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Autoantibodies in chronic delta virus infection recognize a common protein of 46 kD in rat forestomach basal cell layer and stellate thymic epithelial cells

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SUMMARY

Chronic hepatitis delta virus infection is associated with the presence of autoantibodies to rat forestomach and thymus in approximately 60% of patients' sera. We have characterized the antigen against which these autoantibodies are directed as a protein of 46 kD by immunoblotting studies on rat forestomach and thymus extracts. Normal human sera or sera from patients with other hepatic or non-hepatic autoimmune disorders did not bind to this protein. The immunoblott assay was more sensitive than immunofluorescence. Maximal titre was 1:10 000 *versus* 1:5120. By techniques of elution of specific antibodies from immunoblots, our results showed that the same antigen was present in both tissues. This antigen did not share common epitopes with hepatitis delta virus (HDV). Patients' sera depleted of basal cell layer and thymic stellate epithelial cell antibodies by absorption with the corresponding tissue extract maintained the HDV antibody titres. The autoimmune phenomena observed in patients with HDV infection seems to be a colateral process induced by the replication of delta virus in the host.

Keywords delta infection autoantibodies autoantigens

INTRODUCTION

Chronic liver disease associated with hepatitis delta virus (HDV) infection is characterized by a wide spectrum of autoantibodies. An antibody to microsomal membranes of human liver and kidney (LKM3) has been described in 13% of these patients (Crivelli *et al.*, 1983), being specific for HDV infection and different from LKM1 (Rizzetto, Swana & Doniach, 1973) and LKM2 (Homberg, Andre & Abuaf, 1984; Homberg *et al.*, 1987) detected in other liver diseases. Association of an antibody reacting with the basal cell layer (BCL) of rat forestomach has also been detected (Zauli *et al.*, 1984), and originally described in 20% of patients with hepatitis B virus chronic infection (Lenkei *et al.*, 1983). Antibodies reacting with thymic stellate epithelial cells (SEC) have also been found, in close relation to the BCL antibody (Magnius *et al.*, 1985; Lenkei *et al.*, 1985). Furthermore, a high prevalence of antibodies directed to cytoskeleton, primarily to intermediate filaments, has recently been detected in 61% of BCL antibody positive sera (Zauli *et al.*, 1985).

Pisi, Zauli & Crespi (1987) have suggested that BCL may be a cytokeratin, since 61% of sera positive for BCL antibody recognized keratin-containing intermediate filaments of cultured epithelial cells; however, this remains to be demonstrated.

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In the present study we have partially purified the antigens BCL and SEC by fractionation of rat forestomach and thymus extracts. Immunoblot analysis showed that the same antigen is present in both tissues. The relation between this antigen and HDV is investigated.

MATERIALS AND METHODS

Patients' sera

One hundred and two sera were obtained from patients with HDV infection. The diagnosis of hepatitis delta was based on the presence of the hepatic antigen or anti-HDV antibody or both. As controls, 145 sera without serological markers for HDV infection were also tested. These were from 22 patients with primary biliary cirrhosis (PBC), 22 patients with autoimmune chronic active hepatitis (ACHA), 30 patients with HDV-negative, acute hepatitis B (HBV), 14 patients with non-A non-B hepatitis (NANB), 22 patients with systemic lupus erythematosus (SLE) and 25 normal human sera (NHS).

Immunofluorescence studies

For indirect immunofluorescence, unfixed 3-μm cryostat sections of rat forestomach and thymus were collected on glass slides and incubated for 25 min with the patient or control sera diluted 1:40 in 0·15 M phosphate-buffered saline (PBS) pH 7·4. The slides were washed, treated for 25 min with fluorescein-conjugated IgG from rabbit anti-human immunoglobulins or anti-IgG, anti-IgA or anti-IgM (Dakopatts, Copenhagen, Denmark), diluted 1: 50 in PBS and examined with an epifluorescence microscope (Leitz, Wetzlar, FRG).

