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Anti-neutrophil nuclear antibody in ulcerative colitis, Crohn's disease and primary sclerosing cholangitis

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SUMMARY

We have previously described circulating autoantibodies to a portal tract antigen in patients with primary sclerosing cholangitis. In this study the antigen has been shown by double-labelling studies to be specifically located in the nuclei of tissue neutrophils. Using isolated peripheral blood neutrophils and an immunoperoxidase technique, anti-neutrophil nuclear antibody (ANNA) was found in the serum of 84% of patients with primary sclerosing cholangitis (PSC: $n=32$) with a median titre of 1/1000 and a peak titre of 1/500 000. ANNA was also detected in 86% of patients with inflammatory bowel disease alone (IBD: $n=76$) with a median titre of 1/10 and a peak titre of 1/10 000. In contrast, only 12% of controls had ANNA, and in none was the titre greater than 1/10. In PSC the ANNA titre correlated with the serum aspartate transaminase concentration, suggesting that it is related to disease activity. In IBD the titre of ANNA was significantly higher in patients with recently active disease. There was no significant difference between the titres seen in ulcerative colitis and Crohn's disease. ANNA was not associated with neutropaenia. The results provide further evidence of involvement of autoimmune mechanisms in inflammatory bowel disease and primary sclerosing cholangitis.

Keywords ulcerative colitis Crohn's disease cholangitis anti-nuclear antibodies immunoperoxidase techniques

INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic inflammatory disorder of the biliary tree with characteristic cholangiographic appearances. The majority of cases occur in subjects who also suffer from ulcerative colitis but the mechanism underlying this association is unclear (Chapman *et al.*, 1980; Wiesner *et al.*, 1985). The aetiology of PSC is unknown but the association with the HLA B8/DR3 haplotype favours an autoimmune basis (Chapman *et al.*, 1983). However, PSC shows no striking association with any of the conventional autoantibodies found in the more established autoimmune disorders (Chapman *et al.*, 1980; Wiesner *et al.*, 1985).

We have shown previously that a proportion of patients with PSC have circulating antibodies directed against an antigen in the portal tracts of human liver with bile duct obstruction (Chapman *et al.*, 1986). In this study evidence is presented that this autoantigen is located within the nuclei of tissue neutrophils. Using isolated peripheral blood neutrophils, a more sensitive assay for anti-neutrophil nuclear antibody (ANNA) has been developed, and its prevalence in PSC and related disorders assessed.

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MATERIALS AND METHODS

Patients

Serum samples were obtained from six groups of patients (Table 1). Group 1 consisted of 25 healthy subjects and two with extrahepatic bile duct obstruction. Group 2 was composed of 14 patients with coeliac disease; 12 were untreated and 10 were HLA-B8-positive. Group 3 consisted of 21 subjects with autoimmune chronic liver disease (CLD): 10 with primary biliary cirrhosis (PBC) and 11 with autoimmune chronic active hepatitis (CAH). Seven of those with CAH had a positive tissue-non-specific ANA.

Groups 4 and 5 consisted of 76 subjects with inflammatory bowel disease (IBD) and no evidence of liver disease; 22 were taking a steroid preparation and/or azathioprine. Forty-three (group 5) had current or recent (i.e. within the last year) evidence of active disease, while 33 (group 4) did not.

Group 6 consisted of 32 subjects with a diagnosis of PSC made on the basis of conventional cholangiographic and histological criteria. All but two had associated ulcerative colitis or Crohn's disease although in only two had this been recently active. Thirteen (41%) had experienced symptoms attributable to PSC and three had established cirrhosis on liver biopsy. The tissue-non-specific ANA was positive (titre $\geqslant 1$ in 40) in four (13%). Fifteen (47%) were HLA-B8$^+$.

Table 1. Details of the study groups

Group	*n*	M/F	Age range (years)	UC	Crohn's
Control	27	15/12	21–83		
Coeliac	14	3/11	19–68		
CLD	21	3/18	36–76		
Inactive IBD	33	17/16	23–77	23	10
Active IBD	43	23/20	15–78	31	12
PSC	32	23/9	21–82	27	3

CLD autoimmune chronic liver disease; IBD inflammatory bowel disease; PSC primary sclerosing cholangitis.

