7 S subunits. In contrast to the WHO studies [10], this method also includes an independent physico-chemical check on the calibration without using any isolated IgM as a reference.

In practice, the classical method gave values for the control group which were comparable to those of other investigators, and significantly higher values for all patients with PBC. Precision was good with an inter-assay variation of 11%. The very different values (in g/l) obtained with the procedure of De Bruyn and Klein, however, demonstrate the importance of properly calibrated standards or sera in the radial immunodiffusion technique. Although the classical method appears theoretically to be inferior to the method including reduction, the reduction step had no influence on the average IgM levels for either group; individual values, however, were sometimes quite different.

The observed difference in IgM-ratios could be due to variations in the IgM subgroups between PBC patients and controls; it also could be explained by the in vivo presence of monomeric IgM in some patients with PBC. Recently Fakunle and others [14] detected monomeric IgM by polyacrylamide/agarose

TABLE I

SERUM IgM CONCENTRATION IN PBC (MANCINI TECHNIQUE)

Controls			PBC			
Author	No.	Type	IgM g/l range	No.	IgM g/l range (median)	% abnormal
Feizi [5] 1968	60	asymptomatic volunteers	0.25—1.8	16	1 —28 (4.5)	81
Bevan [3] 1969	478	asymptomatic volunteers	<1.9	13	0.91—11 (5.0)	85
McSween [4] 1972	73	age- and sex-matched patients with non-hepatic disease	0.25—1.75	73	0.50— 5.75 (2.75)	85
Epstein [6] 1979	?	asymptomatic volunteers	0.60—2.80	30	0.20—14 (4.50)	73
This study 1980						
classical method	25	age- and sex-matched patients without organic disease	0.60—1.90	25	2.0 —15.7 (4.70)	100
alternative method			0.30—1.00		0.90— 8.10 (3.10)	96

gel immunodiffusion and Sephadex G200 gel filtration in 33% of the sera from patients with PBC. Its presence was ascribed to incomplete polymerization of 7 S IgM because of an increased rate of synthesis of the IgM protein. Whatever its cause, the observed differences suggest that quantitation of IgM in the 7 S form might be the most reliable method for determining the level of total IgM in these sera.

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MONOMERIC (7S) IgM IN PRIMARY BILIARY CIRRHOSIS

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Running title: 7S IgM in primary biliary cirrhosis.

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Abstract

Monomeric (7S) IgM was measured in 25 patients with primary biliary cirrhosis and 25 age- and sex-matched controls by simple double immunodiffusion in a polyacrylamide gel. 7S IgM was detected in a quarter of the patients and none of the controls.

The presence of 7S IgM was related to the elevation of total IgM as measured by the radial immunodiffusion techniques. Patients with 7S IgM had significantly higher levels of cryoglobulins and immune complexes in their sera than 7S IgM-negative patients. In addition, serum alkaline phosphatase concentrations were more elevated in the 7S IgM-positive patients than in the 7S IgM-negative patients, reflecting more extensive bile duct damage. The presence of monomeric (7S) IgM as well as other differences in the IgM molecule may be significant for the pathogenesis of primary biliary cirrhosis.

Key words: primary biliary cirrhosis, immunoglobulin M, pathogenesis.

INTRODUCTION

In primary biliary cirrhosis (PBC) the serum IgM concentration is almost always elevated (1), probably due to increased synthesis (2). The increased synthesis rate of IgM may be associated with abnormalities in size or structure of the IgM molecule, since in PBC both 7S IgM (3) and 19S IgM with variable properties have been demonstrated with the radial immunodiffusion technique (4).

This study was performed to determine the frequency of 7S IgM in PBC and its possible significance in relation to other immunological and biochemical features.

MATERIALS AND METHODS

Blood was drawn from twenty-five consecutive PBC patients (21 women, 4 men; age 38-72 years) and twenty-five age- and sex-matched controls without overt somatic abnormalities. PBC was defined as elevation of serum alkaline phosphatase, a positive antimitochondrial antibody test, a liver biopsy showing portal lymphoplasmocellular infiltration with destruction of bile ducts, and no abnormalities on the cholangiogram of the extrahepatic bile ducts.

The serum samples were processed at room temperature; the IgM concentration was then measured by the classical Mancini radial immunodiffusion technique, using Partigen^{R} immunoplates (Behringwerke, Marburg, F.R.G.) and by an alternative method involving reduction to 7S IgM monomers with 1,4-dithioerythritol and a IgM-standard calibrated by independent physical methods (1,5).

7S IgM was detected by double immunodiffusion in a polyacrylamide gel, prepared by dissolving 5 g cyanogum 41 (Serva, Heidelberg, F.R.G.), 0.4 ml tetramethylethylendiamine (Serva, Heidelberg, F.R.G.) and 0.1 g ammonium persulphate (Merck, Darmstadt, F.R.G.) in 100 ml distilled water. The resulting solution was immediately poured into a glass dish to form a layer 2.2 mm thick and then left to polymerize for thirty minutes. Sample wells, containing 20 µl serum in two dilutions, and antiserum wells were arranged in parallel rows rather than in the form of rosettes; all determination were carried out in duplicate on two different plates. Rabbit anti-human IgM (Dako, Copenhagen, Denmark) was used as an antiserum. Reference 7S IgM, in a dilution ranging from 25 to 250 mg/l, and 19S IgM (dilution range: 5 to 10 g/l, comparable to the total IgM content in PBC sera) served as positive and negative controls. Reference 19S IgM was isolated from a serum sample containing a 19S IgM paraprotein by gel filtration through Sepharose 6B and dissolved in phosphate buffered saline (PBS). Reference 7S IgM was obtained by reducing the 19S IgM solution with 1,4-dithioerythritol, blocking with ethyleneimine and removing excess reagent by dialysis against PBS. After incubation for two days at room temperature, the gel was washed with PBS, coloured with amidoblack and inspected for the presence of a precipitation line indicating the existence of 7S IgM.

From similar serum samples processed at 37° C, cryoglobulins were isolated (6) and their immunoglobulin M content determined by the classical Mancini immunodiffusion technique. The ^{125}I-C1q binding percentage (7) was also assayed.

Serum alkaline phosphatase and bilirubin were measured with the Technicon SMA 12-60, while serum glutamic oxalacetic transaminase (SGOT or serum aspartate aminotransferase) was determined by means of the LKB Reaction Rate Analyzer (UV kinetic measurement). For statistical analysis of the data the unpaired Wilcoxon test was used; differences were considered significant when the p-value was less than 0.05.

RESULTS

All PBC patients had elevated serum IgM, ranging from 2.0 to 15.7 g/l when measured by the classical Mancini method and from 0.9 to 8.1 g/l when measured by the alternative method; the IgM ratio (concentration obtained with classical method divided by that obtained with the alternative method) ranged from 0.9 to 2.2.

The polyacrylamide double immunodiffusion technique yielded a precipitation line when the 7S IgM concentration exceeded 25 µg/ml. For 6 out of 25 patients a precipitation line was found, while none of the controls showed such a line. The amount of 7S IgM did not exceed 100 µg/ml in the positive sera. The immunological and biochemical features of both 7S IgM-positive and 7S IgM-negative PBC patients and controls are summarized in table I.

All PBC patients had cryoglobulins, mainly consisting of IgM, in amounts varying from 5-228 mg/l, as determined by the classical radial immunodiffusion method. Results of the reduction method were not available for all samples. Moreover most PBC sera (23 of 25) showed abnormal ^{125}I-C1q binding of up to 60%. These immunological abnormalities were considerably more prominent in 7S IgM-positive patients.

