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Nuclear antigens recognized by antibodies present in liver disease sera

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SUMMARY

Nuclear and nuclear matrix proteins of HeLa cells were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and subsequently transferred onto nitrocellulose. Antibodies present in sera of patients with primary biliary cirrhosis and autoimmune chronic active hepatitis reacted with some of the blotted proteins. The antibodies were mainly directed against chromatin-associated proteins and protein constitutents of discrete RNP particles. In addition, antibodies found in autoimmune liver disease sera detected a hitherto undescribed nuclear protein of 54 kD, and a nuclear matrix protein of approximately 150 kD. Antibodies recognizing a nuclear 25 kD doublet apparently constituted a marker antibody for autoimmune liver disease. Those directed at the 17 kD centromere protein were associated with the primary biliary cirrhosis-related CREST syndrome, while those recognizing La antigen were related to cases of sicca syndrome associated with autoimmune liver diseases.

Keywords liver disease nuclear antibodies immunoblotting

INTRODUCTION

Antibodies specific for intracellular, predominantly nuclear macromolecules, are frequently observed in association with inflammatory rheumatic disorders (Tan, 1982), autoimmune chronic hepatitis (CAH) and primary biliary cirrhosis (PBC) (Mackay *et al.*, 1980).

In rheumatic diseases, most of these antibodies are either restricted to, or appear in high titres in certain syndromes, and are thus of considerable diagnositic value. Antibodies to the Sm antigen are uniformly found in systemic lupus erythematosus (SLE) (Tan & Kunkel, 1966). Anti-Sm antibodies recognize the small nuclear RNAs U1, U2, U4, U5, U6 and two proteins of 25 and 13 kD, termed B/B′ and D respectively (Lerner & Steitz, 1979). Antibodies to U1-RNP, when occurring in high titres, are typical of mixed connective tissue disease (Sharp *et al.*, 1972). Anti-U1-RNP antibodies react with U1 RNA, they also identify a protein of 70 kD, and proteins of 31 and 19 kD, termed A and C (Lerner & Steitz, 1979). La antibodies characterize subsets of SLE and sicca syndrome patients (Mattioli & Reichlin, 1974). These antibodies precipitate a highly banded pattern of RNAs (Lerner *et al.*, 1981), and recognize a protein of 50 kD (Habets *et al.*, 1983a). On the other hand, antibodies to a nuclear matrix protein termed Scl-86, define patients with progressive systemic sclerosis (Douvas, Achten & Tan, 1979; van Venrooij *et al.*, 1985), antibodies against a chromatin-associated centromere protein of 19·5 kD appear to be specific for the CREST variant of systemic sclerosis (Moroi *et al.*, 1980; Guldner, Lakomek & Bautz, 1984).

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Thus, while considerable information is available as to the nuclear antigens seen by connective tissue disease sera, little is known about nuclear proteins detected by sera of patients with liver disease (Kurki *et al.*, 1984; Bernstein *et al.*, 1984). In the present study we have used immunoblotting (Towbin, Staehlin & Gordon, 1979; Habets *et al.*, 1983b) to define the nuclear proteins recognized by autoimmune CAH and PBC sera. The data obtained in immunoblotting were then correlated with clinical features.

MATERIALS AND METHODS

Patients. Thirty autoimmune CAH and 38 PBC sera, 15 liver disease controls, and 14 normal control samples were tested. Disease controls included seven sera from patients with alcoholic liver disease, and eight specimens obtained from patients with hepatitis B virus-associated CAH. All diagnoses based on accepted clinical, morphologic and whenever appropriate serologic criteria (Leevy, Popper & Sherlock, 1976). The normal control samples were obtained from age and sex matched individuals. All samples were stored at $-20^{\circ}C$ until testing.

Serology. Hepatitis B virus-associated markers were detected using commercially available solid-phase radioimmunoassays (Abbott Laboratories, North Chicago, IL, USA).

Indirect immunofluorescence tests for antinuclear antibodies were performed on HEp-2 cells (Kallestad Laboratories Inc., Austin, TX, USA), according to the instructions of the manufacturer. Titres of $\geq 1:40$ were recorded as positive; at this titre $< 5\%$ normal controls gave a positive reaction.

Isolation of HeLa cell nuclei and nuclear matrices. The fractions were prepared by modification of the methods of Hodge *et al.* (1977), Capco, Wan & Penman (1982), Habets *et al.* (1983b) and van Eekelen *et al.* (1982).

