Clinical significance of autoantibodies directed against nuclear and cytoplasmic antigens in autoimmune liver disease

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ABSTRACT

Previous research carried out in the area of autoimmune liver disease and autoantibody specificity has not been conclusive, and correlation between the two in most situations remains unclear. This study aims to facilitate a consensus on these autoantibodies in an unselected patient population and their relationship to autoimmune liver disease (AILD). The study detected two autoantibodies that show reasonable specificity for primary biliary cirrhosis (PBC), anti-sp100 and anti-gp210, and it may be clinically useful to report any antimitochondrial antibody (AMA) detected, as this may be a sign of very early PBC. The inter-methodological differences in assays available for detection of the autoantibodies were also noted. Care must be taken when selecting methods to detect these autoantibodies.

KEY WORDS: Autoantibodies.
Autoantigens.
Hepatitis, autoimmune.

Introduction

Previous research has resulted in conflicting opinions on the relationship between autoimmune liver disease (AILD) and autoantibody specificity; this has not been conclusive and correlation in most situations remains unclear. Some studies report a correlation between antibodies to sp100 and primary biliary cirrhosis (PBC),[1] systemic lupus erythematosus (SLE) and collagen disease,[2] while others have reported that antibodies to sp100 are highly specific for PBC.[2–6] Many reports suggest that antibodies to gp210 are found in patients with PBC, but the prevalence ranges from 9.5% to 41%.[7–11]

The current literature is confusing, with different studies concluding opposite findings. It should be noted that none of these studies have addressed the frequency and significance of these antibodies outside the context of preselected disease groups, and so no comment can be made about their significance in an unselected patient group.

This study aims to facilitate consensus on the importance of these autoantibodies in an unselected patient population, and their relationship to AILD, by identifying positive sera for a number of different antibodies associated with AILD, and establishing the autoantigen involved. The autoantibodies and clinical diagnoses will be studied to determine any correlation, ultimately attempting to conclude if there is a valid clinical reason for detecting and reporting these antibodies.

Materials and methods

Over the course of 15 months, 22,298 samples were tested for antinuclear antibody (ANA) on HEp-2 cells in the rheumatology laboratory at Charing Cross Hospital, London. Sera were selected following indirect immunofluorescence (IIF) on HEp-2 cells using the criteria for ANA patterns according to the European consensus document. Samples positive for multiple nuclear dot/nuclear dot (MND/ND; n=21), punctate nuclear membrane/smooth nuclear membrane (PNM/SNM; n=6), antimitochondrial-like (AMA-like; n=52) and cytoplasmic filament staining (n=21) were identified and selected for further evaluation to identify antibody specificity and clinical disease. For comparative studies, sera from PBC (n=5), SLE (n=50), rheumatoid arthritis (n=50) and healthy blood donors (n=50) were also obtained. Of the 100 serum samples, 67 patient clinical notes were obtained and examined as part of an audit carried out in immunology. The specificity, sensitivity, positive predictive value (PPV) and negative predictive value (NPV) were determined using 2x2 contingency tables for the autoantibodies detected on enzyme-linked immunosorbent assay (ELISA), immunoblot and liver/kidney/stomach (LKS) slides.

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Indirect immunofluorescence: HEp-2 ANA
Sera were screened for ANA using commercial HEp-2 slides (DiaSorin, Italy) at a 1:80 dilution. The secondary antibody used was a fluorescein isothiocyanate (FITC)-conjugated polyclonal rabbit anti-rabbit IgG (Dako, Denmark). The patterns were observed under a fluorescence microscope (Nikon, UK) and designated according to the European consensus document.

Indirect immunofluorescence: LKS
Sera were tested on rat LKS slides (The Binding Site, UK) at

a dilution of 1:40. The secondary antibody used was an FITC polyclonal rabbit anti-human IgG (Dako, Denmark). The patterns were observed under a fluorescence microscope and designated according to standard guidelines.[12]

Enzyme-linked immunosorbent assay
Sera selected on the basis of ANA pattern were evaluated using an ELISA method (IMTEC, Germany). The wells of the microtitre plate were coated in a synthetic peptide representing the immunodominant region of cytochrome P450-2D6, gp210, recombinant sp100 and lamin B receptor (LBR) antigens and native M2 antigen (pyruvate dehydrogenase). Any antibodies directed against the antigens present in the serum were bound to the microtitre plate. Bound antibody was detected by adding a secondary antibody (horseradish peroxidase [HRP]-labelled anti-human IgG) which hydrolyses a chromogenic substrate to give a colour change. Acid was used to stop the reaction and the intensity of colour was measured photometrically. The level of colour is directly proportional to the amount of antibody present in the serum. Samples revealing an AMA pattern on IIF were tested for anticardiolipin antibodies (ACA) using a commercially available ELISA (Orgentec, Germany)