Double labelling studies

Cryostat sections of obstructed human liver were fixed in alcohol and double-labelled using standard immunoperoxidase and alkaline phosphatase/anti-alkaline phosphatase (APAAP) techniques (Cordell *et al.*, 1984; Erber, Pinching & Mason, 1984). Briefly, sections were incubated for 30 min with serum (diluted 1/10) from subjects previously shown to possess 'portal tract' antibody. Following blocking with normal swine serum (1/5) the sections were then incubated with peroxidase-labelled rabbit antibody to human immunoglobulins G, A and M (Dako Laboratories) (1/200) and developed with 3,3'-diaminobenzidine hydrochloride/hydrogen peroxide (DAB).

The same sections were then incubated with either E29 or Np57, mouse IgG monoclonal antibodies to epithelial membrane antigen and neutrophil elastase respectively (courtesy of Dr D.Y. Mason, Oxford). This was followed by incubation with rabbit antibody to mouse immunoglobulin (Dako Laboratories) (1/25) and then with APAAP complex. Finally the sections were developed using substrate containing fast red TR salt. Appropriate controls were performed to exclude non-specific binding.

Anti-neutrophil nuclear antibody (ANNA) assay

Peripheral blood polymorphs were isolated by centrifuging fresh blood samples from healthy volunteers through Mono-Poly Resolving Medium (Flow Laboratories). The polymorph layer was carfully pipetted off, washed in a 5% solution of fetal calf serum in PBS, applied to microscope slides using a cytospin technique and finally fixed in absolute alcohol for 5 min.

Serial dilutions of patient serum were incubated with the polymorph cytospin preparations for 30 min at room temperature. The preparations were then incubated with peroxidase-labelled rabbit antibody to human immunoglobulins G, A and M (Dako Laboratories) (1/100) and finally developed with DAB. The titre of ANNA was taken as the last dilution at which specific nuclear labelling was discernible under light microscopy.

Statistical analysis

The results were analysed using Student's *t*-test and the Chi-squared test (with Yates' correction) as appropriate. Where multiple comparisons were made, the *P* values obtained were corrected by multiplying by the number of comparisons (Bonferroni's adjustment).

RESULTS

Double labelling studies

The location of the antigen recognized by PSC autoantibodies in the portal tracts of obstructed liver was determined by means of double labelling. Studies with E29, a monoclonal antibody to epithelial membrane antigen, indicated that the 'portal tract antigen' was not related to bile ductules. On the basis of the multilobular appearance of immunoperoxidase-labelled 'portal tract antigen', it was suspected that the antigen might be located in the nucleus of tissue neutrophils. Using Np57, a monoclonal antibody to neutrophil elastase, a florid neutrophil infiltrate was demonstrated in the portal tracts of obstructed liver. The 'portal tract antigen' was consistently shown to be surrounded by a ring of neutrophil cytoplasm.

Anti-neutrophil nuclear antibody (ANNA)

The presence of ANNA in serum known to contain 'portal tract' antibody was confirmed using isolated peripheral blood polymorphs (Fig. 1). There was complete concordance between the presence of 'portal tract' antibody and high titre ANNA for sera from 15 subjects with PSC tested in both systems.

Examination of other white blood cells in mixed leucocyte preparations revealed that most PSC and IBD sera labelled neutrophil nuclei only. Labelling of monocytes and lymphocytes appeared to occur only in the presence of significant titres of tissue non-specific ANA, and was seen mostly in sera from the subjects with chronic active hepatitis. Labelling of monocytes and lymphocytes without co-existent labelling of neutrophils was never seen. Although both neutrophil-specific and tissue-non-specific ANA could contribute to the ANNA identified in this study, formal absorption studies to distinguish the two were not employed because the prevalence of tissue-non-specific ANA was so low in the PSC and IBD populations. Only one subject, from the PSC group, had serum containing antibody to the nuclei of both neutrophils and eosinophils.

Peripheral blood neutrophils from subjects with high titre ANNA, washed throroughly prior to fixing, were negative for bound ANNA unless they were re-exposed to ANNA-positive serum after fixation. There was thus no evidence of binding of ANNA to intact circulating neutrophils *in vivo*.

ANNA prevalence studies

The distribution of ANNA titres is shown in Fig. 2. Only 12% of the control group had detectable ANNA, none with a titre of greater than 1/10. In contrast, 84% with PSC possessed ANNA, with a median titre of 1/1000 and a peak titre of 1/500 000. Similarly, 86% with IBD were ANNA-positive, though the median titre was somewhat lower at 1/10. In the CLD group, 52% (seven with CAH, four with PBC) had detectable ANNA. There was no difference between the groups in the pattern of nuclear labelling.