Bilirubin levels were still normal in the majority of our PBC patients. Liver function tests were comparable in 7S IgM-positive and 7S IgM-negative PBC patients, with the exception of significantly higher serum alkaline phosphatase in 7S IgM-positive patients.

DISCUSSION

Recently Fakunle (3) found 7S IgM in one third of his patients. We could detect 7S IgM in a quarter of the patients with PBC using a double immunodiffusion technique in a polyacrylamide gel.

The presence of 7S IgM may cause a larger precipitation ring when the classical radial immunodiffusion technique is used, leading to spuriously elevated IgM values(8). Errors of the classical radial immunodiffusion technique may also be caused by the presence of IgM subgroups (4). For these reasons we also quantified IgM after reduction to uniform 7S subunits (5). The IgM ratio, obtained by dividing the IgM concentration obtained with the classical method by that obtained with the alternative method, did not differ significantly between the 7S-negative and 7S-positive PBC-groups. The observed difference in the IgM ratio between PBC patients and controls (1) is therefore more likely to be due to different subgroups of IgM than to the presence of monomeric 7S IgM.

The presence of 7S IgM corresponded to the high elevation of total IgM and the presence of immune complexes as measured by cryoglobulins and ^{125}I-C1q binding. A possible hypothesis is that an increased or accelerated immunoglobulin synthesis (9) is associated with production of IgM of abnormal size and structure, followed by the formation of immune complexes. The finding of significantly higher serum alkaline phosphatase concentrations in 7S IgM-positive PBC supports the idea that bile duct damage, which is the key to the PBC syndrome, might be related to the presence of large circulating immune complexes.

Table I. Immunological and biochemical features of 7S IgM-negative and -positive patients with PBC, and age- and sex-matched controls.

		Controls n=25 median(range)	PBC 7SIgM-neg.* n=19 median(range)	PBC 7SIgM-pos.* n=6 median(range)	Wilcoxon test p-value
IgM classical	g/l	1.1 (0.6 -1.9)	4.1 (2.0 -7.8)	9.6+ (7.7 -15.7)	0.0004
IgM alternative	g/l	0.7 (0.3 -1.0)	2.7 (0.9 -6.1)	7.1+ (6.9 - 9.2)	0.0003
IgM ratio: $\frac{\text{classical}}{\text{alternative}}$		1.75 (1.00-2.33)	1.59 (0.91-2.22)	1.36 (1.08- 1.71)	0.610
cryo IgM classical	mg/l	0 (0- 2)	8 (5- 34)	79+ (66-228)	0.0003
^{125}I-C1q binding	%	5 (3-16)	12 (5- 40)	23+ (12- 60)	0.024
alkaline phosphatase	U/l	27 (18-45)	135 (54-329)	282+ (167-450)	0.044
bilirubin	mmol/l	6 (3-22)	16 (6-497)	15 (7- 65)	0.226
SGOT	U/l	15 (9-30)	78 (33-162)	96 (57-156)	0.171

*all measurements for both PBC groups differed significantly from those for the control group, with the exception of the IgM ratio in 7S IgM-negative PBC.

+significantly increased if compared to the 7S IgM negative PBC patients, according to the unpaired Wilcoxon test. ($p < 0.05$)

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CRYOGLOBULINS IN PRIMARY BILIARY CIRRHOSIS: PREVALENCE AND MODULATION BY IMMUNOSUPPRESSIVE THERAPY.

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Running title: cryoglobulins in PBC.

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SUMMARY

In the pathogenesis of primary biliary cirrhosis (PBC) immunological abnormalities such as the presence of immune complexes might be of significance. In this study cryoglobulins were measured in 25 patients with PBC and, for comparison purposes, in 25 age- and sex-matched normal individuals as well as 25 patients with chronic active hepatitis (CAH). Cryoglobulins were present in all patients with PBC(median protein content 18 mg/l, range 8-233) and consisted predominantly of IgM (80%), while none of the normal controls and only 20% of the patients with CAH had cryoglobulins.

In PBC, a statistically significant correlation was found between cryoglobulin M concentration and other immunological measurements, such as the serum IgM level ($p=0.003$) and ^{125}C1q binding ($p < 0.001$). Cryoglobulin M also correlated significantly with alkaline phosphatase ($p=0.002$) and liver fibrosis ($p=0.013$), but only in a larger group of patients with PBC.

In a longitudinal study of patients with PBC, no changes in the cryoglobulin concentration were found following treatment with D-penicillamine alone or a placebo, but the cryoglobulin M level decreased significantly during low-dose combination therapy of D-penicillamine and prednisone (median 15→4 mg/l); this was accompanied by a statistically significant improvement in the cholestatic features (alkaline phosphatase 240→139 U/l).

The relation between cryoglobulin M and the features of prolonged liver damage as well as the decrease in cholestasis after disappearance of these immune complexes is supporting evidence for its pathogenetic role in PBC.

Keywords: primary biliary cirrhosis, cryoglobulins, treatment.

INTRODUCTION

Primary biliary cirrhosis (PBC), a disease predominantly seen in middle-aged women, is characterized by destruction of intrahepatic bile ducts and fibrosis leading to cirrhosis (1). Although the etiology is unknown, abnormalities in both cellular and humoral immunity have been connected with the pathogenesis of the disease.

Antibodies to the inner membrane of mitochondria (AMA) are almost always present, but are more likely to be of diagnostic value rather than pathogenetic significance (2). Antibodies to biliary ductular cells have also been found, but they are not specific for PBC (3,4). An elevated serum IgM (5), but more probably circulating IgM containing immune complexes (6-10), may be important in the mediation of the destruction of bile ducts.

To investigate further the pathogenetic role of immune complexes, we measured the cryoglobulin concentrations and determined their composition in PBC patients and in age- and sex-matched controls as well as in patients with various chronic liver diseases. We found that in PBC cryoglobulins consisted mainly of IgM and that the cryoglobulin concentration can be correlated with the level of serum alkaline phosphatase; in addition, disappearance of cryoglobulin M after treatment with immunosuppressive drugs was associated with a decrease in cholestasis.

MATERIALS AND METHODS

Patients: The diagnostic criteria for PBC were an elevated serum alkaline phosphatase level, a positive antimitochondrial

antibody test, an elevated serum immunoglobulin M concentration, a liver biopsy showing portal lymphoplasmacellular infiltration with destruction of bile ducts with or without granuloma and no abnormalities of the extrahepatic bile ducts on the cholangiogram.

Twenty-five consecutive patients with PBC were matched for age and sex with controls who had no overt somatic abnormalities. In addition, twenty-five patients with various chronic liver diseases were studied: eight patients with HBsAg-positive chronic active hepatitis, eight patients with HBsAg-negative chronic active hepatitis (serum glutamic oxalocetic transaminase at least three times the upper limit of normal and a liver biopsy showing moderate or severe piecemeal necrosis) and nine patients with biopsy-proven cirrhosis (six alcoholics and three with unknown etiology). Two patients with PBC received corticosteroids when blood was drawn for the measurement of cryoglobulins. Some correlations were tested in an additional group of fifteen consecutive patients with PBC; two of these latter patients were also treated with corticosteroids.

Eighteen symptomatic patients with PBC participated in the longitudinal study. Sixteen took part in our randomized trial and received either a placebo (N=7) or D-penicillamine (N=9) for twelve months in a dosage increasing within 4 months from 250 mg to 1000 mg daily. After six months without therapy, the first seven patients (four of the placebo group and three of the D-penicillamine group) and two new patients were placed on a low-dose combination therapy consisting of 250 mg D-penicillamine and 10 mg prednisone daily for twelve months.