Immunoblotting
All sera were tested on anti-liver antigen immunoblot (Euroline-WB ([gG] Euroimmun, Germany) at a dilution of 1:51. The test strips contained separated extracts of primate liver, M2 antigen, liver cytosol (LC-1), liver/kidney microsomal (LKM-1), cytokeratin, glutathione-S-transferase (GST), and a line of recombinant soluble liver antigen/liver pancreas antigen (SLA/LP). Any antibodies present in the sera bound to one or more of the antigens on the strip. Bound antibody was detected using an alkaline phosphatase labelled anti-human IgG and a chromogenic substrate which forms a complex, identified by a coloured band on the nitrocellulose. The strips were then interpreted visually following the manufacturer's guidelines.

Results

HEp-2 patterns and autoantigens
Out of the 100 sera tested, 21 had an ND/MND staining pattern on HEp-2 IIF, of which 71% had sp100 as the target autoantigen. Six sera had a PNM/SNM pattern on HEp-2. These was no correlation between the nuclear membrane group and the expected autoantigens (LBR and gp210), although one serum had anti-sp100 antibodies. Of the sera which exhibited nuclear membrane pattern fluorescence, 85% of the autoantigens were unidentified by ELISA or immunoblot.

Twenty-one sera showed cytoplasmic filament staining on HEp-2, Again, there was no correlation between these sera and anti-smooth muscle antibody (ASMA) and 90% of the autoantigens in this group were unidentified by ELISA or immunoblot. In the cytoplasmic filament group, one serum had anti-sp100 antibodies and one had anti-lamin B receptor antibodies.

Fifty-two sera had an AMA-like staining pattern on HEp-2, of which 65% were found to have antibodies to M2 by ELISA, 11.5% had antibodies to sp100, 7.6% had antibodies to gp210 and 2% had antibodies to ASMA; 33.8% of the autoantigens in the AMA-like group were unidentified. Anticardiolipin antibody (ACA) levels were tested on the positive AMA-like sera found using IIF but negative on ELISA for M2. Of these 18 sera, 16 were negative for ACA and two had positive IgM ACA (one low positive, one moderate positive).

Patterns: LKS versus HEp-2
The LKS and HEp-2 slides showed poor correlation in most of the staining patterns seen. The LKS picked up just 33% of the AMA-like patterns seen on HEp-2 cells, and 27% of the positive AMA-like sera on HEp-2 were negative on LKS. Only 9.5% of the cytoplasmic positive sera were positive for ASMA on LKS, and 38% were negative on LKS.

Immunoblot
Of the 52 AMA-like positive sera by HEp-2, 17 were anti-M2 positive by Western blot (WB); of the anti-M2 antibodies revealed by ELISA ($n=34$), only 11 were positive on WB. The blot detected five anti-M2 antibodies in the other three groups (cytoplasmic filaments, ND and NM) which had not been anti-M2 positive by any other method. Of the positive AMA-like antibodies by HEp-2, 63% were found to be negative by WB. Three sera were found to be positive for anti-LKM-1 antibodies by WB and one for GST, all of which had not been identified by other methods. Western blot did not detect any anti-LC, anti-cytokeratin or anti-SLA/LP antibodies in any of the serum samples.

Controls
In order to see if the four patterns of interest occurred in diseases other than AILD, control samples were screened on HEp-2 slides, comprising 50 sera each from rheumatoid arthritis (RA) and SLE patients, and from healthy blood donors; 8% of the RA controls gave an AMA-like staining pattern and 4% gave a cytoplasmic pattern on HEp-2. None of the other patterns of interest were observed in the RA group. In the SLE group, 4% gave an ND staining pattern and 6% gave an AMA-like pattern. None of the other patterns of interest were observed in this group. In the healthy blood donors, 4% gave an AMA-like pattern. None of the other patterns of interest were observed in this group.

