Immunomorphological characterisation of antinuclear antibodies in chronic liver disease

FABIO CASSANI, FRANCESCO BIANCO BIANCHI, MARCO LENZI,
UMBERTO VOLTA, EMILIO PISI

From the Istituto di Patologia Medica I, Università di Bologna, Policlinico S Orsola, 40138 Bologna, Italy

SUMMARY Two immunofluorescence procedures to evaluate antinuclear antibodies were compared in a series of 221 patients with chronic liver disorders of various aetiologies. The use of HEp-2 cells allowed us to discriminate with more confidence between the homogeneous and speckled patterns, to show the presence of associated patterns in the same serum, and, above all, to identify two specificities, unrecognisable on tissue sections. The anticentromere antibody was found in 10% of cases of primary biliary cirrhosis and occasionally in other conditions; the antibody staining multiple nuclear dots was strictly confined to primary biliary cirrhosis (17%). With the exception of autoimmune chronic active hepatitis the prevalence of antinuclear antibodies increased in all groups, particularly in primary biliary cirrhosis. Homogeneous antinuclear antibody was associated by both immunofluorescence procedures with autoimmune chronic active hepatitis. The multiple nuclear dot antinuclear antibody turned out to be an additional marker of primary biliary cirrhosis, helpful for the positive diagnosis of primary biliary cirrhosis in a proportion of cases negative for antimitochondrial antibody. Absorption experiments showed that multiple nuclear dot and antimitochondrial antibody are antigenically distinct. Moreover, multiple nuclear dot antinuclear antibody was associated with the finding of a dry Schirmer's test.

Antinuclear antibodies are found in the serum of patients with chronic liver disorders. The method routinely used to detect them is the indirect immunofluorescence procedure with frozen tissue sections as substrate, which also allows recognition of different patterns of positivity. The diagnostic relevance of the antinuclear antibodies detected by this method is confined to the identification of the autoimmune subgroup of chronic active hepatitis, where antinuclear antibodies, mainly giving a homogeneous staining pattern,¹ are to be found at a high titre (1/20-1/80).

It is now known from rheumatological studies that a more detailed characterisation of antinuclear antibody patterns can be achieved and new patterns have been described by the use of immunofluorescence on a substrate of dividing cells such as the HEp-2 cell line, instead of tissue sections. Recently, immunofluorescence on HEp-2 cells was applied to serum samples from patients with chronic liver disorders: the most striking findings were in primary biliary cirrhosis,^{2,4} a prototype autoimmune disease.^{5,6} In addition to M₂-antimitochondrial anti-

bodies,⁷ two new antinuclear antibody specificities were identified in primary biliary cirrhosis: the anticentromere antibody, already known as a marker of the CREST variety of scleroderma,² and antibody giving a pattern described as multiple nuclear dots³ or atypical discrete speckled staining.⁴

We report a comparison of immunofluorescence on tissue sections and on HEp-2 cells in a large series of Italian patients with chronic liver disease of various aetiologies. The clinical and diagnostic implications of new antinuclear antibody specificities are discussed.

Material and methods

PATIENTS

Two hundred and seventy one patients with chronic liver disease of various aetiologies (Table 1) were studied. The diagnoses were made by internationally accepted clinical, serological, and histological criteria.⁸ Of the patients with primary biliary cirrhosis, 71 (86%) were women; in 22 of them Schirmer's test for tear secretion was carried out. Patients with chronic active hepatitis who were positive for liver kidney microsomal antibody were

negative for all serological markers of hepatitis B virus. Autoimmune chronic active hepatitis was defined by the detection of serum antinuclear antibody or smooth muscle antibody, or both at a titre equal to or higher than 1/40 in the absence of any other cause of the liver disease. Cryptogenic chronic active hepatitis cases had no serological markers of hepatitis B virus infection, no antinuclear antibodies or smooth muscle antibodies, and no history of alcohol abuse. Patients with alcoholic liver disease had alcohol intakes of more than 150 ml/day and were negative for hepatitis B virus markers. Of the 25 patients with hepatitis B surface antigen (HBsAg) positive chronic active hepatitis, 14 were positive for the hepatitis B e antigen and 11 for the corresponding antibody. Among patients with liver kidney microsomal antibody, autoimmune, cryptogenic, and HBsAg positive chronic active hepatitis the numbers and percentages of women were as follows: 12 (60%), 25 (81%), 13 (68%), and 7 (28%), respectively. Twenty three (53%) of the patients with alcoholic liver disease were women.

INDIRECT IMMUNOFLUORESCENCE

Immunofluorescence studies using cryostat sections of rat liver, kidney, and stomach and commercially available HEP-2 cells preparations (Kallestad) were performed according to standard procedures.⁹ Serum samples were stored at -20°C until used and tested at a 1/40 dilution. A fluorescein isothiocyanate conjugated sheep antihuman F(ab)₂ (Wellcome) was used as second antibody. Slides were read blindly by two independent observers under a Leitz Orthoplan microscope with vertical illumination. Staining patterns were defined according to Bernstein *et al.*³

ABSORPTION EXPERIMENTS

Eight serum samples from patients with primary biliary cirrhosis, three positive for anticentromere antibody and five for multiple nuclear dot antibodies, were absorbed at 1/40 dilution with a rat liver mitochondrial fraction at a protein concentration¹⁰ of 5 mg/ml. This fraction was prepared and sonicated according to Sayers *et al.*¹¹ Absorption

Table 1 Results of antinuclear antibody characterisation by immunofluorescence on tissue sections and HEP-2 cells in chronic liver disease (serum dilution = 1/40).