The studies were approved by the local institutional Medical Ethics committee on September 26th.,1977.

Cryoglobulin isolation and quantification: cryoglobulins were isolated according to the method described by Wands (8,11) with only minor modifications. Venous blood, drawn into warm polystyrene tubes (Vacuplast ^{R}, Greiner, Nürtingen, F.R.G.), was allowed to clot for 3 hours at 37°C and was then centrifuged for 30 minutes at 200xg at 37°C. Ten ml of serum were incubated at 4°C for 10 days. The serum was then centrifuged for 1 hour at 8000xg at 4°C. The precipitate was resuspended in 10 ml of phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA), pH 7.6, and 0.01% sodium azide, and left overnight at 37°C. After centrifugation for 15 minutes at 2000xg at 37°C the supernatant was left for 3 days at 4°C. After centrifugation for 1 hour at 8000xg at 4°C, the precipitate was washed three times with PBS and finally redissolved in 500 ul 0.1 M sodium acetate, pH 5.0, and 0.01% sodium azide.

The cryoprecipitate was tested using the Ouchterlony immunodiffusion technique for the presence of albumin to check the washing procedure. The protein content was measured by Lowry's method (12) and the immunoglobulins G, M and A were determined by the radial immunodiffusion technique (13) using special low-level immunoplates (Behringwerke, Marburg, F.R.G.); the concentrations were expressed in milligrams per liter original serum.

Other measurements: serum bilirubin and alkaline phosphatase were measured with the Technicon SMA 12-60, while glutamic oxalocetic transaminase (SGOT or serum aspartate aminotransferase) was determined by the UV kinetic method (LKB reaction rate analyzer, Stockholm, Sweden). Serum immunoglobulin G and M were

measured by the radial immunodiffusion technique using Partigen ^{R} immunoplates (Behringwerke, Marburg, F.R.G.). The ^{125}I-C1q binding percentage was assayed according to Zubler (14).

Liver biopsies of patients with PBC were evaluated by a pathologist who had no knowledge of the clinical features. Many features were scored semi-quantitatively: for instance, the degree of fibrosis was scored as absent, mild (minor deposition in the portal tracts), moderate (some portoportal septa) or severe (overt septa formation connecting the portal triads).

Statistics: for statistical analysis of the initial data of patients with PBC, controls and patients with various chronic liver diseases, the unpaired Wilcoxon test was used. The data of patients with PBC before and after treatment were tested by the paired Wilcoxon test. For the correlation of biochemical, immunological and histological data of the initial PBC group the non-parametric correlation test according to Spearman was used. Differences were assumed to be significant when the p-value was less than 0.05.

RESULTS

The series of 25 patients with PBC comprised 21 women and 4 men with a median age of 54 years (range 38-72). The "normal" control group was adequately matched as far as age and sex are concerned. The group of 25 patients with various chronic liver diseases included 10 women and 15 men with a median age of 51

years (range 24-68).

The initial cryoglobulin analyses are summarized for the three groups in Table I. In the controls , only a few precipitates were observed, so that the protein concentration in the final solution often could barely be measured. Cryoglobulins were detected in all patients with PBC and in 20% of the patients with various chronic liver diseases. In PBC the cryoglobulins consisted mainly of IgM with little or no IgG or IgA. The cryoglobulins in the four patients with various chronic liver diseases, however, were mixed, i.e. containing both IgM and IgG. The distribution of cryoglobulins in PBC is depicted in Figure I; there is no normal distribution, but two subpopulations might be discernible in the PBC group: 6 patients had a rather high concentration (more than 35 mg/l), and 19 patients a moderate concentration (less than 35 mg/l) of cryoglobulin M.

The relationship between the initial cryoglobulin M concentration and several other immunological (serum IgM, ^{125}I-C1q binding, AMA titer), biochemical (serum bilirubin, alkaline phosphatase, SGOT) and histological (fibrosis, granuloma, piecemeal necrosis) features was tested for the group of patients with PBC. There was a statistically significant relationship between cryoglobulin M and both serum IgM ($p=0.003$) and the ^{125}I-C1q binding percentage ($p<0.001$) as shown in Figure 2. The correlation of cryoglobulin M with both serum alkaline phosphatase ($p=0.082$) and the degree of liver fibrosis ($p=0.104$) was not significant for our group of 25 patients with PBC (Figure 2 and 3), but was highly significant for a larger group of 40 patients with PBC (serum alkaline phosphatase: $p=0.002$ and liver fibrosis: $p=0.013$).

Data from the longitudinal study on cryoglobulins in PBC patients are summarized in Table II. The three groups were comparable as far as age, sex and symptoms as well as biochemical, immunological and histological features are concerned. A significant decrease in serum IgM was seen during treatment with D-penicillamine, but no variations in either cryoglobulin M or alkaline phosphatase were noted. During low-dose treatment with D-penicillamine plus prednisone a significant decrease in both serum IgM and cryoglobulin M was found. Cryoglobulins even disappeared in 6 out of 9 patients. A significant decrease in serum alkaline phosphatase was only observed during this latter therapeutic regimen.

DISCUSSION

Cryoglobulins were detected in 90% of 20 patients with PBC by Wands and co-workers (8); in our study of 40 patients with PBC about the same prevalence was found. With regard to the levels of cryoglobulins, patients with PBC do not seem to be a homogeneous group. The group with high levels of cryoglobulins, however, did not differ in symptoms (fatigue, pruritus, arthritis) from the group with mild cryoglobulinemia, but differences in biochemistry (alkaline phosphatase) and immunology (^{125}I-C1q binding, serum IgM) were found. Berg (16) also postulated the existence of subgroups in PBC, the classical form and the form with features of chronic active hepatitis. He based his subdivision on two different types of antimitochondrial antibodies. The clinical significance of these obser-

vations, however, is not clear.

The cryoprecipitate was composed almost exclusively of IgM; reduction of the cryoglobulin M with 1,4-dithioerythritol to 7S monomers did not change this finding. In contrast, IgG was present in the precipitates found in 4 out of 5 patients with other types of chronic liver disease. The hypothetical role of cryoglobulins in the mediation of liver damage is based on the concept that cryoglobulins are circulating immune complexes. Evidence that cryoglobulins represent immune complexes was provided by Wands (8), but this will not be convincing until it is demonstrated that they consist of antigens, antibodies and complement components. The size and composition of the immune complexes (in particular the role of complement) are probably related to the type of immune-complex disease (15). It has been suggested that the presence of IgG in cryoglobulins is associated with inflammation, while IgM-containing cryoglobulins could be harmless.

We observed IgG-containing cryoglobulins in patients with chronic parenchymal liver disease, but there was no correlation between piecemeal necrosis and cryoglobulin M in PBC. In PBC cryoglobulins may be harmful for bile ducts because of their size. We found a positive correlation with liver fibrosis, which is probably the result of persistent destruction of the bile ducts. A direct effect of cryoglobulins on the formation of collagen, which also occurs in associated diseases such as Raynaud's phenomenon and scleroderma, remains a second possibility.

