ANTINEUTROPHIL CYTOPLASMIC ANTIBODIES IN RHEUMATOID ARTHRITIS PATIENTS

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SUMMARY

We determined the occurrence of antineutrophil cytoplasmic antibodies (ANCAs) and their specificities in 77 rheumatoid arthritis (RA) patients and compared them with 25 patients with psoriatic arthritis (Pso), 19 with drug-induced lupus erythematosus (DI-LE) and 11 with systemic lupus erythematosus (SLE). Thirty-two per cent of RA patients had positive indirect immunofluorescence (IF) stains (P or atypical ANCA). Twenty-nine per cent of patients with rheumatoid vasculitis (RAV), 48% with long-standing RA (LSRA) and 20% with early RA (Ely RA) had positive ANCAs compared with 4% of Pso patients, 47% of DI-LE patients and 45% of SLE patients. Western blotting (with polymorphonuclear cell extracts or alpha-granules) and alpha-granule enzyme-linked immunosorbent assay (ELISA) yielded variable results and proved unhelpful for characterizing the specificities of ANCAs. ELISAs based on commercial purified lactoferrin (LF), myeloperoxidase (MPO), human elastase (HLE) and cathepsin G (CG) showed that anti-HLE antibody was the most prevalent (14%) antibody in RA, followed by anti-MPO antibody and anti-LF antibody (10% each). Statistical analysis of antibody prevalence by clinical presentation showed that LSRA patients were more likely to have anti-HLE antibody and that DI-LE patients were more likely to have anti-CG antibody compared with the other patient groups. In lupus patients serial ELISA titration of ANCAs (LF and MPO) was found to be reliable for predicting the outcome. The overall incidence of ANCAs in RA patients was 33% by IF.

KEY WORDS: Rheumatoid arthritis, Antineutrophil cytoplasmic antibodies, Rheumatoid vasculitis, Systemic lupus erythematosus, Drug-induced lupus, Psoriatic arthritis.

ANTIBODIES to neutrophil cytoplasmic antigens (ANCAs) have been extensively studied as markers for systemic vasculitis and crescentic glomerulonephritis [1-3]. Two major patterns of ANCAs can be identified using indirect immunofluorescence (IF): the classic cytoplasmic pattern (C-ANCA) is seen mainly in Wegener's disease and reflects the presence of anti-proteinase 3 antibody [4], whereas the perinuclear pattern (P-ANCA) is mainly produced by anti-myeloperoxidase (MPO) antibody. Although P-ANCAs directed against MPO are associated with microscopic polyarteritis and crescentic glomerulonephritis, other target antigens of P-ANCAs have been identified, including human elastase (HLE), cathepsin G (CG) and lactoferrin (LF). ANCAs have been recently detected in sera from patients with seropositive and seronegative rheumatoid arthritis (RA), Felty's syndrome, systemic lupus erythematosus (SLE) and drug-induced lupus (DI-LE) [5-10]. The purpose of this study was to determine the occurrence of ANCAs and their antigenic targets in the sera of RA patients [with early RA (Ely RA), rheumatoid vasculitis (RAV) and/or long-standing RA (LSRA)]. Data from RA sera were compared with data from three different control groups [patients with psoriatic arthritis (Pso), DI-LE and SLE] in order to evaluate ANCAs as potential markers for RAV and LSRA.

METHODS

Patients and sera

Sera from 162 patients were studied. Eighty-four patients met the 1987 American Rheumatism Association (ARA) criteria for RA. We divided these patients into three subgroups, composed respectively of 25 patients with RA of less than 1 yr duration (Ely RA), 31 patients (31 sera) with seropositive RA of more than 2yr duration (LSRA) and 21 patients (28 sera) with RAV. All the 52 patients with RA or RAV were positive for rheumatoid factor (RF). Among the 25 patients with Ely RA 12 were RF+ and others were RF— but positive for at least one of the following autoantibodies: anti-RA33 antibody (Western blotting), anti-perinuclear factor (IF) or anti-keratin antibody (IF). Six RA patients had positive anti-nuclear antibodies (ANA). Of the 30 lupus patients studied 19 had DI-LE and 11 SLE with glomerulonephritis, World Health Organization (WHO) class IV (SLE-GN); there were positive anti-DNA antibodies in all cases. Twenty-five patients with Pso were also investigated. Patients with SLE were studied longitudinally with time: the 95 available sera were obtained just before the diagnosis of lupus nephritis, then during and after monthly intravenous cyclophosphamide treatment. Control sera were obtained from 30 healthy subjects (all nurses or other members of the medical staff) and from patients with ANCAs of defined specificity (MPO, proteinase 3, HLE, CG, LF) or other antibodies (anti-SSA, anti-SSB, anti-dsDNA, anti-Sm).