	Primary biliary cirrhosis (83)	Chronic active hepatitis					Controls (50)
		Liver kidney microsomal antibody positive (20)	Autoimmune (31)	Cryptogenic (19)	HBsAg positive (25)	Alcoholic liver disease (43)	
Immunofluorescence on tissue sections							
Antinuclear antibody positive (all patterns)	21 (25%)	4 (20%)	25 (81%)	—	3 (12%)	12 (28%)	4 (8%)
Speckled	20 (24%)	3 (15%)	18 (58%)	—	3 (12%)	9 (21%)	3 (6%)
Homogeneous	—	—	6 (19%)	—	—	1 (2%)	—
Nucleolar	—	1 (5%)	—	—	—	2 (5%)	1 (2%)
Peripheral*	1 (1%)	—	1 (3%)	—	—	1 (2%)†	—
Antimitochondrial antibody positive	69 (83%)	—	—	—	—	—	—
Immunofluorescence on HEp-2 cells							
Antinuclear antibody positive (all patterns)	48 (58%)	5 (25%)	25 (81%)	5 (26%)	8 (32%)	20 (47%)	6 (12%)
Speckled	28 (34%)‡	1 (5%)	15 (48%)**	3 (16%)††	6 (24%)§§	16 (37%)‡‡	5 (10%)
Homogeneous	2 (2%)§	1 (5%)¶	8 (26%)	—	1 (4%)	1 (2%)	—
Nucleolar	10 (12%)	3 (15%)	4 (13%)	3 (16%)	4 (16%)	4 (9%)	3 (6%)
Anticentromere antibody	8 (10%)	1 (5%)·	1 (3%)	—	—	1 (2%)	—
Multiple nuclear dot	14 (17%)	—	—	—	—	—	—

*Never detected on HEP-2 cells.

†Associated with nucleolar staining.

‡Associated with nucleolar in seven and with multiple nuclear dots in five samples.

§Associated with multiple nuclear dots in one sample.

¶Associated with nucleolar staining in one sample.

||Associated with anticentromere antibody.

**Associated with nucleolar staining in three samples.

††Associated with nucleolar staining in one sample.

‡‡Associated with nucleolar staining in two samples.

§§Associated with nucleolar staining in three samples.

||Associated with nucleolar staining in two samples.

tests were carried out at 4°C overnight.

STATISTICS

Statistical analysis was performed using Fisher's exact test and the χ^2 test with Yates' correction.

Results

The results of the immunofluorescence studies are shown in Table 1. On tissue sections antimitochondrial antibody was found only in serum samples from patients with primary biliary cirrhosis (83%). Antinuclear antibody was found in 81% of autoimmune chronic active hepatitis samples, but much less often (12%–28%) in the other conditions. Among the antinuclear antibody patterns, speckled staining was the commonest in all groups; homogeneous staining, though not common, was associated with autoimmune chronic active hepatitis ($p < 0.001$, χ^2 test). In some cases it was difficult to distinguish between the speckled and homogeneous patterns.

With the HEP-2 cells as substrate for immunofluorescence, the frequency of antinuclear antibody positive serum samples increased in all groups except autoimmune chronic active hepatitis. In particular, 58% of primary biliary cirrhosis samples were antinuclear antibody positive, chiefly because of the recognition of two antinuclear antibody patterns not detected on tissue sections: anticentromere antibody and multiple nuclear dot antinuclear antibody. Anticentromere antibody was found in eight primary biliary cirrhosis samples (10%), of which seven were antimitochondrial antibody positive, and in one case each of alcoholic liver disease, and autoimmune and liver kidney microsomal antibody positive chronic active hepatitis. Multiple nuclear dot staining was confined to primary biliary cirrhosis, where it occurred in 14 cases (17%), of which two were antimitochondrial antibody negative. Among the other antinuclear anti-

body patterns the homogeneous pattern was again associated with autoimmune chronic active hepatitis ($p < 0.001$, χ^2 test).

A comparison of immunofluorescence patterns on frozen tissue sections and HEP-2 cells in primary biliary cirrhosis and autoimmune chronic active hepatitis cases is given in Table 2. These two conditions were selected because of the high prevalence of antinuclear antibody, displaying the whole range of relevant patterns. From its analysis it appears that 15 of the 23 anticentromere antibody and multiple nuclear dot positive samples were negative, and the remaining eight were scored as speckled on tissue sections. Of the 10 samples giving a homogeneous pattern on HEP-2 cells, five showed a speckled and three a homogeneous staining and two were negative on tissue sections. Moreover, three of the six homogeneous antinuclear antibody positive samples on tissue sections turned out to give a speckled pattern on HEP-2 cells.