Preparation of rat forestomach and thymus extracts
Forestomach and thymus were obtained from male or female, 8-12 week old Sprague-Dawley rats.

The internal layer was separated from the rest of the forestomach and resuspended in 20 volumes of PBS, pH 7·4, supplemented with 5 mM EDTA, 0·1 mM phenyl methyl sulphonide fluoride (PMSF) and 200 U/ml trasylol (buffer E).

The thymus was partially depleted of thymocytes by intrathymic infusion of PBS dispersed and resuspended in buffer E (1:20).

Both suspensions were homogenized in a Virtis (Gardiner, NY) homogenizer and centrifuged at 10 000 g for 60 min. The pellet was extracted in buffer E with 1% octylglucoside (Sigma Chemical Co., St Louis, MO) overnight, and centrifuged at 100 000 *g* for 1 h. The pellet was resuspended in buffer E and sonicated. NaCl and urea were subsequently added at a final concentration of 2 M and 6 M, respectively, and centrifuged at 100 000 *g* for 1 h. Supernatants were discarded and the pellet was resuspended in buffer E and stored at −20°C until use.

Enzymatic treatments
The extract was treated with trypsin in PBS at 5 mg/ml final concentration for 30 min at 37°C; and with DNAse at 0·1 mg/ml final concentration, in 10 mM Tris-HCl, pH 7·4; 0·15 M NaCl; 1 mM $CaCl_2$; and 5 mM $MgCl_2$ for 30 min at 37°C. The treated extracts were centrifuged in a microfuge at 10 000 *g* for 2 min, and the pellet was resuspended in buffer E.

Immunoblot analysis
Forestomach and thymus extracts were electrophoresed under reducing conditions on 10% sodium dodecyl sulphate–polyacrilamide gels (Laemli, 1970). Immunoblot analysis was performed as described by Towbin, Staehlin & Gordon, (1979). Nitrocellulose was blocked with 1% gelatin in 0·15 M NaCl–5 mM Tris, pH 7·4, for 1 h and incubated with patient or control sera at a dilution of 1:100 for 2 h in the diluting buffer (0·15 M NaCl: 5 mM Tris, pH 7·4; 5 mM EDTA; 0·05% NP-40; 0·25% gelatin). The second antibody, rabbit anti-human immunoglobulins (Dako), was applied at a dilution of 1:100 and incubated for 1 h. The third antibody, a peroxidase-conjugated swine anti-rabbit IgG (Dako), diluted 1:500, was incubated for 1 h. The washed nitrocellulose sheets were developed with α-naphtol (Bio-Rad, Richmond, CA) in 0·1 M Tris, pH 7·4, containing H_2O_2 at a final concentration of 0·01%

Table 1. Frequency of anti-basal cell layer (BCL) and anti-stellate epithelial cells (SEC) antibodies in patients with hepatitis delta virus infection (HDV) and control subjects

	No. of individuals	BCL antibodies	SEC antibodies
HDV	102	69	50
Control			
PBC	22	0	0
ACAH	22	0	0
HBV	30	0	0
NANB	14	0	0
SLE	22	0	0
NHS	25	0	0

PBC, primary biliary cirrhosis; ACAH, autoimmune chronic active hepatitis; HBV, acute B hepatitis; NANB, non-A non-B hepatitis; SLE, systemic lupus erythematosus; NHS, normal human serum.

Table 2. Prevalence of autoantibodies to 46 kD protein extracted from rat forestomach and thymus in patients with chronic hepatitis delta virus (HDV) infection and in control subjects

				46 kD	
	No. of individuals	BCL antibody	SEC antibody	Fore-stomach	Thymus
HDV	22	16	10	17	17
Control					
PBC	22	0	0	0	0
ACAH	22	0	0	0	0
HBV	12	0	0	0	0
NANB	14	0	0	0	0
SLE	22	0	0	0	0
NHS	25	0	0	0	0

BCL, basal cell layer; SEC, stellate epithelial cells; PBC, primary biliary cirrhosis; ACAH, autoimmune chronic active hepatitis; HBV, acute B hepatitis; NANB, non-A non-B hepatitis; SLE, systemic lupus erythematosus; NHS, normal human serum.