The role of cryoglobulins in the pathogenesis of PBC may

also appear from the effects of treatment. Recently, Epstein et al (17) reported a reduction of the immune complex concentration, as measured by the ^{125}I-Clq binding test, during treatment with D-penicillamine. In our study, three treatment regimens (placebo, D-penicillamine, or a low-dose combination therapy of D-penicillamine and prednisone) were used. During therapy with D-penicillamine the level of the cryoglobulins did not change conspicuously, although a significant decrease in serum IgM was found. Only combined therapy gave rise to a statistically significant decrease in cryoglobulin M as well as a drop in alkaline phosphatase and bilirubin and only the therapy associated with a decrease in cryoglobulins led to biochemical improvement in cholestasis. This finding supports the hypothesis that cryoglobulins may damage bile ducts and that removal of cryoglobulins is beneficial. We realize that the correlation between the decreased cryoglobulin levels and biochemical improvement in cholestasis is only supporting evidence. However, direct proof of the pathogenetic role of cryoglobulins will be very difficult to obtain in humans.

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Table I.

Initial cryoglobulin composition in PBC patients, age and sex-matched controls and patients with various liver diseases. The values are expressed in mg per liter of original serum.

	Cryoglobulin analysis				
diagnosis	total protein mg/l median (range)	IgM mg/l median (range)	IgG mg/l median (range)	IgA mg/l median (range)	number of positive patients *
controls (n=25)	3 (1-12)	0 (0-2)	0 (1)	0 (1)	0
PBC (n=25)	18 (6-233)	11 (5-228)	1 (0-4)	1 (0-2)	25
various chronic liver disease (n=25)	4 (0-220)	2 (0-77)	0 (0-14)	not measured	5

* The cryoglobulin analysis was considered positive when the cryoglobulin M or G content was at least 4 mg/l.

Table II.

Biochemical and immunological features (median values) in primary biliary cirrhosis before and after six months of various therapies.

Treatment group	Month	Cryo IgM (< 2 mg/l) median (range)	Serum IgM (< 2.8 g/l) median (range)	alk. phosph. (< 45 u/l) median (range)
Placebo (N=7)	0	9 (4-71)	4.8 (2.6-15.7)	212 (125-543)
	6	15 (3-170)	4.9 (3.1-16.0)	209 (149-530)
	12	9 (2-150)	6.1 (3.2-18.6)	208 (153-387)
D-penicillamine (N=9)	0	11 (6-228)	6.4 (3.2-11.6)	203 (96-365)
	6	10 (1- 47)	4.0* (2.3- 8.3)	199 (94-475)
	12	4 (1- 50)	5.2 (2.5-16.6)	234 (62-415)
D-penicillamine and prednisone (N=9)	0	15 (2-116)	8.9 (3.2-12.8)	240 (95-558)
	6	4* (0- 12)	5.7* (1.6- 8.0)	139* (46-581)

* significant decrease according to the paired Wilcoxon test ($p < 0.05$) when compared with month 0.

B.G.Taal

LEGEND TO FIGURES.

Figure 1. The distribution of cryoglobulin M in 25 patients with PBC.

Figure 2. The relationship between levels of cryoglobulin M and serum IgM (Spearman coefficient 0.57, $p<0.001$) as well as ^{125}I-C1q binding percentage (Spearman coefficient 0.68, p=0.003) and alkaline phosphatase (Spearman coefficient 0.35, p=104) in sera of 25 patients with PBC, before treatment.

Figure 3. The relationship between levels of cryoglobulin M and the degree of fibrosis in the liver biopsy (Spearman coefficient 0.13,p=104) in 25 patients with PBC, before treatment.

Fig. 1.

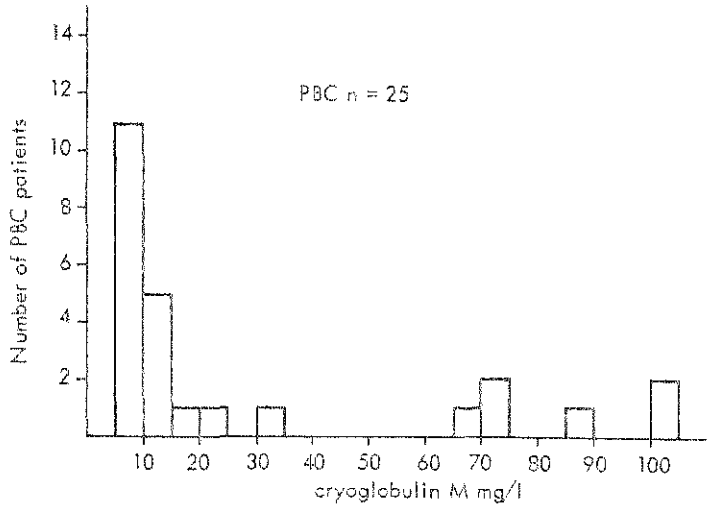


Fig. 2.

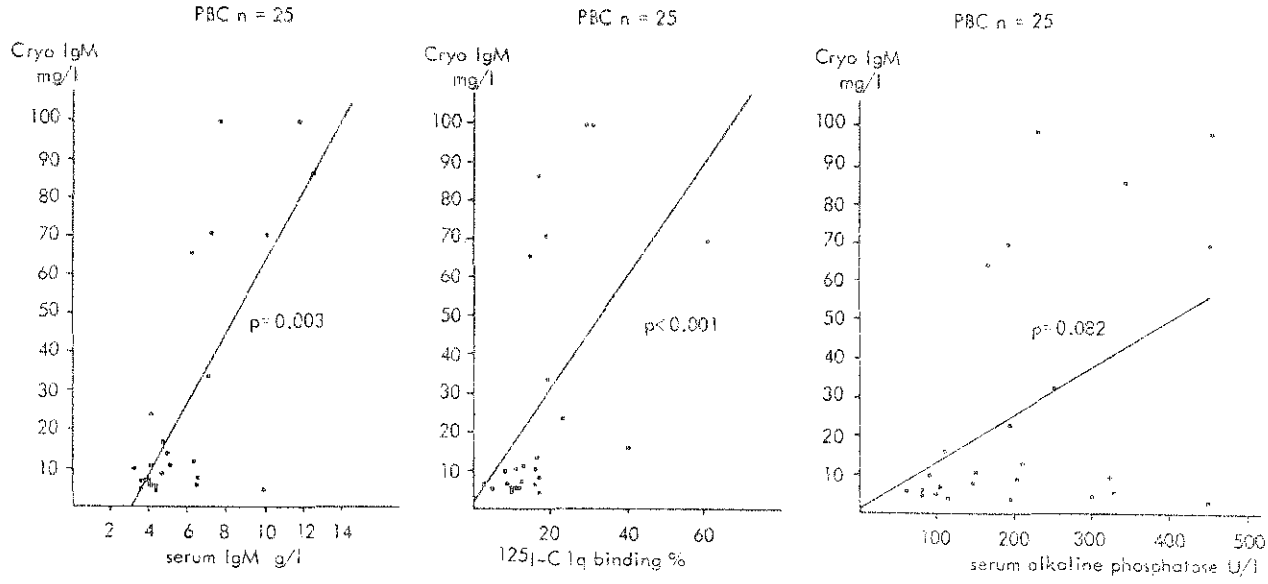
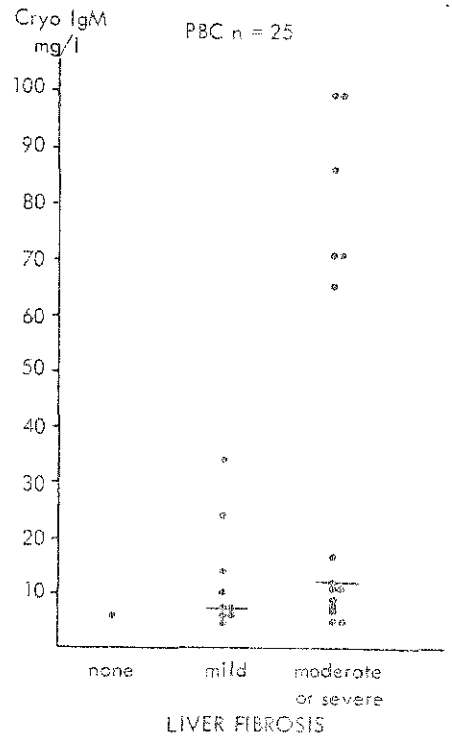


Fig. 3.



