Characterisation of autoantibodies to neutrophil granule constituents among patients with reactive arthritis, rheumatoid arthritis, and ulcerative colitis

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Abstract

Objective—To study the frequency and distribution of antineutrophil cytoplasmic autoantibodies (ANCA) among patients with reactive arthritis (ReA), rheumatoid arthritis (RA), and ulcerative colitis (UC) using different immunological methods.

Methods—Fifty serum samples from patients with reactive arthritis (26 with acute disease and 24 with chronic disease—that is, disease of more than one year) were analysed for ANCA with indirect immunofluorescence, enzyme linked immunosorbent assay (ELISA) with six different neutrophil granule proteins as antigens, and immunoblotting on whole neutrophil extract and extracts of azurophil and specific granules. Thirty serum samples from patients with RA and UC served as controls in ELISA and indirect immunofluorescence.

Results—Sixteen per cent of patients with ReA were positive in immunofluorescence compared with 30% of patients with RA, and 70% of patients with UC. Thirty-two per cent of patients with ReA were positive in ELISA. Antibodies directed against lactoferrin occurred in 20%, antibodies against bactericidal permeability increasing protein (BPI), elastase, cathepsin G, myeloperoxidase, and proteinase 3 were found in 8%, 2%, 2%, 8%, and 6%, respectively. Overall, 50% of RA sera and 53% of UC sera were positive in one or more ELISA assays, the corresponding figures for antibodies against individual antigens were for RA 7%, 3%, 0%, 13%, 47%, 17% and for UC 13%, 20%, 0%, 23%, 10%, and 17%. In immunoblotting, bands corresponding to lactoferrin and BPI were recognised in 44% and 22% of ReA sera.

Conclusion—Antibodies against neutrophil granule antigens are often found in patients with ReA, primarily among those with chronic disease. The different methods detect various subsets of antibodies, with immunoblotting being the most and immunofluorescence the least sensitive.

Antineutrophil cytoplasmic antibodies (ANCA) are diagnostic hallmarks of various forms of small vessel vasculitides.1-3 ANCA directed against proteinase 3 (PR3) are a reliable seromarker for Wegener’s granulomatosis and produce a coarse granular cytoplasmic staining pattern (C-ANCA) on ethanol fixed polymorphonuclear leucocytes.7 The artefactual perinuclear (P-ANCA) staining pattern seen when antibodies are directed against myeloperoxidase (MPO) occurs in other small vessel disease, such as microscopic polyangiitis or the Churg-Strauss syndrome.1 2 Other neutrophil granule proteins may also be targeted by ANCA and give rise to a P-ANCA pattern—for example, lactoferrin (Lf), human leucocyte elastase (HLE), or cathepsin G (Cat G).1 These types of P-ANCA may occur in non-vasculitic conditions like rheumatoid arthritis (RA), systemic lupus erythematosus, and inflammatory bowel disease (IBD).3,5 Antibodies against bactericidal permeability increasing protein (BPI) have been found in IBD, especially ulcerative colitis (UC), and to a lesser extent in Crohn’s disease.10 11 A number of clinical features are shared between IBD and the seronegative spondyloarthropathies, and ANCA, predominantly directed against Lf, have also been shown in sera from patients with reactive arthritis (ReA) as well as in UC.12

This study aimed at investigating the prevalence, possible clinical relevance, and antigen specificity of ANCA in ReA, using three different methods: indirect immunofluorescence (IIF), enzyme linked immunosorbent assay (ELISA), and immunoblotting (IB). Thirty serum samples from patients with UC and RA served as disease controls.

Patients and methods

PATIENTS

Serum samples from 50 consecutive outpatients with ReA were included. They comprised 37 men (mean age 34 years, range 18–52) and 13 women (mean age 40 years, range 22–56). The diagnosis of ReA was based on the following criteria: a mono- or oligo-arthritis, predominantly of the legs, after an enteric or urogenital infection. The triggering microorganisms were identified by culture or serology, routinely performed at the Department of Clinical Microbiology. In cases where no preceding infection could be disclosed the diagnosis of ReA was based on a combination of arthritis and one or more of the following manifestations: acute anterior uveitis/conjunctivitis, urethritis, sacroiliac joint arthritis, circinate balanitis, keratoderma blennorrhagia, or dactylitis. Patients with ReA were divided into acute or chronic (that is, disease of more than one year).
Sixty randomly selected deep frozen serum samples (from the Serumbank, Department of Autoimmunology, Statens Serum Institute, Copenhagen, Denmark) were used as disease controls. Thirty samples were from patients with UC and 30 from patients with RA.