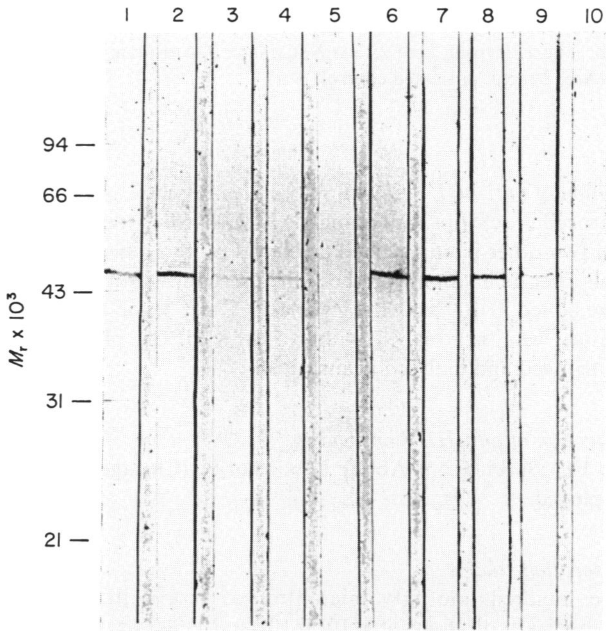

Fig. 1. Immunoblotting analysis of four different hepatitis delta virus (HDV) sera against rat thymus (lanes 1–5) and forestomach (lanes 6–10) extracts. Lanes 5 and 10 correspond to normal human serum; 4 and 9 show the reactivity of a basal cell layer (BCL) antibody negative, stellate epithelial cell (SEC) antibody negative serum.

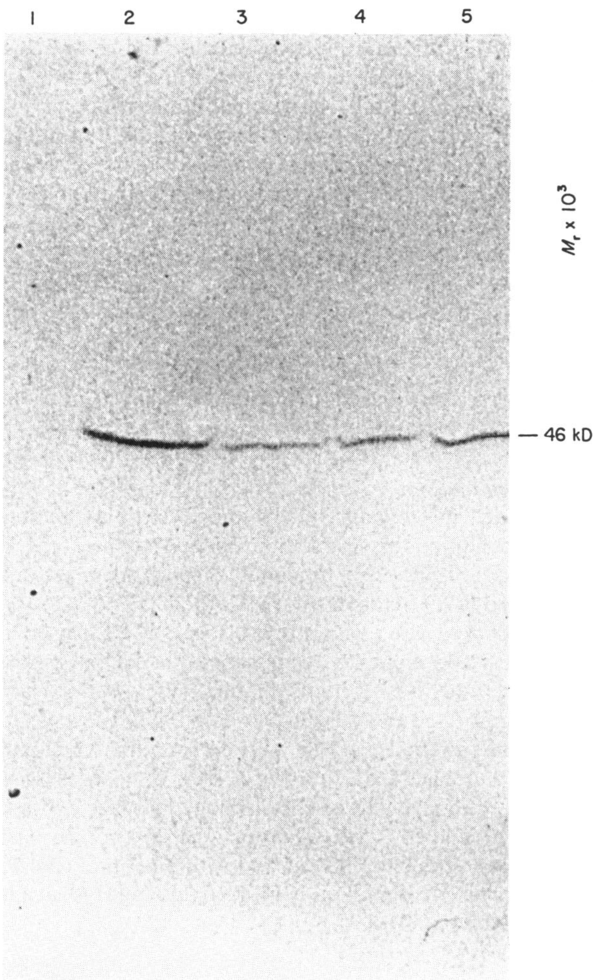

Fig. 2. Sensitivity of the basal cell layer (BCL) antigen to treatments with: lane 1, trypsin; lane 2, 2 M NaCl; lane 3, 6 M urea; and lane 4, DNAse. Lane 5, untreated control.