In the IBD group, there was no relationship between ANNA titre and the type of IBD (UC v Crohn's) or the time since initial diagnosis. However, 65% of those with recently active IBD possessed ANNA at a titre of greater than 1/10, compared to only 27% of those with inactive disease ($\chi^2 = 12{\cdot}3$, corrected $P < 0{\cdot}002$).

The distribution of ANNA between the major classes of immunoglobulin was assessed in 10 ANNA-positive subjects with IBD alone and 10 with PSC, using the appropriate

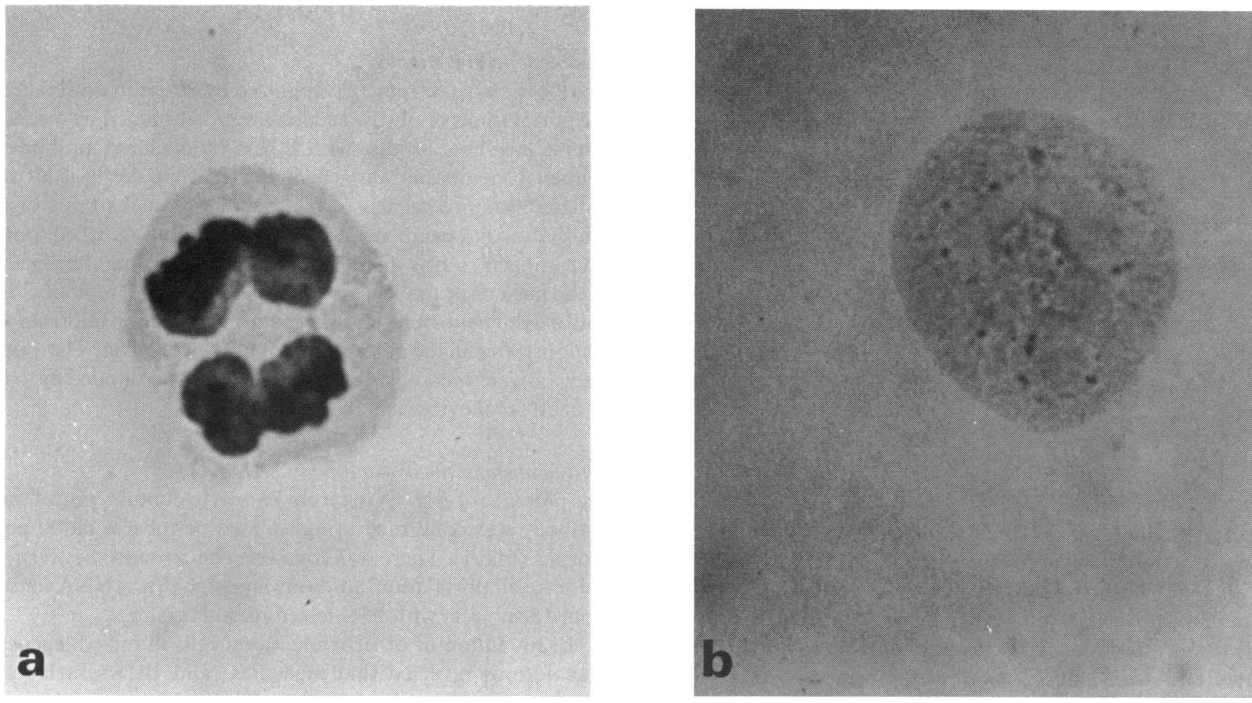

Fig. 1. Immunoperoxidase demonstration of ANNA. Normal peripheral blood neutrophils previously incubated with serum from a patient with PSC (a) and control serum (b).

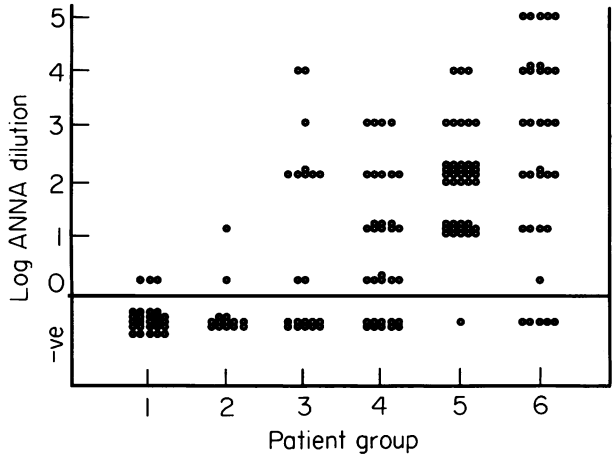

Fig. 2. The distribution of ANNA titres in healthy controls (1), coeliac disease (2), autoimmune chronic liver disease (3), inactive IBD (4), active IBD (5) and PSC (6).

peroxidase-labelled rabbit antibody. In all but one case IgG, IgA and IgM ANNA were detectable, though IgG was generally the dominant class.