**ANTIBODIES**

Mouse monoclonal antibodies with the following specificities were used: human Lf (2.88 mg/ml) and PR3 (1.4 mg/ml) (Department of Immunology, Statens Serum Institute, Copenhagen, Denmark), BPI, and HLE (1 mg/ml) (Pharmingen, San Diego, CA, USA). We also used rabbit polyclonal antibodies directed against MPO or Cat G (DAKO, Glostrup, Denmark). As secondary antibodies the following were used: alkaline phosphatase conjugated goat antihuman IgG (γ chain specific) (Sigma, St Louis, MO, USA), goat antimouse IgG (Sigma) and goat antirabbit IgG (Sigma), peroxidase conjugated F(ab’), rabbit antihuman IgG (γ chain specific) (DAKO).

**IIF MICROSCOPY**

Freshly isolated human leucocytes were ethanol fixed on microscope slides and used for ANCA detection by IIF microscopy as described in detail elsewhere. Initial dilution of sera was 1:20 in phosphate buffered saline (pH 7.2). Sera were scored as P-ANCA/C-ANCA or negative.

**ENZYME LINKED IMMUNOSORBENT ASSAYS (ELISA)**

**PR3-ANCA ELISA**

Microtitre plates (Maxisorp, Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 µl/well human PR3 (1 µg/ml), in 0.015 M Na2CO3, 0.035 M NaHCO3, 0.02% (w/v) NaN3, pH 9.6) purified from azurophilic granules as described. The purified PR3 was tested in our laboratory and did not react with antibodies against HLE, Cat G, MPO, or Lf. After washing, patient or control sera diluted 1:100 in incubation buffer and incubated for 30 minutes at 37°C on a precoated (PR3, MPO, or Lf respectively) ELISA plate. After 30 minutes sera were aspirated and passed over to the adjacent coated well. This procedure was repeated eight times. Inhibition was considered positive if the difference in U/ml between inhibited or uninhibited serum was >20%.

**MPO-ANCA ELISA**

ELISA kits for the detection of HLE-ANCA and Cat G-ANCA were purchased from Shield Diagnostics Ltd (Dundee, UK) and tests were carried out according to the protocol provided with the kit. Positive cut off value was determined as the mean + 3SD of 20 normal sera.

An ELISA kit for the demonstration of BPI-ANCA was obtained from DLD Diagnostica GmbH (Hamburg, Germany) and used according to the manufacturer’s instructions. The positive cut off value was 4 U/ml.

**INHIBITION ELISA**

A solid phase inhibition experiment was performed on sera positive for antibodies against PR3, MPO, or Lf. Five randomly chosen serum samples from each specificity were diluted 1:100 in incubation buffer and incubated for 30 minutes at 37°C on a precoated (PR3, MPO, or Lf respectively) ELISA plate. After 30 minutes sera were aspirated and passed over to the adjacent coated well. This procedure was repeated eight times. Inhibition was considered positive if the difference in U/ml between inhibited or uninhibited serum was >20%.

**ISOLATION OF NEUTROPHIL GRANULOCYTES AND PREPARATION OF ANTIGEN EXTRACTS**

Human neutrophil granulocytes were isolated from buffy coats as described elsewhere. The neutrophil suspension (3 × 10^6 cells/ml) was sonicated four times for 20 seconds each in 1 M NaCl containing 0.5 mM phenylmethylsulphonyl fluoride (Sigma). Insoluble constituents were removed by centrifugation. Neutrophils were lysed by nitrogen cavitation and subfractionated on Percoll density gradients to yield three distinct bands of azurophilic granules, specific granules, and a membrane fraction. Percoll was removed by ultracentrifugation at 100 000 × g for three hours. The azurophilic and specific granules were extracted by sonication as described for whole cells. These extracts were used in order to identify the reactivities on blots of whole neutrophil extract.