Elution of anti-BCL and anti-SEC autoantibodies

Nitrocellulose strips containing the BCL- or SEC-specific bands and two other bands, named as blanks, were excised from the blots after incubation with the patient serum. Bound antibodies were eluted in 100 μl 0·2 M glycine-HCl, pH 3, for 1 min. The eluates were neutralized with 0·5 M K_2HPO_4, pH 8 (v/v), centrifuged and dialysed against PBS.

Detection of anti-HDV antibodies

An ELISA kit from Abbot Laboratories (Chicago, IL) was employed.

Absorption studies

One hundred and fifty microlitres of sera with anti-delta antibodies at dilutions of 1:10, 1:100 and 1:200 were absorbed with the pellet corresponding to 500 μl of rat forestomach or thymus extract for 2 h at room temperature. After centrifugation, the absorbed sera were tested for BCL and SEC antibodies by immunofluorescence on cryostat sections of rat forestomach and thymus, and for anti-HDV antibodies by ELISA (Abbot).

RESULTS

Immunofluorescence studies

We studied 102 serum samples from patients with HDV infection by indirect immunofluorescence on cryostat tissue sections; 69 (68%) were antibody-positive for the BCL of the squamous epithelium in the gastric mucosa and 50 (49%) were antibody-positive for SEC in the thymus (Table 1). The same fluorescence pattern was obtained when serum was incubated on human thymus sections. Only sera with BCL antibodies were positive for SEC, at the same or lower titers. NHS or sera from patients with other hepatic or autoimmune disorders were BCL- and SEC-negative; some of these sera gave a diffuse fluorescence on the rat thymus but this differed clearly from the specific SEC pattern.

The highest titre was 1:5120.

Immunoblotting analysis

Sera from HDV patients were incubated with thymus and forestomach extracts as described in Materials and Methods. A specific band with M_r of 46 kD was recognized by BCL- and SEC-positive sera on cryostat sections (Fig. 1). Sera from one of six patients who were BCL-negative and seven of 12 who were SEC-negative by immunofluorescence bound to the 46-kD band on the immunoblots (Table 2). The highest titre was 1:10 000. No NHS recognized the specific band. Sera from patients with autoimmune connective tissue disorders and other hepatic diseases (CBP, ACAH, HBV and NANB) were also negative.

