

## INFLAMMATORY BOWEL DISEASE

## Serum antineutrophil cytoplasmic autoantibodies in inflammatory bowel disease are mainly associated with ulcerative colitis. A correlation study between perinuclear antineutrophil cytoplasmic autoantibodies and clinical parameters, medical, and surgical treatment

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### Abstract

Perinuclear antineutrophil cytoplasmic antibodies have recently been demonstrated in the sera of patients with inflammatory bowel disease. Three hundred and sixty six sera obtained from 120 patients with ulcerative colitis, 105 patients suffering from Crohn's disease and 49 non-inflammatory bowel disease controls were tested in two laboratories, using an indirect immunofluorescence assay. In addition, a fixed-neutrophil enzyme linked immunoabsorbent assay (ELISA) was evaluated in one of the two laboratories. The results in the immunofluorescence test showed a high degree of correlation between the two laboratories (Kappa coefficient=0.8). Ninety five of the 120 (79%) ulcerative colitis patients had a positive test whereas only 14 of the 105 (13%) patients with Crohn's disease were positive. Sera from four patients suffering from primary sclerosing cholangitis were positive as well as four of the 45 control sera (9%). The sensitivity of the perinuclear antineutrophil cytoplasmic antibody immunofluorescence test for the diagnosis of ulcerative colitis was 0.75 with a specificity of 0.88 and a positive predictive value of 0.88 (all sera). In the ELISA technique 37 of 94 ulcerative colitis sera and one of the 68 Crohn's disease sera were positive. In the control group only one of the patients suffering from primary sclerosing cholangitis reacted positively (32 non-inflammatory bowel disease sera tested). The ELISA technique had a high specificity (0.97), but a low sensitivity (0.39). There was no relation of perinuclear antineutrophil cytoplasmic antibodies in ulcerative colitis patients or in Crohn's disease patients with disease activity, duration of illness, localisation, extent of disease, previous bowel operations or medical treatment. The clinical significance of perinuclear antineutrophil cytoplasmic antibody positive and negative subsets in both groups of patients thus remains unexplained. Our study confirms that determination of serum anti-

neutrophil cytoplasmic antibodies in patients with inflammatory bowel disease may differentiate ulcerative colitis from Crohn's disease. Further immunological studies are needed to explain the absence of these antibodies in a subset of ulcerative colitis patients and their role in the pathogenesis of the disease.

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Ulcerative colitis and Crohn's disease belong to the group of idiopathic inflammatory bowel diseases and could theoretically be extremes of

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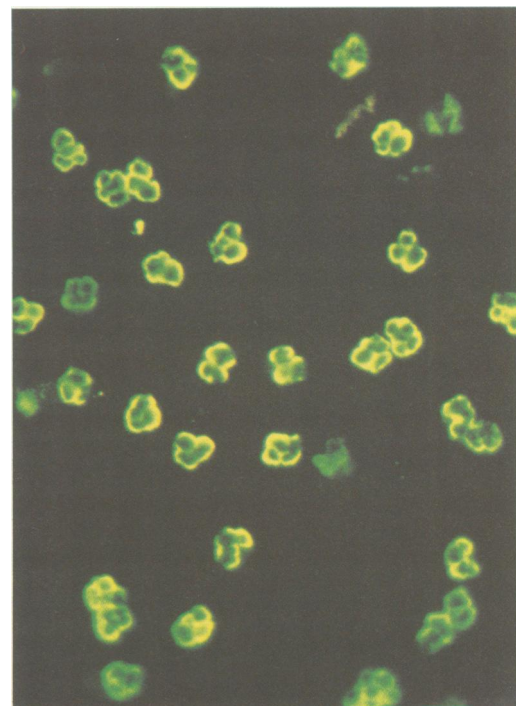


Figure 1: Immunofluorescence perinuclear binding pattern in serum from a patient with ulcerative colitis.