Human lymphocytes (3 × 10^6 cells/ml) were isolated from buffy coats by centrifugation through Lymphoprep (Nycomed Pharma, Oslo, Norway) at 400 g for 15 minutes. The lymphocyte-rich interphase was harvested and resuspended in RPMI containing 10% fetal calf serum. After incubation in a plastic flask (Nunc) for one hour at 37°C in a CO2 incubator to let monocytes adhere, the lymphocytes were pelleted, extracted by sonication in 1 M NaCl, and centrifuged as described for whole neutrophil extract.

**IMMUNOBLOTTING**

Extracts of neutrophil granulocytes (3.0 mg/ml), azurophilic and specific granules (700 µg/ml) were suspended in non-reducing sample buffer and boiled for five minutes. Separation was carried out by sodium dodecyl sulphate-polyacrylamide gel electrophoresis using 12%
polyacrylamide gels (Novex, San Diego, USA). The separated antigens were blotted onto 0.2 mm nitrocellulose membranes in a semidry blotting system. Blots were blocked for two hours in immunoblot incubation buffer (0.01 M Tris-HCl, 0.15 M NaCl, pH 7.4) containing 3% horse serum (v/v). They were then washed three times for five minutes and incubated for two hours with sera or specific antibodies diluted in incubation buffer containing 1% horse serum (v/v) and 0.1% Tween 20 (v/v). The sera were diluted 1:100 and secondary \( \gamma \) chain specific peroxidase conjugated \( F(ab')_2 \) rabbit antihuman IgG antibodies were diluted 1/3000 (DAKO). After washing, blots were developed with the ECL western blotting detection system (Amersham, Aylesbury, UK). Monoclonal and polyclonal antibodies directed against PR3, MPO, Lf, HLE, BPI, Cat G, or a pool from seven healthy blood donors were used as controls.

To ascertain myeloid cell specificity, extract from human lymphocytes handled according to the same procedures was used.

OTHER LABORATORY ANALYSES
IgM rheumatoid factor and HLA-B27 were also analysed. Blood samples to record haemoglobin and C reactive protein (CRP) were also analysed. Blood samples to record haemoglobin and C reactive protein (CRP) were drawn at the same time as those for the ANCA tests.

STATISTICS
The \( \chi ^2 \) test and Fisher’s exact test, two sided, were used to compare the frequency of ANCA in the various disease groups.

Results
Twenty six patients had acute disease (mean six months, range 1–12) and 24 had chronic disease (mean 7.4 years, range 1.2–19). The triggering infection was *Yersinia enterocolitica* in 10, *Salmonella* species in four, *Campylobacter jejuni* in seven, and *Chlamydia trachomatis* in nine patients. In 20 cases culture or serology could disclose no triggering infection. However, six of these patients reported symptoms of enterocolitis preceding the arthritis.

Forty per cent had extra-articular manifestations, predominantly acute anterior uveitis/conjunctivitis (12 patients), urethritis (10), circinate balanitis (three), erythema nodosum (three), and keratoderma blennorrhagia (one). The percentages of patients with affected joints at the time of ANCA testing were: knee (65%), ankle (61%), sacroiliac joint (42%), hip (24%), metatarsophalangeal/dactylitis (20%), wrist (8%), elbow (8%), metacarpophalangeal/proximal interphalangeal (4%).

Overall, 88% carried the HLA-B27 antigen. Numbers of patients found positive for IgM rheumatoid factor were for the patients with ReA/UC/RA respectively one, two, and 28.

INDIRECT IMMUNOFLUORESCENCE (IIF)
Serum samples from the 50 patients with ReA, 30 with UC, and 30 with RA were screened for the presence of IgG antineutrophil cytoplasmic autoantibodies by IIF microscopy on ethanol fixed leucocytes. Among the patients with ReA eight sera (16%), all of which were from patients with chronic disease, gave a P-ANCA pattern. None of the samples from the 26 patients with acute ReA gave positive results in IIF (p<0.005). The corresponding figures for the patients with UC were 21 positive (70%), of whom 20 had P-ANCA and one had a C-ANCA pattern. Nine patients with RA (30%) were P-ANCA positive.