The titre of anticentromere antibody and multiple nuclear dot staining was unaffected by preabsorption of the serum with sonicated mitochondrial fraction, whereas the cytoplasmic staining seen with most primary biliary cirrhosis sera samples was always affected.

Tear secretion was reduced in eight of the 22 primary biliary cirrhosis patients tested (36%). Multiple nuclear dot antinuclear antibody occurred in four (50%) of these eight patients, but in only one (7%) of the 14 patients without sicca syndrome ($p < 0.01$, Fischer's exact test).

Discussion

Immunological tests have an established place in the classification and differential diagnosis of chronic liver disease. Besides M_2 -antimitochondrial antibody, found in primary biliary cirrhosis, and liver kidney microsomal antibody, which identifies a sub-

Table 2 Comparison of antinuclear antibody patterns detected by immunofluorescence on tissue sections and HEP-2 cells in primary biliary cirrhosis and autoimmune chronic active hepatitis serum samples (diluted 1/40).

		Immunofluorescence on tissue sections			
		Speckled (38)	Homogeneous (6)	Peripheral (2)	Antinuclear antibody negative (68)
Immunofluorescence on HEP-2 cells	Speckled	24	3	2	14
	Homogeneous	5	3	—	2
	Nucleolar	8	—	—	6
	Anticentromere antibody	3	—	—	6
	Multiple nuclear dot	5	—	—	9
	Antinuclear antibody negative	3	—	—	38

For associations of patterns see footnotes to Table 1.

set of chronic active hepatitis, smooth muscle antibody and antinuclear antibody are generally regarded as markers of the autoimmune form of chronic active hepatitis.¹² This study, and two previous ones,^{3,4} show that by using HEP-2 cells as immunofluorescence substrate antinuclear antibody can be detected much more frequently in chronic liver disorders. In primary biliary cirrhosis this is largely due to the detection of two new specificities: anticentromere antibody and multiple nuclear dot antinuclear antibody. Additional advantages of HEP-2 cells are that the homogeneous pattern may be differentiated with much more confidence from the speckled one on the basis of mitotic cells chromatin positivity and that different patterns, occurring simultaneously in the same serum, are individually recognised. Such considerations may not apply to all cell lines at present used as immunofluorescence substrate. In a recent report on antinuclear antibody characterisation in liver disease by immunofluorescence, using human embryonic fibroblasts as a substrate,¹³ no anticentromere antibody was found, and a peripheral nuclear staining, similar to that found on tissue sections but never detected to our knowledge on HEP-2 cells, was reported. Although the small number of primary biliary cirrhosis serum samples tested could partially explain such discrepancies, a different expression of antigenic nuclear components in different substrates cannot be ruled out.

Using immunofluorescence on both tissue sections and HEP-2 cells, the homogeneous pattern was associated with autoimmune chronic active hepatitis. No correlation, however, was found between homogeneous antinuclear antibody positive cases detected on tissue sections and HEP-2 cells. This further suggests that antinuclear antibody patterns detected on tissue sections must be confirmed on HEP-2 cells. The low prevalence of antinuclear antibody in liver kidney microsomal antibody positive chronic active hepatitis¹⁴ was confirmed on tissue sections and was only slightly increased by testing on HEP-2 cells, where a centromere positive case was detected. The overall increased sensitivity of antinuclear antibody testing recorded in our series (from 29% on tissue sections to 50% on HEP-2 cells) was associated with a negligible decrease of specificity (antinuclear antibody prevalence in controls from 8% on tissue sections to 12% on HEP-2 cells).

It has already been shown that anticentromere antibody identifies primary biliary cirrhosis cases with elements of the CREST syndrome.² We found anticentromere antibody in 10% of primary biliary cirrhosis serum samples and occasionally in other chronic liver disorders, but the features of

scleroderma were not reviewed in our study. As in Bernstein's study³ multiple nuclear dot antinuclear antibody on HEP-2 cells seems to be highly specific for primary biliary cirrhosis and can be considered a serological marker of this disease, albeit a much less sensitive one than antimitochondrial antibody. The multiple nuclear dot pattern was given by two of the 14 antimitochondrial antibody negative primary biliary cirrhosis serum samples included in this study. Since primary biliary cirrhosis can be difficult to diagnose in the absence of antimitochondrial antibody, the finding of multiple nuclear dot antinuclear antibody may be helpful in such cases.⁸ It remains to be seen whether patients with multiple nuclear dot antinuclear antibody become antimitochondrial antibody positive later. Our absorption studies show that multiple nuclear dot antinuclear antibody and antimitochondrial antibody are antigenically distinct. In terms of clinical relevance this study confirms that multiple nuclear dot antinuclear antibody is associated with the sicca syndrome in primary biliary cirrhosis.

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Requests for reprints to: Professor FB Bianchi, Istituto di Patologia Medica I, Policlinico S. Orsola, Via Massarenti, 9, 40138 Bologna, Italy.

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