Clinical correlates of ANNA in PSC

In the PSC group the presence of high-titre ANNA was examined for correlation with a range of clinical parameters. ANNA titre correlated significantly with serum levels of aspartate transaminase (AST), alkaline phosphatase and total immunoglobulin, but only the correlation with AST remained significant after correction for the number of comparisons made (corrected $P < 0{\cdot}05$). Although there was a trend towards association of high-titre ANNA with symptomatic PSC, major cholangiographic changes, the HLA B8 allele, and the presence of piecemeal necrosis and a major degree of fibrosis on biopsy histology, none were statistically significant. The mean peripheral blood neutrophil count was normal in both subsets, and there was no significant difference between the two.

DISCUSSION

Since first reported (Calabresi, Edwards & Schilling, 1959), circulating autoantibody to neutrophil-specific nuclear antigen has been described in patients with rheumatoid arthritis, in particular those with Felty's syndrome, systemic lupus erythematosus, autoimmune chronic active hepatitis, myasthenia gravis and some seronegative arthritides (Calabresi, Thayer & Spiro, 1961; Faber & Elling, 1966; Smalley, Mackay & Whittingham, 1968; Rosenberg, Johnson & Holborow, 1979; Whittingham *et al.*, 1981). There are grounds for supposing that autoimmune mechanisms may contribute to the pathogenesis of all of these disorders, but ANNA is not simply a non-specific manifestation of autoimmune disease because it is not a feature of pernicious anaemia (Faber a Elling, 1966) or idiopathic thrombocytopaenic purpura (Calabresi *et al.*, 1959), and in this study none of 14 patients with coeliac disease had significant titres. Nor is it an entirely non-specific marker of inflammation or tissue destruction because it is not seen in alcoholic liver disease, sacroidosis, psoriasis or neoplasia (Calabresi *et al.*, 1959; Calabresi *et al.*, 1961; Faber & Elling, 1966; Rosenberg *et al.*, 1979; Whittingham *et al.*, 1981).

The high prevalence of an autoantibody in PSC and IBD is evidence that autoimmune mechanisms may be of importance in their pathogenesis. This suggestion is strengthened by the selective association of ANNA with other conditions, mentioned above, that are more firmly established autoimmune

disorders. However, it should be stressed that ANNA itself has yet to be shown to be of any direct pathogenetic importance; in view of the relative lack of disease specificity it may well have none.

The stimulus to the generation of ANNA is unknown; if the situation resembles that for tissue-non-specific ANA, the immunogen may be one of a diverse range of antigens with which the antibody cross-reacts (Isenberg & Shoenfeld, 1987). While the intriguing possibility of cross-reactivity with a gut bacterial antigen remains, it seems most likely that the immunogen is generated by breakdown of tissue neutrophils at the site of active disease. The nature of the neutrophil antigen to which ANNA binds is also unknown. The assumption that it is a nucleic acid and/or nuclear protein may be invalid, because it has been shown that with most methods of in-vitro preparation constituents of the neutrophil cytoplasmic granules such as lactoferrin may migrate to give the immunocytochemical appearance of a nuclear distribution (Briggs *et al.*, 1981).

The literature contains four studies of ANNA in IBD. In a selected population of 24 colitics with a high prevalence of systemic manifestations, ANNA was present in 18 while only four of 13 post-colectomy patients had ANNA (Calabresi *et al.*, 1961). Studies of unselected colitics have provided prevalence figures for ANNA of 0% (Faber & Elling, 1966), 44% (Thayer & Spiro, 1963) and 45% (Nielsen, Wiik & Elmgreen, 1983), all rather lower than our figures of 86% overall and 98% for recently active disease. This disparity presumably reflects differences in sensitivity and specificity of the techniques used to display ANNA.

In conclusion, we have identified a circulating autoantibody, with affinity for a neutrophil-specific nuclear antigen, in both primary sclerosing cholangitis and inflammatory bowel disease. Antibody titres appear to be related to disease activity. Although the antibody itself may be an epiphenomenon, the finding provides further evidence for the involvement of autoimmune mechanisms in primary sclerosing cholangitis and inflammatory bowel disease.

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