SPECIFICITIES OF ANCA DETECTED BY ELISA
All serum samples were analysed by ELISA for their ability to detect six different neutrophil cytoplasmic antigens—that is, PR3, MPO, BPI, Lf, Cat G, and HLE. Sixteen ReA sera (32%) were positive in ELISA. Antibodies against Lf were found in 10, MPO-ANCA in four, BPI-ANCA in four, PR3-ANCA in three, Cat G-ANCA and HLE-ANCA in one serum each (table 1). Five of the 16 serum samples reacted with more than one antigen. Eleven sera were from the group of 24 patients with chronic or chronic intermittent ReA disease (46%) and five (19%) from the remaining 26 with acute ReA disease. This difference between patients with chronic \( v \) acute disease reached significance (p<0.05). Of the 16 sera, four gave rise to a P-ANCA pattern in IIF. Four sera were positive in IIF (all P-ANCA), but failed to react with any of the six ELISA antigens.

In the group of patients with UC 16 (53%) were positive in ELISA. Antibodies against Lf were found in four, MPO-ANCA in three, BPI-ANCA in six, PR3-ANCA in five, Cat G-ANCA in seven, no patient had HLE-ANCA. Eight patients had antibodies to more than one antigen. Of the 16 ELISA positive sera, 11 were also positive in IIF (10 P-ANCA and 1 C-ANCA). The remaining ten sera which gave a P-ANCA pattern in IIF did not, however, react with any of the six antigens in ELISA.

Among the control patients with RA 15 were positive in ELISA (50%). Two had Lf-ANCA, 14 MPO-ANCA, one BPI-ANCA, five PR3-ANCA, four Cat G-ANCA, and none had HLE-ANCA. Eight patients had antibodies to

Table 1  Results of IgG ELISA determinations with six neutrophil granule constituents as antigens, IgM rheumatoid factor, and IgG indirect immunofluorescence (IIF) antineutrophil cytoplasmic antibodies (ANCA) among 50 patients with reactive arthritis (ReA), 30 control patients with ulcerative colitis (UC) and 30 controls with rheumatoid arthritis (RA). Results are shown as numbers (%)

<table>
<thead>
<tr>
<th></th>
<th>Anti-PR3*</th>
<th>Anti-MPO*</th>
<th>Anti-Lf*</th>
<th>Anti-HLE*</th>
<th>Anti-Cat G*</th>
<th>Anti-BPI*</th>
<th>IgM RF*</th>
<th>ANCA (IIF)</th>
</tr>
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<tbody>
<tr>
<td>Acute ReA (n=26)</td>
<td>2 (4)</td>
<td>2 (4)</td>
<td>1 (2)</td>
<td>1 (2)</td>
<td>4 (8)</td>
<td>8 (16)</td>
<td>1 (2)</td>
<td>8 P* (16)</td>
</tr>
<tr>
<td>Chronic ReA (n=24)</td>
<td>1 (2)</td>
<td>4 (8)</td>
<td>8 (16)</td>
<td>1 (2)</td>
<td>4 (8)</td>
<td>6 (20)</td>
<td>2 (7)</td>
<td>20 P 1 C* (70)</td>
</tr>
<tr>
<td>UC (n=30)</td>
<td>4 (13)</td>
<td>7 (23)</td>
<td>4 (13)</td>
<td>1 (3)</td>
<td>28 (93)</td>
<td>9 P (30)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RA (n=30)</td>
<td>14 (47)</td>
<td>5 (17)</td>
<td>2 (7)</td>
<td>1 (3)</td>
<td>28 (93)</td>
<td>9 P (30)</td>
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*PR3 = proteinase 3; MPO = myeloperoxidase; Lf = lactoferrin; HLE = human leucocyte elastase; Cat G = cathepsin G; BPI = bactericidal permeability increasing protein; RF = rheumatoid factor; P = P-ANCA; C = C-ANCA.