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IIF

IIF was carried out according to the method agreed on [11] at the first international ANCA workshop held in 1988. The sera were diluted to 1/20th and revealed using a goat anti-human IgG, IgA and IgM fluorescein-conjugated antibody (Cappel, Organon Teknika Corporation). Fluorescence patterns were classified as classic C-ANCA, P-ANCA or atypical (neither C-ANCA nor P-ANCA). Our laboratory has been involved in ANCA testing since 1989 [12-14] and the assay is performed routinely.

Alpha-granules extract

Alpha-granules were obtained from human polymorphonuclear cells by nitrogen cavitation followed by Percoll gradient density ultracentrifugation, as described by Borregaard in [11]. A Triton extract from alpha-granules was used for microplate coating, as described by Wiik et al. [11].

ELISAs with purified antigens

Antibodies to MPO, LF, HLE and CG were sought using direct-binding ELISAs, all of which were performed and evaluated using the same procedure. Briefly, the antigens (HLE and MPO from Elastin Product Company Inc. and LF and CG from Sigma Chemicals) were diluted in 0.05 M sodium carbonate buffer, pH 9.6, and coated onto polystyrene microtitre plates 200 µl of solution, i.e. 1 µg of each antigen, per well (Maxisorp immuno plates, Nunc). The plates were saturated by transferring 100 µl of phosphate-buffered saline (PBS)/bovine serum albumin (BSA)/Tween saturated by transferring 100 µl of phosphate-buffered saline solution with 0.15 M sodium chloride solution (PBS) to each well. After intensive washing 100 µl of alkaline phosphatase substrate solution (PNPP, Sigma Chemicals) were added and optical densities were read at a wavelength of 405 nm. Results were expressed in arbitrary units. Titration curves were obtained using the D-Soft program (Biometaflicas Inc.) after subtraction of background binding of individual sera to wells not coated with antigens. For each plate a standard curve was constructed using an internal positive reference control serum at various dilutions (1/50 to 1/1600). Sera from 30 healthy subjects were used to determine the normal reference range: values more than 2 s.d. above the normal mean were considered positive.

Western blotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the method of Laemmli. Fresh neutrophils from normal human blood donors were adjusted to 20 million cells/ml and extracted using sonication, Triton lysis (1%) or acidic extraction [15]. Supernatants were separated by centrifugation (10 000 r.p.m. for 15 min) and used as antigens for migration. Alpha-granules were also used for Western blotting. The extracts were used either with or without prior boiling or reduction [16]. Subsequent Western blotting was performed according to standard procedures.

RESULTS

ANCA detection using immunofluorescence (Table I)

None of the 30 healthy controls had positive IIF staining for ANCA. Among the 84 sera from RA patients 56 gave negative stains and 28 positive stains (mostly P-ANCA or atypical patterns; see Table I). There were 24 negative stains and one P-ANCA pattern in the PsO group; 10 negative stains, six P-ANCA patterns and three atypical ANCA patterns in the DI-LE group; and six negative stains, one P-ANCA pattern, and four atypical ANCA patterns in the SLE-GN group.