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LOW THERAPEUTIC VALUE OF D-PENICILLAMINE IN PRIMARY BILIARY CIRRHOSIS.

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Summary

A small double-blind controlled trial to evaluate D-penicillamine therapy was carried out; the dose-responses in twenty-four patients with PBC were related to beneficial results and side-effects. The daily dose of D-penicillamine was increased monthly by 250 mg until a total of 1 gram daily was reached. Two out of 11 patients (18%) were withdrawn because of side-effects, but also 4 out of 13 (31%) patients receiving the placebo. One gram of D-penicillamine induced no improvement in symptoms, and was associated with a mild improvement in alkaline phosphatase and serum IgM as well as a marked reduction in hepatic copper. Lowering the dose of D-penicillamine to 500 mg daily abolished the effect on the liver and the immunological tests, but the low levels of hepatic copper persisted.

The clinical effect of D-penicillamine therapy appears to be small and dose-related; side effects should not prevent its widespread use but the drug must be introduced slowly. Because the observed improvements in the liver tests were probably related to improved immunological abnormalities, a combination of D-penicillamine and another immunosuppressive agent may have increased therapeutic potential.

Introduction

Primary biliary cirrhosis (PBC) or chronic cholangiolytic hepatitis is a slowly progressive disease characterized by portal lymphoplasmocellular infiltrates surrounding destroyed bile ducts which leads to fibrosis and cirrhosis. The etiology is unknown, but several immunological abnormalities in both humoral (1-3) and cellular immunity (4) have been reported. Effective therapy is currently not available: in particular the administration of prednisone or azathioprine has been without proven value (5,6).

Recently, D-penicillamine was introduced as a therapeutic agent because of its copper chelating properties since hepatic copper in PBC may reach concentrations comparable to those seen in Wilson's disease (7). D-penicillamine also has immunosuppressive properties which may even be a better explanation of its possible beneficial effect in PBC (8); the toxicity, however, appears to be a limiting factor for its widespread use.

In this double-blind controlled study, D-penicillamine was introduced slowly in order to lower the incidence of initial side-effects. In addition the maximum dose of 1 gram per day was decreased after six months to a maintenance dose of 0.5 grams in order to avoid serious side-effects,which often appear after prolonged use (9). To evaluate whether the presumed beneficial effects of D-penicillamine persisted after discontinuation of the drug, therapy was terminated after one year and all patients were observed for another six months. The purpose of this protocol was to investigate, in addition to the dose-responses, whether a favourable effect is achieved by reducing hepatic copper or normalizing the immunological disturbances, such as reduction of serum IgM and immune complexes.

Material and methods

Patients. The diagnostic criteria for PBC consisted of an elevated serum alkaline phosphatase level, a positive antimitochondrial antibody test, a liver biopsy showing lymphoplasmocellular infiltrates with destruction of interlobular bile ducts, or a paucity of bile ducts and no demonstrable abnormalities of the extrahepatic bile ducts on the cholangiogram. Only symptomatic patients (fatigue, pruritus, and/or jaundice) with an alkaline phosphatase concentration of at least twice the upper limit of normal were included in the study. Patients were excluded when they presented with ascites, hepatic encephalopathy, or one of the following conditions: the use of a cholestatic drug within the past 6 months; histologically proven ileitis or colitis; a neoplasm within the past 5 years; or pregnancy.

The studies were approved by the local institutional Medical Ethics Committee on September 26th 1977.

Experimental design. Patients from two centres participated in the study, in each centre they received at random either D-penicillamine or the placebo (lactose). Both tablets were kindly provided by Gist-Brocades (Delft, the Netherlands) and were identical in appearance. The initial dose was one tablet a day (250 mg D-penicillamine or placebo); the dose was increased every month by one tablet until the maximum dose of 1000 mg daily was reached. Six months after the initiation of therapy this maximum dose was decreased abruptly to 500 mg daily for the following period of six months. After therapy for one year, all patients stopped taking tablets; they were then followed for a third period of six months. When side-effects appeared the dose was usually reduced, followed by slow re-introduction of the full dose. When side-effects recurred or were fairly severe, medication was discontinued. All patients received a prophylactic

dose of 25 mg of pyridoxine daily. Concomitant use of cholestyramine was allowed, but not together with the trial tablets because of potential interference with absorption.

Measurements. Each patient underwent a detailed examination before entry and at six-month intervals; this included clinical, biochemical and immunological determinations as well as a liver biopsy when feasible. To monitor side-effects, blood tests and an urinanalysis were initially performed monthly and thereafter at three-month intervals.

Symptoms of fatigue were scored as none, mild or severe (i.e. inability to perform normal daily activities) and pruritus as absent, intermittent or continuous (necessitating the use of cholestyramine). Upon physical examination special attention was given to scratch marks, spider naevi, palmar erythema, xanthelasma, the size of liver and spleen, ascites and oedema. Tear production was measured by the Schirmer test (SMP Division Cooper Laboratory, New York, U.S.A.).

Haematological studies were performed using EDTA blood (Coulter Counter). Serum bilirubin and alkaline phosphatase were measured with the Technicon SMA 12-60, while serum glutamic oxaloacetic transaminase (SGOT or serum aspartate aminotransferase), glutamic pyruvic transaminase (SGPT or alanine aminotransferase) and gamma-glutamyl transpeptidase (γ-GT) were determined by an UV kinetic method using the LBK Reaction Rate Analyzer (Stockholm, Sweden). Albumin and gamma-globulin were measured by standard cellulose acetate electrophoresis. Fasting bile acids were measured by means of a radioimmunoassay (10).

Immunoglobulin M and G were determined by the radial immunodiffusion technique using Partigen^{R} immunoplates (Behringwerke, Marburg, F.R.G.) at one centre (16 patients) and by rate nephelometry at the other centre (8 patients). An indirect immunofluorescent technique using fluorescein

isothiocyanate-conjugated sheep-anti-human immunoglobulin was chosen for detection of antibodies against nuclear antigens and mitochondria (on rat kidney) and antibodies against smooth muscles (on rat stomach) in serum at various dilutions (11). Serum samples were processed at 37°C, cryoglobulins were isolated (3) and their immunoglobulin content determined by the classical Mancini technique; in addition the ^{125}I-Clq binding percentage was also assayed according to the method of Zubler (12). These last two methods were only performed at one centre (16 patients).

Liver biopsies were obtained with copper-free Tru-Cut^{R} needles (Travenol Laboratories, Deerfield, Illinois, U.S.A.). Portal inflammation, bile duct lesions and granuloma as well as the degree of fibrosis and piecemeal necrosis were scored by an independent pathologist; for fibrosis the following semiquantitative scale was used: absent, mild (minor deposition in the portal tracts), moderate (some porto-portal septa) or severe (overt septal formation connecting the portal tracts). Similarly, piecemeal necrosis was scored as absent, mild, moderate, or severe. From each biopsy, measuring about 2 cm, a 3-mm piece was removed and transferred to a copper-free tube; hepatic copper was measured by neutron-activation analysis (13).