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more than one antigen. The number of MPO-ANCA positive sera was unexpectedly high, but the individual unit values were low or borderline with a mean (SD) of 16 (5) U/ml, which is less than the double cut off level of the assay. Of the 15 ELISA positive sera, four gave a P-ANCA pattern in IIF. Five sera were positive in IIF (all P-ANCA), but failed to react with any of the six ELISA antigens.

**INHIBITION ELISA**

Fifteen serum samples, five each positive for PR3-ANCA, MPO-ANCA, and Lf-ANCA respectively were absorbed by passing the samples over precoated ELISA plates. All but one, a borderline PR3-ANCA positive serum, showed at least 20% reduction in U/ml compared with unabsorbed sera.

**IMMUNOBLOTTING**

Monoclonal or polyclonal antibodies against PR3, MPO, HLE, BPI, Cat G, and Lf all showed reactivity in immunoblotting to proteins of the expected molecular weight when tested on sonicated extracts of whole neutrophils, azurophilic and specific granules.

A double band of 78 kDa corresponding to Lf was recognised by 22 ReA sera, and seen equally in neutrophil and specific granule extract. Eight of these sera were also positive in the Lf-ANCA ELISA. A band corresponding to the 55 kDa antigen, stained with the anti-BPI monoclonal antibody, was recognised by 11 ReA sera on blots of neutrophil granule extract. Four of the 11 sera reacting with the 55 kDa band were positive in the BPI-ANCA ELISA. In the azurophilic granule extract a smear in the 28–34 kDa region was weakly stained by a few sera all of which, however, were negative in the ELISA (fig 1A and B). Furthermore, unknown antigens of 60 kDa and 16 kDa were recognised by seven and four sera respectively in both neutrophil and specific granule extracts. To study the neutrophil specificity of these proteins recognised by ReA sera, they were also tested for reactivity with sonicated and NaCl extracted lymphocytes in immunoblotting. There was, however, no reactivity to antigens of the abovementioned molecular weights.

When the results obtained by ELISA, immunoblotting, and IIF were compared with clinical status and other laboratory data, there was no correlation with triggering infections, numbers of affected joints or extra-articular manifestations, neither was there any correlation between the presence of ANCA and CRP, haemoglobin, or HLA-B27 status, respectively. Patients with longstanding (chronic) ReA disease, however, had a higher prevalence of ANCA than patients with a short disease course. This difference was significant for ANCA as detected by IIF and ELISA. The same trend was observed for Lf-ANCA and BPI-ANCA in immunoblotting, though it did not reach significance (p<0.05 and <0.1 respectively). A comparison of the group of patients with chronic ReA with the group with RA showed that Lf-ANCA was significantly more prevalent in ReA (p<0.02). Cat G-ANCA was found in only one patient with ReA, whereas seven UC sera were positive (p<0.005).

**Discussion**

In this study we found that ANCA were common among patients with ReA. Thirty two per cent had antibodies against at least one of the six antigens tested for in ELISA. Antibodies against Lf were shown to be most prevalent followed by BPI-ANCA in both ELISA and immunoblotting experiments. There was a certain correlation between ELISA and IB results for BPI-ANCA and Lf-ANCA in that almost all ELISA positive sera also showed reactivity with the same antigens in IB. However, significantly more sera stained positive in IB, possibly owing to greater sensitivity of this assay.

To assign the reactivities obtained in IB to antigens of neutrophil subcellular fractions, IB was performed with extracts of whole neutrophils, azurophilic and specific granules. The
major reactivity to a band of 78 kDa corresponding to Lf was seen equally on granulocyte and specific granule extracts, whereas the reactivity to the 55 kDa antigen corresponding to BPI was seen exclusively on whole granulocyte extracts. We failed to show reactivity to this antigen on the azurophilic granule blots. Reaction to unknown antigens of 60 kDa and 16 kDa were noted in a few serum samples in both specific granule and whole cell extracts. A few ReA sera reacted weakly on blots of azurophilic granules in the 27–32 kDa region, though none reacted in ELISA with antigens of the corresponding molecular weight range—that is, PR3, HLE, or Cat G.

