

Antineutrophil Cytoplasmic Antibodies (ANCAs) in Patients with Inflammatory Bowel Disease Show No Correlation with Proteinase 3, Lactoferrin, Myeloperoxidase, Elastase, Cathepsin G and Lysozyme: A Singapore Study

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Abstract

Introduction: The pathogenic importance of antineutrophil cytoplasmic antibodies (ANCAs) in inflammatory bowel disease (IBD) is unclear and target antigen localisation studies may lend insight to the specific pathogenic mechanisms of IBD. In this pilot study, we looked at occurrence of ANCA in Asian IBD patients. In ANCA-positive samples, we analysed for the presence of target antigens i.e. proteinase 3, lactoferrin, myeloperoxidase, elastase, cathepsin G and lysozyme. **Materials and Methods:** This prospective study was carried out from July 1997 to February 1998. Sera were screened for ANCAs with indirect immunofluorescent test and tested with an enzyme immunoassay (ELISA) kit which provides a semi-quantitative assay for human IgG autoantibodies against 6 antigens: proteinase 3, lactoferrin, myeloperoxidase, elastase, cathepsin G and lysozyme. **Results:** A total of 75 patients were studied: 50 with IBD and 25 controls with functional bowel disease. Ten had Crohn's disease (CD) and 40 had ulcerative colitis (UC). There was no racial predilection among the Chinese, Malays or Indians. In CD, 1 was positive for cytoplasmic ANCA (cANCA) and 2 for perinuclear ANCA (pANCA). In UC, 4 were positive for pANCA, 15 for atypical perinuclear ANCA (apANCA) and 1 for cANCA. In the CD and UC population, the proportion positive for ANCA was 30% and 50%, respectively. There was no ANCA detected among the controls. Of those ANCA-positive IBD patients (n:23), only 1 demonstrated anti-myeloperoxidase antibodies. No antibodies were detected against the other 5 antigens tested. **Conclusions:** This pilot Singapore study concludes that there is no significant ANCA association with proteinase 3, lactoferrin, myeloperoxidase, elastase, cathepsin G and lysozyme.

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Introduction

The diagnostic tests currently employed to diagnose inflammatory bowel disease (IBD) and to differentiate between Crohn's disease (CD) and ulcerative colitis (UC) are both expensive and invasive. Serological markers that are easily applied which can distinguish between CD and UC with comparable sensitivity and specificity are lacking. Antineutrophil cytoplasmic antibody (ANCA) was one such serological test that has been extensively studied in CD and UC.^{1,2} With a standard indirect immunofluorescent test, ANCAs give two distinct classic patterns on ethanol-fixed neutrophils—a perinuclear pattern (pANCA) and a cytoplasmic pattern (cANCA). Recently, an atypical perinuclear pattern (apANCA) was also described.³

However, no apparent consistent correlation between the presence and titre of pANCA and IBD duration, activity, disease localisation, patient age, sex or treatment has been demonstrated.⁴ In contrast, cANCA is known as a specific marker for Wegener's granulomatosis.⁵ In general, pANCA has been more associated with IBD than cANCA in addition to being a more frequent serological finding in UC individuals.⁶

ANCA has failed to be the ideal biological disease marker whose properties should include the ability to identify the individual at risk of a given disorder, be disease-specific, mirror the disease activity and be easily applicable for routine clinical purposes. Hence, the search widens for an extended role of the disease-causing antigen

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in ANCA-positive individuals. It is hoped that the target antigen localisation of ANCA-positive individuals might lend more insight to the specific pathogenic mechanisms of IBD, given that the aetiology of IBD remains unresolved with many possibilities including microbial elements, antigen mimicry and a dysregulated cytokine milieu.

Many studies looking at target antigen localisation have not yielded consistent results.⁷⁻¹² Previous studies looking at possible antigens associated with ANCA have not found any leads with myeloperoxidase,^{13,14} proteinase 3¹³ or elastase.^{2,13,14} However, some researchers found an association between pANCA and lactoferrin^{8,9} and cathepsin G,⁷ but this was not reproducible in other studies.