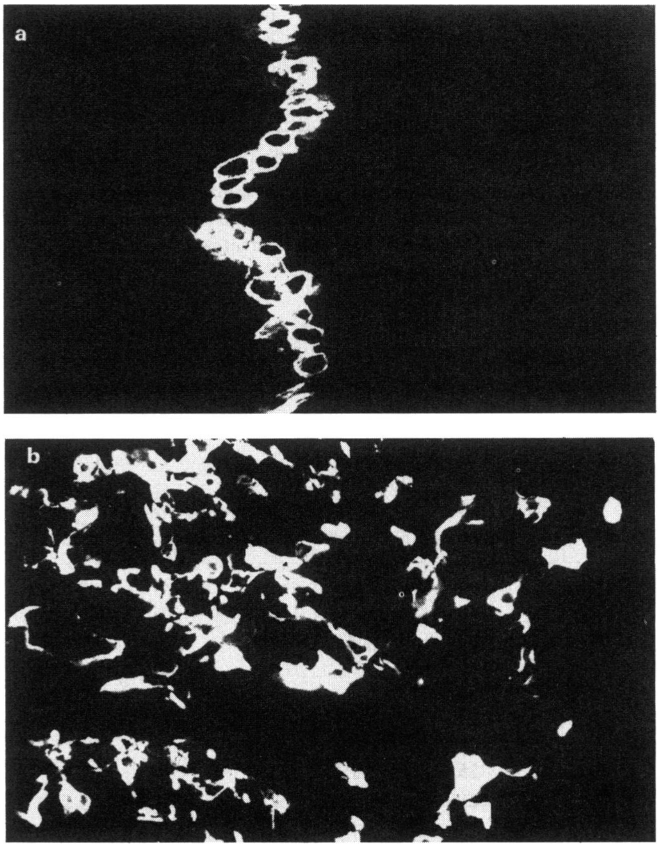

Fig. 3. Indirect immunofluorescence of antibodies eluted from rat forestomach immunoblots on cryostat tissue sections of (a) forestomach; and (b) thymus.

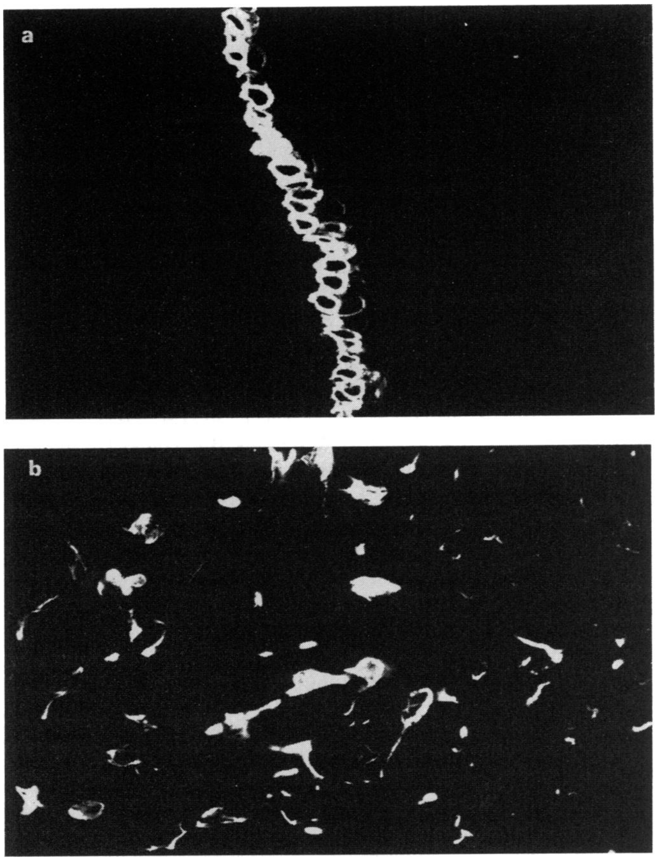

Fig. 4. Indirect immunofluorescence of the antibodies eluted from rat thymus immunoblots on cryostat tissue sections of (a) forestomach; and (b) thymus.

The antigen was sensitive to trypsin and resistant to DNAse and 6 M urea. It was not solubilized by high ionic strength solution (2 M NaCl) (Fig. 2).

Elution of antibodies

In order to determine whether the 46-kD antigen is responsible for the BCL and SEC immunofluorescence pattern, bound antibodies were eluted from immunoblots and tested by immunofluorescence against frozen sections of rat forestomach and thymus. Antibodies eluted from either forestomach or thymus reacted with both cryostat tissue sections (Figs 3, 4). No reactivity was seen with the eluate from other areas of the immunoblots.

The presence of HDV antibody in the eluates was assayed by ELISA (Abbot), and our results showed that BCL and SEC antibodies did not react with the delta antigen. To corroborate the former results, anti-delta serum was incubated with the insolubilized extract, as described in Materials and Methods. The absorbed serum was tested on unfixed cryostat sections of rat forestomach and thymus, and tested for the presence of anti-HDV by ELISA. No fluorescence was detected on the slides, whereas anti-HDV antibody titre was maintained.