In all disease groups 30–50% of sera recognised more than one antigen in ELISA. This is in contrast with ANCA found in primary vasculitic disorders, where only a few distinct antigens seem to be recognised. The levels of antibody against MPO and PR3 were mostly low or borderline. This applied especially to the group of RA sera, where an unexpectedly high number of patients turned out to be positive in the MPO-ANCA ELISA (47%). The 17% PR3-ANCA positive sera in the RA/UC control group is not significantly different from the 14% PR3-ANCA positive sera in the European Commission (EC/BCR) project for ANCA assay standardisation of idiopathic systemic vasculitis.

A similar pattern was found in recent studies, in which Brimnes et al tested RA and UC sera in ELISA for antibodies against different neutrophil autoantigens. A considerable proportion of serum samples reacted in more than one of the ELISAs—for example, 29% of RA sera were found positive in MPO-ANCA and 32% of sera were PR3-ANCA positive; however, most sera were only weakly positive in ReA, unlike most other ANCA associated conditions, the disease can take an acute self-limiting course or develop into a chronic or chronic intermittent disease state with a potentially severe debilitating outcome. Clearly, more ANCA positive sera were found in the group of patients with chronic or acute ReA disease, indicating that the generation of these autoantibodies requires a certain period of time to develop. Whether the presence of ANCA plays a part in the pathogenesis of chronic disease or is merely a consequence thereof is not possible to elucidate from this study.

When the results from the group with chronic ReA were compared with UC/RA disease control sera, there seemed to be greater similarities between ReA/UC than between ReA/RA or UC/RA for Lf-ANCA and BPI-ANCA, both being more prevalent in UC and chronic ReA. Cat G-ANCA, however, were found in significantly more UC than ReA serum samples. P-ANCA is a common finding in IBD, mainly in UC and to a lesser extent in Crohn’s disease. Some investigators have reported a high prevalence of Lf-ANCA and BPI-ANCA in UC. There is a strong link between IBD and the spondyloarthopathies, and gut inflammation (mainly subclinical) resembling IBD has been reported in about 70% of patients with chronic ReA or ankylosing spondylitis. Taking this into account, it is not surprising that ANCA is a common finding not only in UC but also in spondyloarthopathies, as shown in the present study.

There was a discrepancy between results obtained by IIF microscopy and ELISA in that only about 50% of sera with a P-ANCA pattern reacted to any of the six antigens used in ELISA. This applied to sera from all disease groups and may depend upon several factors such as the avidity of the autoantibodies, epitope exposure, conformational changes/denaturation of the antigens, etc. When the eight P-ANCA positive sera from the patients with ReA were analysed by immunoblotting, two samples failed to react with any of the antigen preparations used. This may be due to similar factors.

The practical implications of developing antibodies against neutrophil granule constituents are difficult to determine. Both Lf and BPI are parts of the innate immune system and are released from neutrophils during activation. It has long been known that Lf can act bacteriostatically by binding iron, but more importantly it exerts direct bactericidal activity. Lf can also stimulate phagocytic activity of neutrophils. However, Lf, BPI, and azurocidin (heparin binding protein) can neutralise lipopolysaccharide, thereby preventing Gram negative bacteria from activating neutrophils. By binding catalytic iron, which may be generated through cell destruction in the inflammatory process, Lf may prevent hydroxyl radical mediated tissue injury associated with neutrophil oxidant production. Experimentally it has been shown that anti-Lf antibodies can increase both the magnitude and duration of hydroxyl radical formation. Theoretically, one might expect that the presence of such autoantibodies could impair the immune system, in that Lf/BPI become antagonized to their physiological actions—for example, in protection of the host from invading microorganisms. This may apply especially to ReA, where inadequate handling of foreign bacterial antigens seems to be part of the pathogenesis.

In conclusion, autoantibodies against neutrophil granule constituents occur frequently in ReA. By using different immunological methods it was shown that the dominant reactivity was directed against Lf and BPI. In contrast with primary vasculitic disorders, several sera recognised more than one antigen, indicating that the driving force for ANCA production in ReA and the UC/RA control groups differs from that in vasculitides.
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