Briefly, HeLa cells were harvested, washed in PBS and homogenized in 10 mM Tris-HCl (pH 7·4), 1·5 mM $MgCl_2$, 0·5% Nonidet-P 40, at 4°C. All solutions contained 1 mM phenyl methyl sulphonyl fluoride. The nuclei were sedimented through a layer of 0·8 M sucrose, 10 mM Tris-HCl (pH 7·4), 3mM $MgCl_2$. To remove cytoplasmic constituents, the nuclei were resuspended and agitated in 1% Nonidet-P 40, 0·5% sodium deoxycholate in reticulocyte suspension buffer (RSB) (10 mM NaCl, 10 mM Tris-HCl, pH 7·4; 1·5 mM $MgCl_2$), and then washed in RSB. The isolated nuclei (2×10^8 nuclei/ml) were then digested with 500 μg DNAse-1/ml and 100 μg pancreatic RNAse/ml in buffer A (110 mM NaCl, 10 mM Tris-HCl, pH 7·4; 10 mM $MgCl_2$) at 20°C for 60 min. The mixture was termed total nuclear protein fraction.

For the preparation of the nuclear matrices, the isolated nuclei were digested with both nucleases as described above, except that the incubation period was 15 min. The nuclease-treated nuclei were sedimented through a layer of 1 M sucrose in buffer A and twice extracted with 0·4 M $(NH_4)_2SO_4$, 30 mM Tris-HCl (pH 7·4), 10 mM $MgCl_2$. The nuclear matrices were finally washed in RSB.

Polyacrylamide gel electrophoresis and immunoblotting. The samples were dissolved in 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% mercaptoethanol in 100 mM Tris-HCl (pH 6·8), and electrophoresed on 13% slab gels in SDS buffer (Laemmli, 1970). The separated proteins were electrophoretically transferred onto nitrocellulose sheets (Towbin, Staehlin & Gordon, 1979). The blots were dried, cut into 6–8 mm strips and incubated with properly diluted human sera, as described (Habets *et al.*, 1983b). After exposure to ^{125}I-protein A, the immune complexes were visualized by autoradiography.

Reference sera. Prototype sera specific for anti-Sm, anti-U1-RNP, anti-La, anti-Scl-86, and anticentromere were obtained from our serum bank. An additional anticentromere serum was obtained from H. Guldner (Institute for Molecular Genetics, University of Heidelberg, FRG).

RESULTS

Liver disease sera and control samples were tested by immunoblotting for antibodies to nuclear proteins and nuclear matrices.

Table 1. Incidence of antibodies to nuclear proteins in liver disease

		Antibodies specific for:							
					U1-RNP				
Disease	N	Centromere 17kD	Doublet 25kD	La 50kD	19kD	31kD	70kD	54kD	Nuclear matrix 150kD
Primary biliary cirrhosis	38	11 (29)‡	3 (8)	4 (11)	1 (3)	1 (3)	8 (21)	6 (16)	0
Autoimmune CAH*	30	0	8 (27)	4 (13)	0	1 (3)	8 (27)	3 (10)	2 (7)
Liver controls†	15	0	0	0	0	0	0	0	0
Normal controls	14	0	0	0	0	0	0	0	0

* CAH chronic active hepatitis.
† Alcoholic liver disease, hepatitis B virus-associated CAH.
‡ The number in paranthesis denotes percentage.