There is a paucity of data in the medical literature involving Southeast Asian IBD populations with regards to the above. In the hope of achieving a greater understanding of the aetiopathogenesis of IBD, this pilot study reports the occurrence of ANCA in IBD with a specific interest to localise target antigens. The latter is performed by testing for antibodies to proteinase 3, lactoferrin, myeloperoxidase, elastase, cathepsin G and lysozyme.

Materials and Methods

A prospective study was carried out in the Singapore General Hospital from July 1997 to February 1998. Singapore General Hospital is the largest acute tertiary general hospital in Singapore with a bed complement of 1617 beds. The predominant races in Singapore include Chinese (77.2%), Malay (14.1%) and Indian (7.4%).

We studied 50 patients with IBD, i.e. UC and CD, and 25 controls with functional bowel disease. Of the 50 patients with IBD, 40 had UC and 10 had CD. Consent was obtained from all patients.

All sera from IBD patients and controls were screened for ANCA by indirect immunofluorescence (IFT) using *Euroimmun 6 biochips IFT system* (EUROIMMUN Laboratorium, Lubeck/Germany) for the determination of autoantibodies against the cytoplasm of granulocytes, exocrine pancreas and goblet cells.

The sera obtained were diluted 1:10 in phosphate buffer saline (PBS) and 60 µl of the diluted serum was transferred onto the reagent support. The slides of 6 biochips combination (ethanol-fixed granulocytes/acetone-fixed granulocytes/formalin-fixed granulocytes/monkey liver/monkey intestinal tissue/monkey pancreas) were placed onto the support and incubated for 30 minutes at room temperature.

After being washed with PBS for 10 minutes and incubated for 30 minutes with a 1:5 dilution of fluorescein isothiocyanate (FITC) anti-human immunoglobulin (Ig) G antibodies, the slides were again washed in PBS for 10

minutes and finally mounted under a cover slip with PBS-glycerol pH 8.4. The slides were then examined under a fluorescence microscope with magnifications of x250 and x400. Sera giving a typical perinuclear reaction with the neutrophils fixed with ethanol, and cytoplasmic staining reaction with the neutrophils fixed with formalin were regarded as positive for pANCA. If the sera showed perinuclear staining with ethanol-fixed neutrophils but negative with formalin-fixed neutrophils and the monkey liver cells, atypical pANCA (apANCA) were reported. Sera were examined for goblet cell antibodies with monkey intestinal tissue. The presence of antibodies to acini cells and exocrine glands were determined using monkey pancreas.

Samples giving reaction with the neutrophils, regardless of the patterns seen in the IFT, were tested with the *Euroimmun ANCA profile ELISA test kit* (EUROIMMUN Laboratorium, Lubeck/Germany). The ELISA test kit provided a semiquantitative assay for human auto antibodies of the IgG class against six different types of neutrophilic cytoplasmic antigens: anti-proteinase 3, anti-lactoferrin, anti-myeloperoxidase, anti-elastase, anti-cathepsin G and anti-lysozyme. The reagent wells were each coated with different antigens. Diluted patient samples were added to the wells and incubated. Upon incubation, specific human IgG (also IgA and IgM) antibodies present in the serum would bind to the antigens. Unbound proteins were removed by washing and an enzyme-labelled anti-human IgG conjugate was added to the well. After incubation, the unbound conjugate was removed by washing. The substrate subsequently added underwent colour change in the presence of the conjugated enzyme. After stopping the enzymatic production of coloured product, the concentration of the antibodies in the serum was determined by means of an ELISA reader at 450 nm.

Results

A total of 75 patients were studied. Fifty patients had IBD, diagnosed by standard radiological, endoscopic and/or histologic criteria. Twenty-five patients with functional bowel disease were recruited as controls after being evaluated by a gastroenterologist. There were 15 men and 10 women in the control group with a median age of 41 years. Ten (1 man, 9 women) had CD (median age 35.5 years) and 40 (25 men, 15 women) had UC (median age 39.5 years). There was no racial predilection among the Chinese, Malays and Indians.