Statistics. For statistical analysis of the initial data of the PBC patients in the two treatment groups the unpaired Wilcoxon test was used for the non-continuous data and the chi-square test for the discrete variables. To compare the data of the patients in one group during therapy the paired Wilcoxon test was used. Differences were assumed to be significant when the p-value was less than 0.05.

Results

Between March 1978 and October 1979 twenty-four patients entered the study; 11 patients received D-penicillamine and 13 patients the placebo. The initial features of both groups are summarized in Table I; they were comparable as far as age and sex as well as symptoms and signs were concerned. The subjects were predominantly middle-aged women suffering from pruritus. Fatigue and pain in the region of the liver were frequently mentioned by the patients, but only a minority suffered severe disability. Upon physical examination spider naevi or palmar erythema were only occasionally present (4 patients in both groups), while enlargement of the liver and/or spleen was found in seven patients (29%). With regard to the associated diseases, diminished tear secretion, as measured by the Schirmer test, was observed in fifty percent of the patients. The histological features were comparable. Although frank cirrhosis was present in a minority of the cases (9% and 23% for the D-penicillamine and placebo group, respectively), some fibrosis was often found and was usually quite marked or even severe (50% and 70% for the D-penicillamine and placebo group, respectively). The initial biochemical and immunological determinations for both groups are also shown in Table I. Because the normal values of alkaline phosphatase, γ-GT, SGOT and SGPT used by the two chemical laboratories differed, the concentrations are expressed as a multiple of the upper limit of normal. The most striking features were the high elevation of serum alkaline phosphatase and IgM; only a minority of the patients (17%) exhibited jaundice.

Detailed information about the incidence and nature of side-effects during the one-year period of treatment is provided in Table II. In both groups most of the side-effects, especially nausea and vomiting,

developed during the 3 month introduction period; the loss of taste only occurred in the D-penicillamine group. Finally, the high incidence of side-effects in the placebo group and the subsequent withdrawal of four patients are to be noted. Discontinuation of drug therapy was necessary in two instances in the D-penicillamine group (gastro-intestinal disturbances and tuberculosis plus vaginal neoplasm) and in four cases in the placebo group (gastro-intestinal symptoms, arthralgia, immune complex pneumonitis and cutaneous lesions plus arthralgia).

The effects of drug therapy are summarized in Tables II and III. Fifty percent of the patients treated with either D-penicillamine or the placebo experienced relief of fatigue and/or pruritus after six months. After discontinuation of treatment at 1 year, the beneficial effect disappeared in both groups. None of the patients developed liver failure or died during the 18-month observation period.

Some biochemical measurements, such as alkaline phosphatase and SGOT, showed improvement during the first six-month period when the dose of D-penicillamine was relatively high; these changes, however, were not statistically significant and disappeared in the following treatment period when the dosage was reduced. Serum IgM showed a statistically significant drop in the first six-month period of D-penicillamine therapy. After one year of treatment the median value was still lower than that at the beginning, but the difference was no longer statistically significant. In contrast, the serum IgG concentration remained depressed during D-penicillamine therapy, and increased significantly after cessation of the drug. The titre of antimitochondrial antibodies did not change significantly during the observation period, but increased after discontinuation of

D-penicillamine. The Clq binding did not change. The hepatic copper concentration showed a statistically significant decrease within six months and remained at a relatively low level during further follow-up.

Biochemical and immunological determinations for the patients treated with the placebo were rather stable with the exception of a small but significant increase in serum IgG.

Two or more liver biopsies were obtained from twenty patients (ten from each group). Statistically significant histological improvement following D-penicillamine therapy was not observed. In only one case, a patient with initially mild abnormalities, was the decrease in fibrosis and inflammation striking; another patient showed rapid progression with development of cirrhosis after discontinuation of D-penicillamine therapy. In all other patients the disease was stable or slowly progressive, comparable to the the course noted in the patients treated with the placebo.

Discussion

Preliminary results of D-penicillamine therapy in PBC have recently been reported (14-16). The incidence of side-effects has been particularly disturbing. Withdrawal of D-penicillamine was necessary in seven out of fifty cases when the dose was increased to 1000 mg daily within 6 weeks; withdrawal was 30% when this same dosage was reached within three weeks. As a result of experience with the drug in rheumatoid arthritis (9), D-penicillamine was introduced even more slowly in our study: 1000 mg daily was reached in 12 weeks. In addition, the maintenance dose was decreased to 500 mg after six months in order to avoid the serious side-effects often observed during follow-up (9). In this

respect it was noted that although the side-effects were not suppressed, they were often mild in nature; 2 out of 11 patients on D-penicillamine therapy had to withdraw from the trial (18%). The incidence of withdrawal in this study should be assessed against the high incidence of discontinuation of treatment for patients treated with the placebo (31%). Fear of severe D-penicillamine toxicity on the part of both the patient and the medical staff involved in the trial could be responsible for this latter observation.

The effect of D-penicillamine on symptomatology has never been mentioned in previous reports (14-16). In our study improvement was equal in both treatment groups. The mild improvement in fatigue or pruritus was observed only during the period of medication and was therefore predominantly due to a "placebo effect".

The two initial studies showed a beneficial effect on liver tests associated with a decrease in hepatic copper (14-16). Others found in 56 patients only a decrease in serum IgM and hepatic copper (17), comparable with the findings of this present study. Our study suggests that the effect of D-penicillamine is dose-related. A dose of 1000 mg daily was associated with an improvement in biochemical determinations within six months but, with the exception of hepatic copper, these changes reversed again during the lower maintenance dose.

Improvement in liver tests may be a reflection of decreased levels of hepatic copper or reduction of immunological abnormalities. Our observations support the hypothesis that liver damage is associated with immunological abnormalities rather than copper accumulation, since the drop in hepatic copper was sustained during reactivation of the liver disease. The action of D-penicillamine is probably multi-

factorial (18). By chelation of copper it forms a complex which possesses superoxide dismutase activity and decomposes radicals produced by the phagocytes. By this mechanism, D-penicillamine might have a beneficial effect in inflammatory conditions. In addition, D-penicillamine interferes with carbonyl groups and thus with collagen cross-linking.

This study was not designed to study the effect of D-penicillamine treatment on survival. A recent study (19) reports a significant improvement for patients with PBC stage III or IV, while potential lethal side effect should preclude its use in stage I and II. Since our study showed that the small beneficial effect is only observed with a relatively high dose of D-penicillamine, and that this effect is likely to be related to its anti-inflammatory action, combination of a low dose of D-penicillamine with another anti-inflammatory or immunosuppressive agent might have an increased therapeutic benefit-risk ratio. In a small pilot study, combined therapy with low doses D-penicillamine and prednisone was associated with marked symptomatic and biochemical improvement and excellent tolerance (20), and this approach needs further controlled investigations.

In conclusion, slow introduction of D-penicillamine is well-tolerated by most patients, but in PBC a relatively high dose of this drug may cause only mild overall improvement. Since the beneficial effect is likely to be related to its immunosuppressive action, combination of D-penicillamine with another immunosuppressive agent needs further investigation.

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Table I. Initial features of patients with PBC according to treatment groups.