the same disease. It is more likely, however, that they are fundamentally different. With regard to the diagnosis in approximately 10% of inflammatory bowel disease patients with colonic involvement a definite distinction cannot be made.<sup>1,2</sup> Antineutrophil cytoplasmic antibodies are used in diagnosing vasculitic diseases. In these diseases the related antigens are enzymes in the granules of neutrophils.<sup>3-5</sup> Also in patients with inflammatory bowel disease antineutrophil cytoplasmic antibodies have been detected. The fluorescence pattern of inflammatory bowel disease associated antineutrophil cytoplasmic antibodies is characteristically perinuclear (Fig 1). It has been agreed to call these antibodies perinuclear antineutrophil cytoplasmic antibodies to differentiate them from the classical cANCA present in Wegener's granulomatosis. Saxon *et al* reported that IgG perinuclear antineutrophil cytoplasmic antibodies could be detected in the sera of the majority of patients with ulcerative colitis and in a much smaller percentage of the sera of patients with Crohn's disease.<sup>6</sup> We showed equivalent results in a large group of European patients (n=274) using an indirect immunofluorescent technique.<sup>7</sup> In the present study we have determined the sensitivity and specificity of antineutrophil cytoplasmic antibodies as measured either by immunofluorescence or fixed neutrophil ELISA tests. In addition, we have investigated the possible relation of the presence or absence of these antibodies with clinical and biochemical parameters, disease activity and extent of disease, and medical or surgical treatment.

## Methods

### STUDY POPULATION AND SERUM SPECIMENS

Three hundred and sixty six serum samples were obtained from 274 unrelated patients attending the departments of Gastroenterology and Internal Medicine over a nine month period. The diagnosis of ulcerative colitis or Crohn's disease was based on conventional clinicopathologic criteria according to those described by Lennard-Jones *et al*.<sup>8</sup> We excluded 12 patients because no definite diagnosis of ulcerative colitis or Crohn's disease could be made at the time of testing. Sera from patients positive for antinuclear antibodies were also excluded (15% in ulcerative colitis; 8.5% in Crohn's disease). One hundred and twenty ulcerative colitis patients, 105 Crohn's disease patients and 49 controls were studied. The mean age was 40 years, an equal male/female distribution was found. Control subjects were patients from the same outpatient clinics, matched for sex and age to the inflammatory bowel disease patients without evidence of inflammatory bowel disease. In this particular control group nine patients suffered from diarrhoea, 15 patients had spastic colon, 10 suffered from gastritis, one patient had Wegener's granulomatosis, in 10 patients we diagnosed diverticulosis and five had carcinoma of different origin.

The disease activity in Crohn's disease was assessed using the CDAI according to Best *et al*<sup>9</sup> and van Hees *et al*<sup>10</sup> and in ulcerative colitis the

Sutherland score was used.<sup>11</sup> The patient's disease activity ranged from mild to severe at the time of serum collection. In ulcerative colitis, 114 patients scored 0-3, 33 scored 4-7, 16 scored 8-10, and nine patients over 10 at the time of serum collection (0=quiet; 12=very severe disease activity). All sera were stored at -20°C until assay. Perinuclear antineutrophil cytoplasmic antibody screening was performed in a blind prospective transversal setting. One hundred and seventy two serum samples from 120 patients suffering from ulcerative colitis, 145 from 105 Crohn's disease patients, and 49 control sera were tested in two laboratories. From 84 patients (37 suffering from ulcerative colitis and 47 from Crohn's disease) sera were collected at different timepoints for follow up of antineutrophil cytoplasmic antibody reactivity in relation to clinical parameters.

### INDIRECT IMMUNOFLUORESCENCE ASSAY

The standard antineutrophil cytoplasmic antibodies indirect immunofluorescence assay<sup>12</sup> was performed in two laboratories (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Immunohaematological Laboratory of the Free University Hospital). In short, human peripheral blood neutrophils were smeared on eight well Nutacon slides and fixed in 96% ethanol (15 minutes 4°C). Slides were incubated with 1:16 diluted patient's sera and stained with fluorescein conjugated rabbit anti-human IgG antibodies. The slides were evaluated by fluorescence microscopy. Depending on brightness of immunofluorescence staining pattern, the reactions were graded into negative (-), weak (+), positive (++), and strong positive (+++).

### FIXED NEUTROPHIL ELISA TECHNIQUE

According to the technique described by Saxon *et al*<sup>6</sup> microtitre plates were coated with a monolayer of granulocytes, fixed in methanol, and air dried. Sera were tested at a 1:40 dilution and bound antibody was detected with alkaline phosphatase conjugated goat antihuman gamma chain specific antibody. 3 SD above the mean of the negative controls was considered positive.