Primary biliary cirrhosis group
In the PBC group, 100% of samples were found to have an AMA-like staining pattern on HEp-2 and LKS and also by ELISA. M2 was the only pattern observed on LKS in this group. One serum was found to have an ND pattern on HEp-2 and this was confirmed as having antibodies to sp100 by ELISA. The same serum also had anti-gp210 antibodies, which was not apparent on HEp-2 cells. Western blot detected three samples (60%) which had anti-M2 antibodies, and one of those also had anti-LKM-1 antibodies. Two samples (40%) were negative on WB.

Indirect immunofluorescence patterns and clinical disease
Tables 1–4 show the clinical diseases associated with the four staining patterns found in the study.

Autoantigens by ELISA and clinical disease
Of the positive M2 on ELISA, the most frequent diagnoses were PBC (25.9%), followed by SLE (14.8%). The most

frequent diagnoses seen with positive anti-sp100 antibodies were undifferentiated connective tissue disease (17.6%), RA (11.7%), Sjogren's syndrome (SS; 11.7%) and cancer (11.7%). The most frequent diagnoses for those with anti-gp210 antibodies were PBC (25%), SS (25%), cancer (25%) and non-immunological disease (25%).

Statistics

Of all the methods employed to detect antimitochondrial antibodies (M2 or non-M2) in the test sera, ELISA was the most clinically sensitive for PBC but the LKS slides proved to have the best overall clinical sensitivity and specificity, and also had the best PPV and NPV for PBC (Table 5).

Discussion

The predicted autoantigen specificity in each of the IIF patterns did not always correlate well with the specific autoantibodies detected using immunoassay, and a substantial percentage of each pattern's autoantigens were unidentified by ELISA and immunoblot.

There was quite a range of diagnoses associated with each of the autoantigens defined by IIF; for example, AMA-like pattern was seen mostly in patients with PBC (20.6%) and SLE (11.8%), and 38.2% of these patients had abnormal LFTs. The ND/MND was seen mostly in patients with RA (25%) and cancer (12.5%), and seen in one patient with PBC in the PBC control group; 31.3% of these patients had abnormal LFTs. The nuclear NM pattern was seen mostly in patients with autoimmune thyroid disease (AITD; 16.7%), but not in patients with any type of liver disease; 25% of these patients had abnormal LFTs.

The autoantigens detected by ELISA (i.e., M2, sp100 and gp210) were seen in a range of diseases; M2 was mostly seen in patients with PBC (25.9%) and SLE (14.8%). Sp100 was mostly seen in undifferentiated connective tissue disease (UCTD; 17.6%), SS, cancer and RA (all 11.7%) and in one patient in the PBC control group. gp210 was mostly seen in PBC, SS, cancer and non-immunological diseases (25% each). The immunoblot detected anti-LKM-1 antibodies, which were seen in patients with giant cell arteritis and fibromyalgia, while anti-GST antibodies were detected in patients with hyperthyroidism and newly diagnosed type 1 diabetes.

The ELISA for M2 was found to have good clinical sensitivity (91.6%) but average clinical specificity (68.3%). The NPV is good at 97.6% but the PPV was found to be only 35.5%. These figures would suggest that using this ELISA for the detection of anti-M2 antibodies in the diagnosis of PBC would be a good screening method but not a good confirmatory test (due to the low clinical specificity). The clinical sensitivity of anti-sp100 antibodies for PBC was only 16.6% but there was quite good clinical specificity (72.5%) in this study. The PPV of anti-sp100 antibodies was 10.5% but the NPV was 81.8%. The detection of anti-gp210 in PBC has good clinical specificity (95%) but only 25% clinical sensitivity. The NPV of anti-gp210 for PBC was 86.8% but the PPV was only 50%.

The immunoblot was clinically specific for the detection of anti-M2 antibodies (89.7%), but not a clinically sensitive method. The PPV was 35.5% and the NPV 89.7%. The detection of M2 and non-M2 antibodies on LKS had the best

Table 1. AMA-like pattern and related diseases.

HEp-2 pattern	Clinical disease	Frequency	%
AMA (n=34)	Abnormal LFTs	13	38.2
	PBC	7	20.6
	AIH	0	0
	Hep-C	1	2.9
	SLE	4	11.8
	RA	1	2.9
	SS	2	5.9
	Cancer	1	2.9
	ITP	2	5.9
	Non-immunological	16	47.1

LFTs: liver function tests; AIH: autoimmune hepatitis; SS: Sjögrens syndrome; ITP: idiopathic thrombocytopenic purpura

Table 2. ND pattern and related diseases.