		D-penicillamine (N=11)	Placebo (N=13)
general:	sex, number of females	10	13
	age, years	51 (39-60)	48 (40-64)
	duration of disease, years	2 (1-3)	3 (1-7)
symptoms:	pruritus, intermittent or continuous	10	11
	fatigue, mild or severe	7	10
	pain around liver	6	5
	arthralgia	5	6
signs:	jaundice	2	2
	xanthelasma	3	0
	hepatomegaly	1	5
	splenomegaly	1	3
	Schirmer test < 10 mm	7	5
histology:	cirrhosis	1	3
	fibrosis none or mild	3	5
	moderate or severe	7	5
	granuloma	8	6
	piecemeal necrosis none or mild	7	10
	moderate or severe	4	3

			median (range)	median (range)+
biochemistry:	bilirubin	umol/l	20 (6 - 140)	21 (11 - 68)
	bile acids	umol/l	14 (5 - 99)	17 (3 - 99)
	alk. phosph.	xN*	4.4 (2.3 - 9.9)	4.7 (2.0 - 9.9)
	γ-GT	xN	10.6 (2.0 - 19.9)	15.0 (3.6 - 19.9)
	SGOT	xN	2.9 (1.5 - 5.2)	3.4 (1.7 - 5.0)
	SGPT	xN	3.3 (1.9 - 6.8)	4.1 (2.2 - 9.8)
	albumin	g/l	45 (38 - 55)	43 (35 - 50)
	α-globulin	g/l	16 (11 - 20)	15 (10 - 24)
immunology:	IgM	g/l	6.5 (2.9 - 13.2)	4.9 (2.1 - 9.9)
	IgG	g/l	16 (11 - 20)	14 (11 - 35)
	cryo IgM±	mg/l	11 (6 - 228)	9 (4 - 71)
	^{125}I-C1q±	%	13 (2 - 40)	17 (5 - 25)

x N is the upper limit of normal, defined as the 95th percentile of the normal population of the chemical laboratory.

+ No significant differences were found between the patients treated with D-penicillamine or the placebo according to the unpaired Wilcoxon test ($p > 0.05$).

± For nine patients of the D-penicillamine group and seven patients of the placebo group (see text).

Table II. Frequency of side-effects and subjective effect on symptoms for patients with PBC according to treatment group.

	D-penicillamine (n=11)					Placebo (n=13)				
	frequency				total number with side-effects					total
month	3	6	12	18		3	6	12	18	
dosage, mg	750	1000	500	0						
side-effects										
exanthema	1	2	0	0	2	2	1	0	0	2
gastrointestinal	5	2(1)	0	0	5(1)	3(1)	2	0	0	3(1)
renal or haemapoietic	0	0	0	0	0	0	0	0	0	0
other	0	1(1)	0	0	1(1)	2(2)	1(1)	0	0	3(3)
symptoms*										
improved	-	4	5	0		-	5	3	2	
worsened	-	1	0	3		-	1	0	2	

* Not investigated systematically at three months.

In parentheses the number of patients withdrawn from the trial.

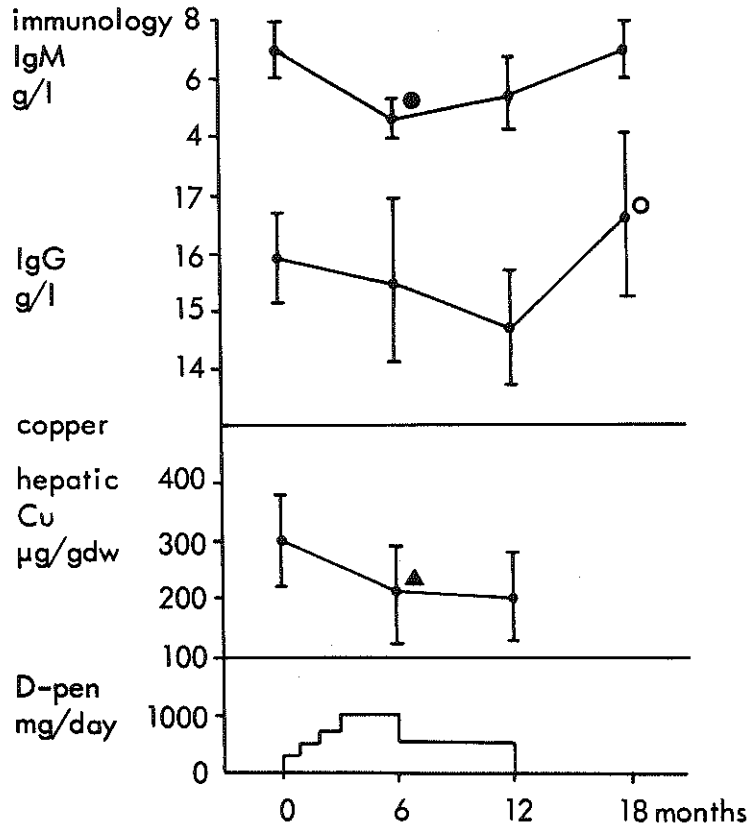
Table III. Biochemical features (median values) of patients with PBC according to treatment group.

		D-penicillamine N=11				Placebo N=13			
month		0	6	12	18	0	6	12	18
dosage	mg	0	1000	500	0	0	1000	500	0
bilirubin	umol/l	20	16	14	23	21	23	23	23
bile acids	umol/l	10	-	14.5	-	17	-	23	-
alk. phosph.	xN*	4.4	4.2	5.0	5.3	4.7	4.6	4.4	4.3
SGOT	xN	2.9	2.4	2.9	3.2	3.4	3.1	3.2	3.5
SGPT	xN	3.3	-	2.5	4.1	4.1	-	4.3	4.8
albumin	g/l	45	46	44	43	43	44	43	44
γ- globulin	g/l	16	-	15	-	15	-	14	-

* N is the upper limit of normal, defined as the 95 percentile of the normal population of the chemical laboratory.

Legend to the figure.

Immunological features and hepatic copper in patients with PBC during treatment with D-penicillamine. Serum IgM ● decreased significantly within 6 months, but rose again during maintenance dose, while the lower hepatic copper concentration ▲ remained lower. Insufficient data were available for the 18th month. After therapy an increase in serum IgG o was found. Data are represented as the mean and the standard error of the mean.


immunology
IgM
g/l
8
6
4
IgG
g/l
17
16
15
14
copper
hepatic
Cu
µg/gdw
400
300
200
100
D-pen
mg/day
1000
0
0
6
12
18 months

(to be published in part in Zeitschrift fur Gastroenterologie)

7

TREATMENT OF PRIMARY BILIARY CIRRHOSIS : A COMPARISON OF D-PENICILLAMINE D-PENICILLAMINE PLUS PREDNISONE AND A PLACEBO.

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Running title: D-penicillamine with prednisone in PBC

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SUMMARY

Auto-immune responses, copper accumulation in the liver and, recently, immune complexes have been considered as pathogenetic factors in primary biliary cirrhosis (PBC). D-penicillamine, which may influence each of the abnormalities, has some beneficial effect but side-effects often appear. Although prednisone is considered to be of no value and is contra-indicated because of aggravation of osteodystrophy, it has never been evaluated properly and the benefits of a low-dose combination of D-penicillamine and prednisone could outweigh the risks of this therapy.

Nine patients with PBC in various histological stages received low-dose combination therapy consisting of 250 mg D-penicillamine and 10 mg prednisone daily, and were compared with patients treated at random with either a placebo (n=7) or high doses of D-penicillamine alone (n=9). Symptomatic improvement(of fatigue and/or pruritus) was observed in 8 out of 9 patients during low-dose combination therapy and in only a minority of the patients treated otherwise. No side-effects were seen during combination therapy, in contrast to a 40% incidence of side-effects during treatment with D-penicillamine. Treatment with low-doses of D-penicillamine and prednisone was also associated with significant biochemical and immunological improvement, which was not found for our small group of patients treated with D-penicillamine alone. Further investigation of the long-term effects of this combination therapeutic regimen is needed.