IgG-specific anti-LF ELISA (Table II)

A positive reaction (more than 2 s.d. above the mean in normal controls, i.e. above 6.21 + 2.42 = 8.64 units)

<table>
<thead>
<tr>
<th>TABLE I</th>
<th>Results of IIF testing</th>
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</thead>
<tbody>
<tr>
<td>ANCA negative</td>
<td>C-ANCA</td>
</tr>
<tr>
<td>Controls</td>
<td>30 (67%)</td>
</tr>
<tr>
<td>RA (all sera) (n = 84)</td>
<td>56 (71%)</td>
</tr>
<tr>
<td>RAV (n = 28)</td>
<td>20 (71%)</td>
</tr>
<tr>
<td>LS RA (n = 31)</td>
<td>16 (52%)</td>
</tr>
<tr>
<td>Ely RA (n = 25)</td>
<td>20 (80%)</td>
</tr>
<tr>
<td>PsO (n = 25)</td>
<td>24 (96%)</td>
</tr>
<tr>
<td>DI-LE (n = 19)</td>
<td>10 (53%)</td>
</tr>
<tr>
<td>SLE (n = 11)</td>
<td>6 (55%)</td>
</tr>
</tbody>
</table>

IIF, indirect immunofluorescence; ANCA, antineutrophil cytoplasmic antibody; RA, rheumatoid arthritis; RAV, RA vasculitis; LSRA, long-standing RA; Ely RA, early RA; PsO, psoriatic arthritis; DI-LE, drug-induced lupus erythematosus; SLE, systemic LE.
ELISA, enzyme-linked immunosorbent assay; RA, rheumatoid arthritis; RAV, RA vasculitis; LSRA, long-standing RA; Ely RA, early RA; Pso, psoriatic arthritis; DI-LE, drug-induced lupus erythematosus; SLE-GN, systemic LE with glomerulonephritis.

for circulating IgG anti-LF antibodies was found in eight RA patients including one of the 28 RAV patients, six of the 31 LSRA patients and one of the 25 Ely RA patients. In addition, one of the 25 Pso patients, four of the 19 DI-LE patients and six of the 11 SLE-GN patients tested positive for IgG anti-LF antibodies. Figure 1 shows the mean titres in the different groups of sera. Statistical analysis (Student's t-test for unpaired data) showed no differences between these values. Anti-LF antibodies did not occur more often in RAV patients than in LSRA patients (Chi-square test = 2.6; NS). Among the 11 SLE-GN patients six had anti-LF antibodies. A longitudinal study of these six patients showed that the anti-LF antibody titre was elevated at the time of diagnosis of lupus nephritis and decreased during treatment.

**IgG-specific anti-MPO antibodies (Table II)**

A positive reaction (more than 2 s.d. above the mean in normal controls, i.e. more than 3.07 + 1.14 = 4.21) for circulating IgG anti-MPO antibodies was found in eight RA patients including five of the 28 RAV patients, two of the 31 LSRA patients and one of the 25 Ely RA patients. IgG anti-MPO antibody was also detected in two of the 25 Pso patients, three of the 19 DI-LE patients and four of the 11 SLE-GN patients. Mean titres are summarized in Fig. 1. Statistical analysis (Student's t-test for unpaired data) did not disclose any differences between these values in all disease groups.

**Fig. 1.**—Mean values (units) and standard deviations for ANCA ELISA. Statistical analysis (Student's t-test for unpaired data) showed that anti-HLE titres were significantly higher in the LSRA group versus the RA (P < 0.01) and Ely RA (P < 0.01) groups. The DI-LE group also had significantly higher titres compared with the SLE-GN (P < 0.01), RAV (P < 0.01) and LSRA (P < 0.01) groups. The rate of occurrence of anti-HLE antibodies was higher in RAV patients than in the LSRA and Ely RA patients (Chi-square test, P < 0.01). Patients with DI-LE had significantly higher levels of anti-CG antibodies than other groups (P < 0.001).
IgG-specific anti-HLE antibodies (Table II)

A positive reaction (more than 2 s.d. above the mean in normal controls, i.e. 11.86 + 3.36 = 15.22) for circulating IgG anti-HLE antibodies was detected in 12 RA patients, including nine of the 28 RAV patients, one of the 31 LSRA patients and two of the 25 Ely RA patients. IgG anti-HLE antibodies were also detected in sera from two of the 25 Pso patients and nine of the 19 DI-LE patients. Mean titres and statistical analysis are summarized in Fig. 1.