### STATISTICAL ANALYSIS

To measure interobserver variation of indirect immunofluorescence testing of the 366 sera Cohen's kappa coefficient was used. Kappa coefficients above 0.75 signify excellent agreement.<sup>13</sup> Sensitivity was defined as the ratio of true

*Comparison of results obtained by ELISA or indirect immunofluorescence assay in 94 ulcerative colitis and 68 Crohn's disease patients*

	ELISA (n)	ELISA (n)
Ulcerative colitis	+	-
IFA (n) +	31	31
IFA (n) -	6	26
Crohn's disease	+	-
IFA (n) +	1	4
IFA (n) -	0	63

n=Number of patients.

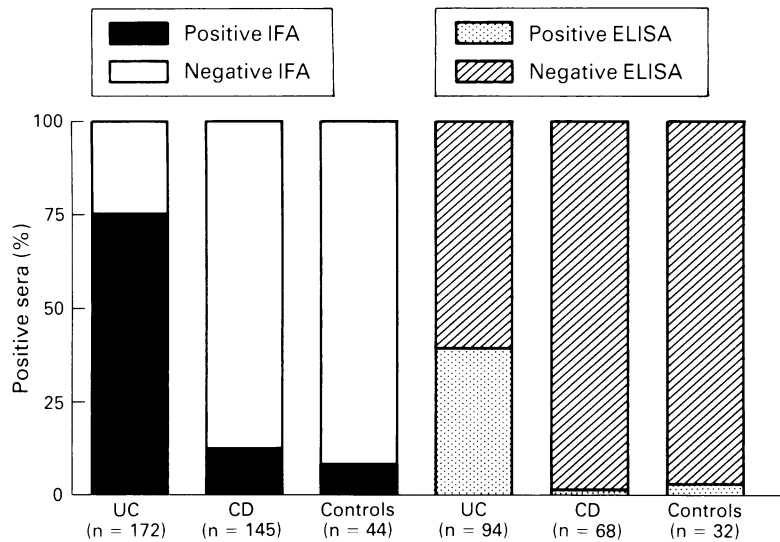


Figure 2: Perinuclear antineutrophil cytoplasmic antibody (pANCA) reactivity of sera from ulcerative colitis (UC) patients, Crohn's disease (CD) patients and normal controls with indirect immunofluorescence assay (left) versus their binding in the fixed whole cell ELISA (right).

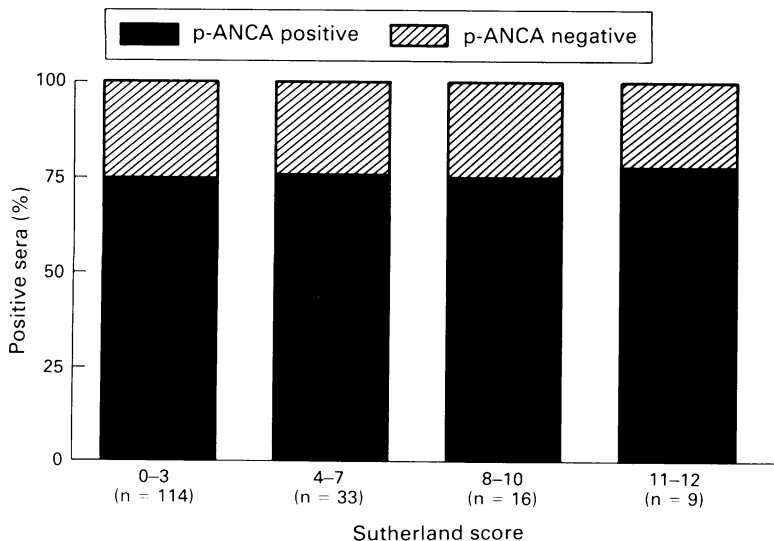
positives to true positives plus false negatives; specificity as that of true negatives to true negatives plus false positives; positive predictive value as that of true positives to true positives and false positives, and negative predictive value as that of true negatives to true negatives plus false negatives.<sup>14</sup> To measure associations, Pearson's correlation coefficient was used.