Keywords: primary biliary cirrhosis, treatment, D-penicillamine

INTRODUCTION

The etiology of primary biliary cirrhosis (PBC) is unknown, but immunological abnormalities may play an important role in its pathogenesis. On the basis of the hypothesis that PBC is an auto-immune disease, various forms of immunosuppressive therapy have been tried during the past two decades.

Azathioprine was tested extensively in two double-blind trials comprising 281 patients (1,2) ; no clear benefit could be demonstrated. D-penicillamine is now being evaluated in several clinical trials (3,4) involving at least 132 patients. Some improvement in liver tests and possibly in survival has been reported; the drug, however, has little effect on symptoms and is frequently associated with side-effects.

Corticosteroids, however, have never been evaluated properly in controlled trials. Only fifteen patients on prednisone therapy were described between 1950 and 1966 (5,6). Symptomatic relief was found in association with improvement in the alkaline phosphatase level, but the drug was later condemned because of aggravation of osteodystrophy in advanced disease (7,8).

Today, many patients with PBC are being diagnosed when the disease is still in the noncirrhotic stage (chronic cholangiolytic hepatitis); the immunological features, including antimitochondrial antibodies, elevated serum immunoglobulin M and associated auto-immune disease, are then often quite marked. Histological examination sometimes reveals overlap with chronic active hepatitis (CAH). Several patients with PBC had received prednisone because of presumed CAH, but this was discontinued upon referral to our centre; the withdrawal of prednisone was subsequently associated with both clinical and biochemical reactivation of the disease. On the basis of these observations, we decided to perform a pilot study to evaluate the effect of prednisone when given together with D-penicillamine (low-dose combination therapy).

MATERIALS AND METHODS

The diagnostic criteria for PBC were an elevated serum alkaline phosphatase level (at least twice the upper limit of normal), a positive antimitochondrial antibody test, an elevated serum IgM concentration, a liver biopsy showing portal lymphoplasmocellular infiltration with destruction of interlobular bile ducts with or without granulomas, and no abnormalities on the cholangiogram of the extrahepatic bile ducts.

After randomisation, sixteen consecutive symptomatic patients with PBC received either the placebo or D-penicillamine in an increasing monthly dosage of 250 - 1000 mg daily for six months, followed by 500 mg daily for another six months. After a third six-month period without therapy, the first seven patients (4 of the placebo group, 3 of the D-penicillamine group) and two new patients were treated with a combination of 250 mg D-penicillamine and 10 mg prednisone daily.

Symptoms were evaluated according to the severity of fatigue and pruritus. The presence of fatigue was scored as none, mild or severe (inability to perform normal daily work), while pruritus was absent, intermittent or continuous(requiring cholestyramine therapy).

Serum bilirubin and alkaline phosphatase were measured with the Technicon SMA12-60, while serum glutamic oxaloacetic transaminase (SGOT or serum aspartate aminotransferase) was determined by means of the LKB Reaction Rata Analyzer (UV kinetic measurement). Immunoglobulin M was measured by the radial immunodiffusion technique using Partigen ^{R} immunoplates (Behringwerke, Marburg, F.R.G.).

For statistical analysis of the data before and after therapy the paired Wilcoxon test was used; the initial features of the three groups were tested with the unpaired Wilcoxon test. Differences were considered significant when the p-value was less than 0.05.

RESULTS

The placebo group consisted of 7 women with a median age of 47 years (range 41-56 years), the D-penicillamine group comprised 8 women and 1 man (median age 51 years, range 39-60) and the D-penicillamine plus prednisone group 9 women (median age 48 years, range 32-56).

The three groups were comparable as far as all initial features at the start of treatment were concerned. Detailed information on symptoms, liver tests and serum IgM are given in table I. Serum IgM was lower in the group receiving combined therapy, but the difference with respect to the other groups was not statistically significant. Histological evaluation showed that all stages of PBC were distributed among the three treatment groups.

The results, summarized in table I, show that the combination of D-penicillamine and prednisone is superior to either D-pencillamine alone or placebo with regard to improvement in biochemical measurements. In addition, the symptomatic improvement with the combined therapy was often impressive. In 8 of the 9 patients symptoms decreased, they even disappeared in 5 patients. In both the placebo and the D-penicillamine group four patients improved, while only two patients treated with D-penicillamine had no symptoms at all after treatment. No side-effects of the combined therapy were observed; in contrast D-penicillamine alone was associated with side-effects of varying severity in four out of nine patients.

DISCUSSION

Recently, we completed a controlled study in two centres, comparing D-penicillamine with a placebo for patients with primary biliary cirrhosis.

Treatment with D-penicillamine did not lead to significant improvement in either symptoms or biochemical measurements, although a significant decrease in serum IgM was observed (9). In the table only the findings for the patients treated in our centre are given.

In this pilot study, the beneficial effect of the combination of prednisone and D-penicillamine, both in low doses, on symptoms was remarkable: severe fatigue diminished rapidly and the patients were often able to recommence normal daily activity. Pruritus also decreased. The relief of symptoms was accompanied by statistically significant biochemical improvements. During treatment with D-penicillamine alone (up to 1000 mg daily) only a significant decrease in serum IgM was noted; there was little improvement in either symptoms or biochemical measurements.

Although none of the patients were in an advanced cirrhotic stage of the disease, many of the liver biopsies showed extensive fibrosis with the formation of septa; these results therefore do not apply exclusively for the early stage of PBC. We did not attempt to evaluate the effect of treatment on liver histology, since the treatment period was considered too short to influence the histology.

Aggravation of osteodystrophy is probably the major contra-indication for long-term coricosteroid therapy for this disease (7,8); recent advances in diagnosis and treatment of osteodystrophy, however, may eventually allow long-term low dose prednisone therapy for selected patients with PBC. Further controlled investigation of the combination of prednisone and D-penicillamine is urgently needed.

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Table I.: Influence of three types of treatment on symptoms as well as biochemical and immunological data in primary biliary cirrhosis.

treatment group		fatigue mild+severe	pruritus interm+cont	bilirubin N*<12 umol/l median(range)	alk.phosph. N*<45 U/l median(range)	SGOT N*<30 U/l median(range)	IgM N*< 2.8 g/l median(range)
Placebo (n=7)	before+	6	5	24 (14-70)	212 (125-543)	102 (50-199)	4.8(2.6-15.7)
	after 6 months	2	6	18 (11-68)	209 (120-530)	126 (63-277)	4.9(3.1-16.0)
D-penicil-lamine (n=9)	before+	6	8	18 (6-140)	203 (96-365)	83 (46-117)	6.4(3.2-13.2)
	after 6 months	3	6	13 (6-142)	199 (94-475)	67 (45-256)	4.0X(2.3-8.3)
D-penicil-lamine and prednisone (n=9)	before+	8	6	16 (9-558)	240 (95-558)	93 (61-162)	8.9(3.2-12.8)
	after 6 months	1	4	9X(3-268)	139X(46-581)	100 (29-136)	5.1X(1.6-8.0)

* N is defined as the 95 percentile of the normal population of the routine clinical chemistry laboratory

+ No significant differences were found for initial features between the three groups

X Significantly decreased after 6 months according to the Wilcoxon test ($p < 0.05$).

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