IgG-specific anti-CG antibodies (Table II)

A positive reaction (more than 2 s.d. above the mean in normal controls, i.e. 9.52 + 2.55) for circulating IgG anti-CG antibodies was found in five RA patients, including two of the 28 RAV patients, one of the 31 LSRA patients and two of the Ely RA patients. IgG anti-CG antibodies were found in one of the 25 Pso patients and three of the 19 DI-LE patients (15.8%) patients. None of the 11 SLE-GN patients had detectable levels of IgG anti-CG antibody. Mean titres (±s.d.) were 7.77 ± 2.33 U in RAV patients, 6.68 ± 3.76 U in LSRA patients, 6.08 ± 2.5 U in Ely RA patients, 8.09 ± 3.1 U in Pso patients, 53.02 ± 201.1 U in DI-LE patients, 7.49 ± 1.59 U in SLE-GN patients and 9.52 ± 1.27 U in healthy controls (Fig. 1). Statistical analysis (Student's t-test for unpaired data) showed that patients with DI-LE had significantly higher levels of anti-CG antibodies than other groups (P < 0.001).

IgG-specific anti-alpha-granule antibodies (Table II)

A positive reaction (more than 2 s.d. above the mean in normal controls, i.e. 2.8 + 3.6) for circulating IgG anti-alpha-granule antibodies was found in two RA patients. One was a man with life-threatening RAV, the other was a woman with LSRA, positive P-ANCA staining and a strongly positive ELISA for HLE; the other was a woman with LSRA, positive C-ANCA staining and a strongly positive ELISA for MPO. None of the patients in the DI-LE, Ely RA or Pso groups had anti-alpha-granule antibodies. Two patients with SLE-GN had anti-alpha-granule antibodies; both had an atypical ANCA pattern and positive anti-MPO antibodies.

Western blotting

Whatever the method used (Triton extraction, acidic extraction, sonication or alpha-granules, with or without boiling or reducing conditions), Western blotting was negative except with a few of the positive C-ANCA control sera (data not shown). In particular, no doublets at 54/63 kDa or 66/67 kDa were detected, even when the test was performed with known positive autoimmune sera (anti-LF, anti-HLE, anti-CG or anti-MPO) from reference laboratories.

DISCUSSION

Granulocyte-specific anti-nuclear antibodies (GS-ANA) were first described in 1969 in patients with Felty's syndrome [2] and are now considered identical with P-ANCA, which have been detected in 50% to 70% of patients with RAV and in 20% to 40% of patients with uncomplicated RA [1, 2, 7]. Neither the usefulness of ANCA detection for differentiating RA from other forms of chronic arthritis nor the prognostic significance of a positive ANCA result (by IIF testing) in RA patients has been determined. In this study, sera from RA patients were screened for ANCA using IIF, ELISA and Western blotting. Results were compared with those obtained in other patient populations and in normal controls.

We found that 67% of RA sera were negative and 33% positive by IIF (P-ANCA 19%; C-ANCA 1%; atypical 13%). In contrast, Mulder et al. found P-ANCA in 70% of RA patients overall, 65% of patients with Ely RA, 64% of patients with 'extra-articular rheumatoid manifestations' and 80% of patients with LSRA [7]. We found lower rates of occurrence in patients with LSRA (48% vs 80%) or systemic RAV (28% vs 64%). These discrepancies may be ascribed to differences between patient populations; in particular, our patients with RA had systemic disease (e.g. polyarthritis, myositis, necrotic skin lesions, ulcers), whereas 65% of the patients studied by Mulder et al. had mainly mild extra-articular symptoms (i.e. pericarditis or nodules). In our study, IIF and ELISA yielded conflicting results. For example, five IIF-positive sera from RA patients were negative by ELISA and seven IIF-negative sera were positive by ELISA. We do not know whether these discrepancies were due to false positive or true positive reactions against unscreened antigens (lysozyme, alpha-enolase, azurocidine) [15] or unknown antigens. This was not related to the presence of RF: we tested RF+ sera with and without denaturation with 2-mercaptoethanol (2-MPE). After reduction of disulphide bonds we observed only a slight decrease (mean 5%) in the optical density. In no case was 2-MPE treatment of the sera associated with a negative ANCA ELISA result. Like many other investigators [17] we think that the IIF test is not the best assay to test for ANCA in patients with chronic inflammatory diseases.