## Results

### INTERLABORATORY CORRELATION OF THE PERINUCLEAR ANTINEUTROPHIL CYTOPLASMIC ANTIBODY IMMUNOFLUORESCENCE TEST

The same immunofluorescence test procedure for the determination of perinuclear antineutrophil cytoplasmic antibodies in the serum of patients and controls was followed in two independent laboratories. Reproducibility in these laboratories showed an interlaboratory variation maximal up to 14% (177 sera perinuclear antineutrophil cytoplasmic antibody positive, 129 sera perinuclear antineutrophil cytoplasmic antibody negative in both laboratories; 48 sera different scored), thus excellent agreement was shown (kappa score 0.80).<sup>13</sup>

Figure 3: Perinuclear antineutrophil cytoplasmic antibody (pANCA) reactivity in 172 sera from ulcerative colitis (UC) patients in relation to the disease activity.



### COMPARISON OF PERINUCLEAR ANTINEUTROPHIL CYTOPLASMIC ANTIBODY MEASUREMENTS BY INDIRECT IMMUNOFLUORESCENCE ASSAY AND ELISA AND THEIR VALUE IN THE DIFFERENTIAL DIAGNOSIS OF INFLAMMATORY BOWEL DISEASE

In the indirect immunofluorescence assay method the sensitivity of perinuclear antineutrophil cytoplasmic antibodies for ulcerative colitis was 0.75 and the specificity 0.88; using the ELISA technique our figures were 0.39 and 0.97 respectively (Fig 2). For ulcerative colitis, the positive predictive value of a positive perinuclear antineutrophil cytoplasmic antibody test in the indirect immunofluorescence assay was 0.88, the negative predictive value of a negative perinuclear antineutrophil cytoplasmic antibody test was 0.75.

The Table gives the exact correlation between ELISA and indirect immunofluorescence assay results in the ELISA tested subgroup. Only 14 out of 105 Crohn's disease patients showed perinuclear antineutrophil cytoplasmic antibody reactivity by indirect immunofluorescence assay. In the ELISA technique only one of 68 Crohn's disease patients was positive for perinuclear antineutrophil cytoplasmic antibodies. In contrast, sera from all patients suffering from primary sclerosing cholangitis show a bright 3+ staining in the indirect immunofluorescence assay.

The ELISA technique proved far less sensitive for identification of ulcerative colitis patients in inflammatory bowel disease but showed a higher specificity than did the indirect immunofluorescence assay.

### RELATIONSHIP OF PERINUCLEAR ANTINEUTROPHIL CYTOPLASMIC ANTIBODIES WITH DISEASE ACTIVITY, HAEMATOLOGICAL, BIOCHEMICAL PARAMETERS, EXTENT, AND EFFECT OF TREATMENT IN ULKERATIVE COLITIS

Disease activity was assessed in 120 ulcerative colitis patients based on the criteria as outlined by Sutherland *et al.* In a longitudinal study of 37 patients a significant difference in activity index before and after treatment was observed, irrespective of the kind of treatment – for example, corticosteroids, 5 ASA, SASP, or surgery. The activity index ranging from 0–12 decreased 3.2 points on average ( $p=0.0046$ , Student's *t* test for paired observations). No significant relationship was found between positivity of perinuclear antineutrophil cytoplasmic antibodies and ulcerative colitis activity. Furthermore, all patients that were perinuclear antineutrophil cytoplasmic antibody positive before treatment remained positive after treatment, and no apparent decrease in staining intensity was noted.

A positive correlation was found between disease activity and the number of peripheral blood leucocytes ( $r=0.44$ ;  $p<0.001$ ). A significant negative correlation was observed between disease activity and haematological and biochemical parameters, in particular haemoglobin ( $r=-0.271$ ;  $p=0.004$ ), serum albumin ( $r=-0.486$ ;  $p<0.001$ ) and total serum protein ( $r=-0.450$ ;  $p<0.001$ ). These haematological and biochemical parameters, however, were not

related to the presence of perinuclear antineutrophil cytoplasmic antibody reactivity, except for a negative correlation between the presence of perinuclear antineutrophil cytoplasmic antibodies and serum albumin concentration ( $r = -0.215$ ;  $p = 0.031$ ).

No difference in fluorescence intensity level (1–3) nor difference in percentage of perinuclear antineutrophil cytoplasmic antibody positive patients among patients suffering from proctitis ( $n = 13$ ), proctosigmoiditis ( $n = 56$ ), and pancolitis ( $n = 38$ ) was found.

In patients with ulcerative colitis who underwent a total (nine) or subtotal (five) colectomy the percentage of perinuclear antineutrophil cytoplasmic antibody positivity remained unchanged regardless of the time passed after colectomy (maximum duration of follow up was over 20 years).