HEp-2 pattern	Clinical disease	Frequency	%
ND (n=16)	Abnormal LFTs	5	31.3
	PBC	0	0
	AIH	0	0
	Hep-C	0	0
	SLE	1	6.3
	RA	4	25.0
	SS	1	6.3
	Cancer	2	12.5
	Non-immunological	8	50

Table 3. NM pattern and related diseases.

HEp-2 pattern	Clinical disease	Frequency	%
NM (n=5)	Abnormal LFTs	3	60
	PBC	0	0
	AIH	0	0
	Hep-C	0	0
	AITD	1	20
	RA	1	20
	ANCA vasculitis	1	20
	Non-immunological	2	40

AITD: autoimmune thyroid disease.

Table 4. Cytoplasmic filament pattern and related diseases.

HEp-2 pattern	Clinical disease	Frequency	%
Cytoplasmic (n=12)	Abnormal LFTs	3	25
	PBC	0	0
	AIH	0	0
	Hep-C	0	0
	AITD	2	16.7
	SS	1	8.3
	Neutropenia	1	8.3
	Non-immunological	8	66.7

Table 5. Clinical sensitivity and specificity of different autoantigens for PBC.

Clinical disease	Autoantigen	Method	Clinical specificity (%)	Clinical sensitivity (%)	PPV (%)	NPV (%)
PBC	M2	ELISA	68.8	91.6	35	97
PBC	sp100	ELISA	72.5	16.6	10.5	81.8
PBC	gp210	ELISA	95	25	50	86.8
PBC	M2	Blot	82.5	50	35.3	89.7
PBC	AMA-like pattern	LKS IIF	90.2	90.9	62.5	98.2

PPV (62.5%) and NPV (98.2%) and was clinically sensitive and specific (90.9% and 90.2%, respectively).

It has been reported by five different studies that detection of antibodies to sp100 is highly specific for PBC,[4,5,7,13,14] but the present study has not reproduced these results, as anti-sp100 antibodies were found in a range of diseases, including PBC, SS, UCTD, cancer, chronic obstructive pulmonary disease (COPD), inflammatory arthritis and pulmonary fibrosis. This correlated with other studies which showed that anti-sp100 antibodies can be found in SLE and collagen disease,[2] and are only present in 30% of PBC patients.[1]

This study determined the prevalence of anti-gp210 antibodies in PBC to be 25%, therefore these data support those of other studies;[7–11] however, it disagrees with another study which reports that anti-gp210 antibodies are only found in PBC,[10] having detected anti-gp210 in PBC, SS, cancer and non-immunological diseases.

The diversity of results seen in this study may be due to a number of factors including the selection of study cohorts. It differed from previous studies in that patients were selected for presence of autoantibody rather that by pre-existence of clinically relevant disease (e.g., selection of an NM pattern on HEp-2 and determining the disease) rather than testing serum from a patient with AILD to determine the autoantibodies involved. This enabled the examination of the occurrence of these autoantibodies in an unselected population and the investigation of clinical significance, if any, of the occurrence of a given autoantibody when detected by IIF screening.

Patients who showed an AMA-like staining pattern on HEp-2 or a non-M2 pattern on LKS and were not diagnosed with PBC had alcoholic sclerosis, irritable bowel syndrome (IBS), sarcoid or idiopathic thrombocytopenia purpura. This may mean that non-M2 mitochondrial antibodies are associated with more diseases than previously reported, or that these patients may have other non-hepatic diseases or perhaps unidentified drug-induced disorders. In addition, the possibility exists that the detection of autoantibodies outside previously defined clinical groups may be a result of the patient population studied. The use of non-disease-selected cohorts may have resulted in the detection of patients in whom an incomplete autoantigenic reactive profile may be present (i.e., limited epitope specificity) or in whom the disease is not yet clinically evident (i.e., prodromal disease).

This study confirmed that two of the autoantigens of interest are reasonably specific for PBC. Anti-sp100 antibodies and anti-gp210 have a clinical specificity for PBC of 72.5% and 95%, respectively. Therefore, it could be argued that detection and reporting of these would be clinically valuable. It has been suggested that reporting any type of antimitochondrial antibody is clinically valuable both in patients with PBC and those not suspected of having PBC, as seen in the clinical prodrome. After detection of antimitochondrial antibodies in those not suspected of having PBC, the patients could be monitored for the possible development of PBC. It remains unclear from the data presented in this study whether or not a similar case could be argued for other autoantibodies reported to be associated with AILD. □

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