To determine the nature of the antigens recognized by ANCA's in RA patients we performed characterization studies. We found that the alpha-granule ELISA lacked sensitivity and specificity. This is in agreement with other studies [4, 18]. We studied ANCA-positive sera using Western blotting with four types of polymorphonuclear cell extracts and found no correlation between the results of ANCA testing by IIF, ELISA and Western blotting. We did not detect the doublets at 54/63 kDa and 66/67 kDa reported by others [2, 7]. Until a technical consensus is achieved, as for IIF [18], and a universally accepted proven method of Western blotting developed, Western blotting appears unsuitable for ANCA testing in RA patients.
ANCA screening failed to discriminate RAV from LSRA because of a high rate of false positive and false negative results (related either to the technical conditions or to the presence of antibodies directed towards unknown targets). ANCA testing also failed to differentiate RA patients from patients with other chronic rheumatic conditions; although RA patients overall were more likely to have a positive IIF stain than PsA patients (Chi-square test = 8.4; P < 0.01), there was no difference between Ely RA and PsA (Chi-square = 3.02; NS). Alpha-granule ELISA and Western blotting results were too variable to be of use. Thus, our data show that alpha-granule ELISA and ANCA Western blotting are not appropriate methods for detecting and characterizing ANCAs in RA patients.

The prognostic significance of positive ANCA tests in RAV patients remains unknown. In our study three RAV patients had serial serum ANCA tests. One was IIF positive and HLE ELISA positive only at the beginning of a clinical remission. Another, who was MPO ELISA positive from the first manifestations of vasculitis, developed a positive IIF stain after 6-monthly cycles of intravenous cyclophosphamide and was still IIF positive after 2 yr of treatment. In the remaining patient, IIF and HLE ELISA were positive at the beginning of the disease and negative after treatment. No conclusions can be drawn from these findings in a small number of patients. In contrast, in patients with SLE-GN we found a consistent pattern of high ANCA ELISA titres at the beginning of the disease, with lowering titres during treatment and rising titres during flares. Further studies are in progress to evaluate the prognostic significance of serial ANCA testing.

Our results provide evidence that ANCAs occur in RA patients without Felty's syndrome or RAV. We found a predominance of P-ANCA in RA patients, and with ELISA testing a predominance of anti-HLE antibodies (14%), followed by anti-LF and anti-MPO antibodies (10% each). Anti-HLE antibody may be a specific marker for RAV. Coremans et al. [8, 10] found ANCA in 43% of RA patients and anti-LF antibody in 45% of RA patients vs 4% of LSRA patients; further differences between these results and ours concern the rates of occurrence of HLE (5% vs 16.9%) and MPO (17% vs 11.8%). Cambridge et al. [6], found that anti-MPO antibodies were significantly associated with RA (12% of patients) and were particularly likely to occur in patients who had long-standing seropositive nodular RA with lung involvement. In our patients, anti-HLE antibody was the most frequent antibody. Possible explanations of these discrepancies include technical differences (and there is an urgent need for standardization of ANCA ELISA testing) and local bias or differences between patient populations which might mask any value of ANCA testing in RA patients. In contrast to our data from RAV patients, results of serial ANCA determinations in lupus patients may be of prognostic value.

In conclusion, positive ANCA testing by IIF was found in 33% of RA patients, usually as a P-ANCA pattern. Our data suggest that IIF is not the best-suited assay for ANCA detection in RA patients, mainly because of a high rate of false negative results and the high rate of undefined 'atypical' ANCA patterns. Alpha-granule ELISA and Western blotting do not seem useful for ANCA testing in RA patients. Our results with ELISAs and Western blotting do not seem useful for ANCA testing in RA patients. Further studies are warranted to evaluate the information generated by serial ANCA testing in RA patients. In contrast to our results from RAV patients, our findings from serial anti-LF and anti-MPO antibody determinations in SLE patients were apparently of prognostic value. Standardization of solid-phase ANCA testing is impatiently awaited.

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REFERENCES