DISCUSSION

We found that sera from patients with HDV infection recognized a protein of 46 kD from both rat thymus and forestomach. No NHS or sera from patients with other hepatic or autoimmune disorders recognized this protein.

The differences found in BCL and SEC antibody titres by immunofluorescence may be a consequence of a lower antigen expression in the thymus. SEC-negative sera had a low titre of BCL antibodies. This discrepancy in titres was bypassed by the immunoblotting technique, because the specific antigen was partially purified and concentrated.

The assay was more sensitive than the immunofluorescence: maximal titre was 1:10 000 *versus* 1:5120 by immunofluorescence; one of six BCL negative sera were positive when analysed on forestomach immunoblotts; and SEC-negative, BCL-positive sera recognized the 46 kD band on thymus immunoblotts.

No correlation was found between the presence of hepatic delta antigen and the BCL and SEC antibody production (data not shown).

Eluted antibodies, bound to the 46-kD band of immunoblots, gave the BCL- and SEC-specific fluorescence pattern of cryostat tissue sections.

It has previously been suggested that the BCL antibody may be an anti-keratin antibody, since 61% of sera positive for BCL antibody were able to bind to keratin-containing intermediate filaments of cultured epithelial cells (Pisi *et al.*, 1987), and monoclonal antibody to acidic keratins stain the same basal cells (Woodcock-Mitchell *et al.*, 1982). However, none of the 22 BCL positive sera recognized any of the proteins from rat forestomach keratin extract, as tested by immnoblot analysis (Pisi *et al.*, 1987).

Our data suggest that BCL and SEC antigens are the same protein since both had the same mol. wt; were difficult to solubilize; did not elute at 2 M NaCl; were resistant to 6 M urea and DNAse; and shared common epitopes, as has been showed by immunofluorescence studies with the eluted antibodies from rat thymus and forestomach immunoblotts.

Anti-HDV antibody and BCL antibody did not seem to be cross-reactive as no strict correlation was found between BCL antibody and antibody titres to HDV, and sera with high titres of BCL antibody failed to block the binding of HDV antibody to its antigen in a radioimmunoassay for antibody to HDV (Magnius *et al.*, 1985). Our findings are in agreement with these results, since the 46-kD eluted antibodies did not recognize the HDV in ELISA, and sera absorbed with tissue extracts maintained the same anti-HDV titres.

Furthermore, the BCL antigen showed a higher mol. wt than two major HDV polypeptides of 24–27 and 27–29 kD previously described (Bergmann & Gerin, 1986; Bonino *et al.*, 1986; Pol *et al.*, 1987).

Nevertheless, the temporal relation of the appearance of BCL antibody and delta seroconversion suggests that HDV is causative of the occurrence of BCL antibody (Lenkei *et al.*, 1987), even though we do not yet know the mechanisms and the significance of such autoantibody production.

Together these data seem to suggest that the antigen eliciting the formation of BCL and SEC antibodies in delta infection is not coded for by the delta virus. The reactivity of BCL-positive sera with fetal liver cells (Magnius *et al.*, 1985) suggest that the production of these autoantibodies may be a secondary event resulting from HDV-induced differentiation of liver antigens.

It is well established that cortical and medullary thymic epithelium induce stages of T cell maturation (McFarland, Scearce & Haynes, 1984). Functional data in rodent models suggest that thymocytes learn to recognize self class I and class II major histocompatibility complex (MHC) antigens within the thymic micro-environment, presumably via contact with micro-environment cells that express MHC antigens. Since SEC antibody stains human thymic cells (Lenkei *et al.*, 1985 and our results) that are HLA-DR positive and morphologically similar to the cells secreting alpha-1-thymosin (Hirokawa, McClure & Goldstein, 1982) we suspect that these antibodies may play some role in interfering with T cell differentiation. This has not been demonstrated to date. Larger studies and further characterization of this antigen may help in clarifying the immune mechanisms involved in chronic HDV infection.

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