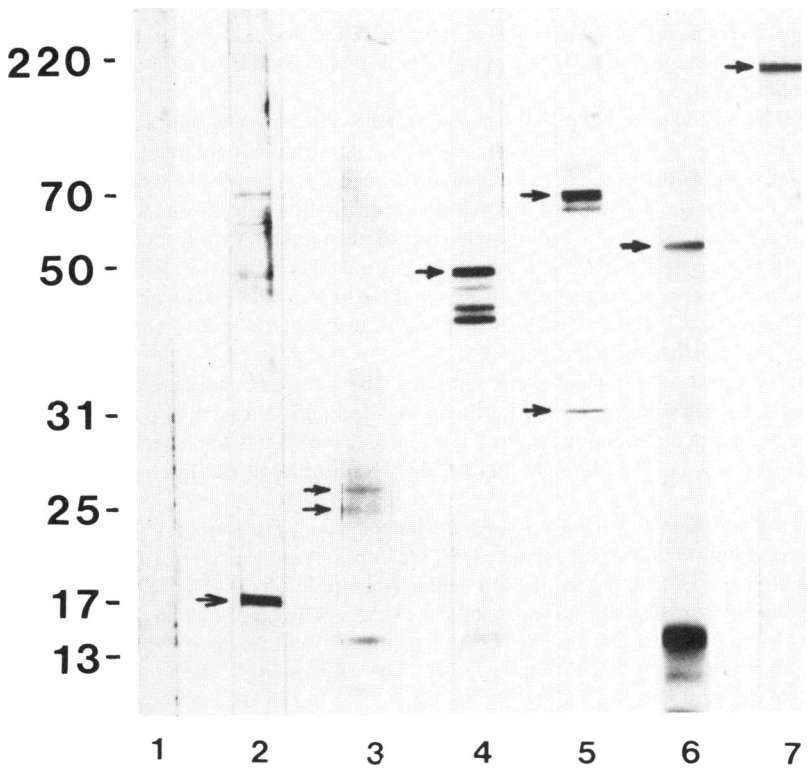

Fig. 1. HeLa cell nuclear proteins and nuclear matrices were separated by polyacrylamide gel electrophoresis in SDS, transferred onto nitrocellulose, probed with liver disease sera, and visualized following autoradiography with ^{125}I-protein A. Lane 1 is a normal human serum, lanes 2–5 represent sera recognizing centromere antigen, a doublet of 25 kD, La and U1-RNP respectively (see arrows). Lane 6 demonstrates the 54 kD nuclear protein and lane 7 a nuclear matrix protein of approximately 150 kD.

Early in our experiments it became apparent that antibodies to nuclear and nuclear matrix proteins were present in sera of autoimmune CAH and PBC patients, but not in liver disease controls or healthy individuals. Antibodies specific for Sm and Scl-86 antigens were not observed in our patients or controls.

Table 2. Features of CREST syndrome in primary biliary cirrhosis, with and without antibodies to the 17 kD centromere antigen

Anti centromere	No.	C	R	E	S	T
Positive	11	5	9	9	4	5
Negative	27	0	0	0	0	0

(C) Calcinosis; (R) Raynaud's phenomenon; (E) oesophageal motility disturbances; (S) sclerodactyly; (T) teleangiectasia.

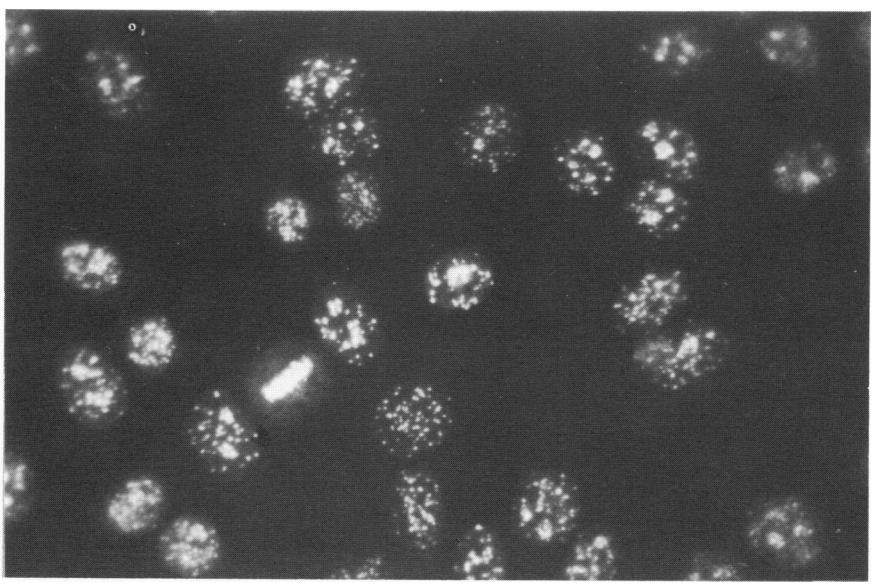

Fig. 2. Indirect immunofluorescence on HEp-2 cells performed with a primary biliary cirrhosis serum and demonstrating centromere staining.

A summary of the immunoblotting results is given in Table 1. Starting with proteins of low molecular weight, a peptide of 17 kD was observed in 11 of 38 PBC sera (29%) (Fig. 1, lane 2). In immunofluorescence, all sera developing the 17 kD band exhibited the characteristic pattern described for the chromatin-associated centromere antibody, showing discrete speckles over the nucleus, either dispersed, or gathered closely together on the chromosomes of cells undergoing division (Moroi *et al.* 1980) (Fig. 2). An identical staining pattern was seen when the 17 kD antibody was eluted from the blot and tested by immunofluorescence (Guldner, Lakomek & Bautz, 1982). Nine of the 11 PBC patients (82%) exhibiting the 17 kD band presented features of complete or incomplete CREST syndrome (Table 2). Anti-17 kD negative PBC patients had no evidence of the CREST variant. Anticentromere antibodies were not observed in patients with autoimmune CAH.