### Discussion

It has been shown that the perinuclear antineutrophil cytoplasmic antibody indirect immunofluorescence test may help to differentiate between Crohn's disease and ulcerative colitis in inflammatory bowel disease patients.<sup>6,7,15,16</sup> In a large blinded study done in two independent laboratories we confirmed the presence of perinuclear antineutrophil cytoplasmic antibodies by the indirect immunofluorescence test in 79% of ulcerative colitis patients whereas only 13% of Crohn's disease patients and 9% of the control group were positive (means from two laboratories).

In contrast with the results of Saxon *et al*<sup>6</sup> our indirect immunofluorescence assay results showed greater sensitivity and a similar specificity compared with the ELISA test. The indirect immunofluorescence assay is suitable for clinical investigation of patients with inflammatory bowel disease: results are reproducible and correlate well in independent laboratories. Our results confirm the presence of a perinuclear antineutrophil cytoplasmic antibody negative subgroup of ulcerative colitis patients. These patients often had clinically, endoscopically, and histologically active disease. They were indistinguishable from perinuclear antineutrophil cytoplasmic antibody positive ulcerative colitis patients (Fig 3).

No satisfactory explanation can be given as to why our ELISA results differ from Saxon *et al*. It is unlikely that the methodology is responsible, because a similar protocol was used. Although we used +3 SD above the mean of negative controls as cut off level for positivity in comparison to the +2 SD used by Saxon *et al* this alone cannot explain the differences. The different source of the neutrophils used in the assay may result in the recognition of different antigens, although Saxon *et al* showed no significant difference in binding with the neutrophils taken from eight normal subjects. The use of a different (European) donor pool can account for the difference.

No correlation was found between perinuclear antineutrophil cytoplasmic antibody positivity and localisation or extent of ulcerative colitis. In contrast with the close relationship of levels of

vasculitis associated antineutrophil cytoplasmic antibodies, no correlation was observed between perinuclear antineutrophil cytoplasmic antibody level and disease activity in inflammatory bowel disease. (Procto-) colectomy did not result in disappearance of perinuclear antineutrophil cytoplasmic antibodies, during a follow up period ranging between two months and two years (mean nine months). Antibodies could still be demonstrated in one patient 20 years after a proctocolectomy.

The reaction of perinuclear antineutrophil cytoplasmic antibody titre to medication (corticosteroids or mesalazine) is controversial. Disappearance of perinuclear antineutrophil cytoplasmic antibodies after treatment with corticosteroids has been reported in a small number of patients.<sup>15,16</sup> In a group of 37 ulcerative colitis patients, however, no effect of medication was found.

The pathophysiological importance of the ulcerative colitis associated perinuclear antineutrophil cytoplasmic antibodies is still poorly understood. Evidence has accumulated that several autoimmune phenomena are present in ulcerative colitis patients.<sup>17</sup> Possibly, HLA heterogeneity or the presence of other not yet identified immunogenetic markers may be responsible for the existence of a perinuclear antineutrophil cytoplasmic antibody positive and negative subgroup in ulcerative colitis. The presence of perinuclear antineutrophil cytoplasmic antibodies in unaffected family members of ulcerative colitis patients favours this view.<sup>18</sup> Of particular interest is the observation that primary sclerosing cholangitis patients are also perinuclear antineutrophil cytoplasmic antibody positive.<sup>19</sup> Possibly, ulcerative colitis and primary sclerosing cholangitis associated neutrophil cytoplasmic antibodies recognise the same antigen(s).

In the past, autoantibodies that have been most consistently detected in inflammatory bowel disease are those reactive to colonic cells and lymphocytes.<sup>6,15,20–26</sup> In our study autoantibodies detected in ulcerative colitis are not only directed towards neutrophils, but also to a very small percentage of monocytes (Ellerbroek *et al*; submitted). No reaction with the antigen(s) recognised by vasculitis associated antineutrophil cytoplasmic antibodies – that is myeloperoxidase, elastase, or proteinase 3, could be demonstrated.<sup>6,7,15,27</sup>

In conclusion, our study confirms that perinuclear antineutrophil cytoplasmic antibodies are indeed demonstrable in the large majority of ulcerative colitis patients. Therefore, antibody determination may have a place in the clinical decision making of inflammatory bowel disease patients, since positivity provides strong arguments for the diagnosis of ulcerative colitis.

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