In patients with CD, 1 had cANCA, 2 had pANCA and another 3 had antibodies to pancreatic exocrine and acini glands. In patients with UC, 4 had pANCA, 15 had apANCA, 1 had cANCA, 7 had antibodies to goblet cells and 2 had antibodies to pancreatic exocrine and acini

glands. In CD and UC, the proportion who had the presence of ANCA antibodies was 30% and 50%, respectively. Among the controls, while none had any ANCA, there was 1 positive for pancreatic exocrine and acini glands and another positive for antibodies to goblet cells. Of all those with IBD and positive for ANCA ($n = 23$), only 1 patient had antibodies against myeloperoxidase. No antibodies were detected against the other 5 antigens tested. pANCA and apANCA are the predominant ANCA patterns seen in IBD. There was a greater number of ANCA-positive patients seen in UC; however, the P value of this finding was 0.2. Statistical significance was demonstrated when comparing ANCA positivity between CD and normal individuals ($P < 0.05$), and between UC patients and controls ($P < 0.001$). The sensitivity and specificity of ANCA in our patients with IBD were 46% and 100%, respectively (Table I). There was no correlation between disease activity in our IBD patients with ANCA reactivity.

TABLE I: DISTRIBUTION OF IBD PATIENTS AND CONTROLS WITH CYTOPLASMIC, PERINUCLEAR AND ATYPICAL PERINUCLEAR ANTINEUTROPHIL CYTOPLASMIC ANTIBODIES

Diagnosis (n)	cANCA	pANCA	apANCA	ANCA
CD (10)	1	2	0	3
UC (40)	1	4	15	20
Controls (25)	0	0	0	0
Sensitivity	-	-	-	46%
Specificity	-	-	-	100%

Sensitivity and specificity values for ANCA-positive IBD patients as compared to controls

Discussion

Many previous studies have alluded to different target antigens in IBD. To this day, the true pathogenic significance of autoantibodies in IBD remains in doubt as conflicting reports continue to surface. This study is the first work in this respect from Southeast Asia studying Chinese, Indian and Malay patients. In a Swedish study by Peen et al,⁹ high frequencies of IgG anti-lactoferrin antibodies (50%) were reported in sera from patients with UC. Another European report found 22% of UC associated pANCA reacted with lactoferrin, and another 19% with cathepsin G.⁷ In the search for antibodies against β -glucuronidase, Nassberger et al¹⁰ showed, in a Swedish population subgroup, that as many as 57% of their patients with UC had a positive reaction. In contrast, Kaneko et al,¹¹ looking at Japanese UC patients, found no such reaction to the same antigen in any of their samples. However, a more recent Japanese report that looked at both UC and CD patients reported reactivity of their ANCA-positive patients with lactoferrin,

myeloperoxidase and cathepsin G.¹⁵ Nonetheless, the paper could not assign any clinical correlation to the discovery of those putative antigens.

Only 1 of our patients with UC, who was positive for ANCA, demonstrated antibodies to myeloperoxidase. None of our patients who were positive for ANCA had any antibodies against the other 5 antigens tested for, i.e. proteinase 3, lactoferrin, elastase, cathepsin G and lysozyme. The antigens responsible for the positive ANCA reactions remain unknown. There have been speculations that these may be related to bactericidal-permeability increasing protein (BPI),^{16,17} tropomyosin,¹⁸ catalase or α -enolase.¹⁹ In addition, newer antigens, such as histone H1 protein²⁰ and azurocidin,¹⁷ have been ascribed to react with ANCA-positive sera. Cohavy et al²¹ reported that a pANCA monoclonal antibody detects a recurrent epitope expressed by colonic bacteria and this, in turn, implicates colonic bacterial proteins as targets of the disease associated immune response.

The sensitivity and specificity of ANCA in our patients with IBD are comparable with the published literature.^{2,13} There is a fairly wide range of numbers quoted with this regard; but this may be related to the lack of standardisation of the methodology and/or, in part, due to disease heterogeneity.

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