Another set of nuclear proteins recognized was a 25 kD doublet (Fig. 1, lane 3). This doublet was

a target antigen for eight (27%) of the autoimmune CAH sera and three (8%) of the PBC sera. No clinical correlations were apparent in anti-25 kD positive cases.

All PBC and two of the CAH cases with sicca syndrome recognized the 50 kD nuclear antigen La (Fig. 1, lane 4), while none of the anti-La negative cases or controls had sicca syndrome. Thus, La antibody appeared to be a marker for sicca syndrome.

Antibodies specific for the 70 kD protein of U1-RNP occurred in eight (27%) autoimmune CAH and in eight (21%) PBC sera, when tested versus the total nuclear protein fraction (Fig. 1, lane 5). Of the sera reacting with the nuclear 70 kD protein 50% (four autoimmune CAH and four PBC sera) also revealed binding to the 70 kD protein of the nuclear matrix (Data not shown). Some of the sera positive for the 70 kD nuclear protein were also directed to proteins A or C (Table 1).

Six (16%) of the PBC sera and three (10%) of the CAH sera recognized a novel nuclear protein antigen of 54 kD (Fig. 1, lane 6). Two (7%) of the autoimmune CAH specimens contained antibodies to a nuclear matrix protein of approximately 150 kD (Fig. 1, lane 7).

DISCUSSION

In the present study we have employed the immunoblotting technique for detection of specific nuclear and nuclear matrix antigens recognized by antibodies found in PBC and autoimmune CAH sera.

The immunoblotting technique allows for demonstration of antigenic components in complex mixtures of cellular components, and provides information as to their number and molecular size (Towbin *et al.*, 1979). Moreover, this method provides information within a short time and can be adapted to screening large numbers of sera. Thus, it can be usefully introduced into a clinical setting.

The data obtained in this study demonstrate a considerable heterogeneity among the nuclear antigens recognized by PBC and autoimmune CAH sera. Similar results, albeit in differing frequencies have been noted by other authors among connective tissue diseases (Tan, 1982, review), and seem to underline the serological relationship between the two disease categories. Antigens recognized in the present investigation were (a) chromatin-associated proteins (centromere) (Moroi *et al.*, 1980; Guldner *et al.*, 1984) (b) proteins known to reside on different sets of RNP particles (La, U1-RNP) (Sharp *et al.*, 1972; Mattioli & Reichlin, 1974) and (c) previously unreported nuclear matrix antigens. The molecular composition and biological role of these novel antigens is subject of further investigations.

Another novel finding was the presence of antibodies recognizing exclusively a 25 kD doublet. These antibodies might consitute an infrequently occuring marker antibody characteristic for autoimmune liver disease. Whether this doublet is identical to the B/B′ protein recognized by anti-Sm antibodies is unclear. Interestingly, none of our sera (a) recognized the D protein of 13 kD, which along with the 25 kD B/B′ doublet is a common target of anti-Sm antibodies, (b) reacted in immunodiffusion in identity with our anti-Sm sera, or (c) produced the speckled pattern typical of anti-Sm in immunofluorescence (Data not shown). It remains to be shown whether we were dealing with distinct antigens of the same molecular weight, or antibodies of different specificities.

Anticentromere antibodies have been reported to precipitate a protein of 19·5 kD (Guldner *et al.*, 1984). However, our sera and the reference serum obtained from H. Guldner, Heidelberg, FRG, gave a centromere band at 17 kD, probably due to slight differences in electrophoretic conditions.

Antibodies specific for the centromere antigen and La have been previously noted by immunofluorescence in autoimmune liver diseases (Bernstein *et al.*, 1982; Makinen, Fritzler & Sherlock, 1983; Kurki *et al.*, 1984; Bernstein *et al.*, 1984), however with an incidence distinctly lower than reported in this paper. This discrepancy may result from the greater sensitivity of immunoblotting versus conventional serological tests as gel diffusion or immunofluorescence. Indeed, routine tests in our laboratory have shown that, by immunoblotting we are able to increase the positivity for centromere antibodies by 20%, when compared to immunofluorescence (Penner; unpublished results).

In this study we have shown that certain nuclear antibodies serve as disease markers in PBC and autoimmune CAH, or are associated with extrahepatic syndromes observed in these diseases.

However, as in other autoimmune diseases, it is unclear if and how the antibodies relate to the pathogenesis of these disorders. The described antibodies may, in addition, serve as valuable probes for the characterization of nuclear proteins.

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