Anti-neutrophil Cytoplasmic Antibodies (ANCA) in Systemic Lupus Erythematosus: Prevalence, Clinical Associations and Correlation with Other Autoantibodies

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Abstract

Aim: This study was undertaken to clarify the nature of anti-neutrophil cytoplasmic antibodies (ANCA) along with other autoantibodies in lupus nephritis (LN) patients and in systemic lupus erythematosus (SLE) patients without nephritis and to know their correlation with clinical manifestations and presence of other autoantibodies.

Material and Methods: Forty one LN patients and 18 SLE patients without nephritis were studied. LN patients were subdivided into diffuse proliferative glomerulonephritis (DPGN), focal proliferative glomerulonephritis (FPGN), rapidly progressive glomerulonephritis (RPGN) and membranoproliferative glomerulonephritis (MPGN). Anti-neutrophil cytoplasmic antibodies (ANCA) were detected by indirect immunofluorescence and confocal laser scanning microscope using PMN and HL60 cells. ANCA specificities like anti-myeloperoxidase (anti-MPO), anti-protease 3 (anti-PR3), anti-lactoferrin (anti-LF) and anti-cathepsin G (anti-CG) were detected by ELISA. Other autoantibodies like anti-nuclear antibodies (ANA), anti-double stranded DNA (anti-dsDNA), anti-single stranded DNA (anti-ssDNA), anti-ribonucleoproteins (anti-nRNP), anti-Smith antibodies (anti-Sm) and rheumatoid factor (RF) were also tested.

Results: ANCA was detected in 37.3% patients. The predominant ANCA pattern was perinuclear (p-ANCA). ANCA positivity was higher in LN patients and when confirmed by ELISA, 54.5% ANCA positives had anti-myeloperoxidase (anti-MPO). The cytoplasmic ANCA (c-ANCA) pattern was not seen in any patient. Two patients having FPGN with crescents showed atypical 'X-ANCA' pattern with dual specificity to anti-MPO and anti-PR3 by ELISA. The titers of ANCA were more in LN as compared to SLE without nephritis. LN cases having DPGN, FPGN, RPGN with crescents had higher titer p-ANCA positivity with corresponding anti-MPO antibodies, along with ANA, anti-dsDNA, anti-ssDNA and anti-Sm + anti-nRNP and also high SLEDAI scores.

Conclusion: ANCA in SLE may be used as a serological marker along with clinical and histopathological assessment to differentiate vasculitides in LN cases from SLE without nephritis.

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disorder that is characterized by the production of autoantibodies against a variety of nuclear antigens. It is possible that more than one process could contribute to the disease and that different processes may be responsible for different disease manifestations.1 One of these may be vascular injury. The two important mechanisms of vascular injury are direct injury to vessels and immune mediated inflammation.2

Anti-neutrophil cytoplasmic antibodies (ANCA) are a group of autoantibodies directed against the components of neutrophil cytoplasmic granules and are mainly associated with small vessel vasculitides such as microscopic polyangiitis.
(MPA), Churg Strauss syndrome (CSS), ‘pauci immune’
necrotizing crescentic glomerulonephritis (NCGN) and active
Wegener’s granulomatosis (WG). ANCA has been reported
of 3-69% in Western literature. The predominant staining
patterns under immunofluorescence microscopy (IIF) is
perinuclear pattern or p-ANCA, however the c-ANCA pattern
and atypical (X ANCA) pattern have also been reported. Further,
the ANCA subspecificities and the reactivity against
neutrophil cytoplasmic components such as myeloperoxidase
(MPO), proteinase3 (PR3), lactoferrin (LF), elastase (HLE)
and cathepsin G (CG) have also been reported in SLE patients.

Recently ANCA have been incriminated in the
pathogenesis of SLE related vasculitides. Therefore we have
studied the association of ANCA in patients of SLE with and
without nephritis, assessed the diagnostic value of
subspecificities, and their relationship with disease activity.

**MATERIAL AND METHODS**

This prospective study was carried out over a period of
two years. A total of 59 cases including 41 renal biopsy proven
cases of LN and 18 cases of SLE without renal involvement
were studied. All cases satisfied ACR critieria. Details of
clinical, histopathological and laboratory findings were
recorded and characterized for Disease Activity Index
(SLEDAI). Renal biopsies were examined by light microscopy
with haematoxylin, eosin and periodic acid Schiff (PAS)
staining and by immunofluorescent microscopy using anti-
IgG, anti-IgM, anti-IgA, anti-C3, anti-C4 and anti-fibrinogen
FITC conjugates. ANCA was tested by IIF method using
human neutrophils (PMN) as well as a human promyelocytic
leukemic cell line (HL-60) obtained from NCCS (Pune)
and maintained in Minimal Essential Medium (MEM) as
a continuous culture and harvested at log phase of growth. A
cytospun cell substrate was prepared using Hettich Universal
16A cytocentrifuge and was fixed with 96% ethanol and also
formalin separately before coating with patient’s sera. Slides
were probed using FITC tagged polyvalent anti-human
globulin serum using a fluorescent microscope, Nikon,
Optiphot II, Japan and microphotography was done using an
automated photography system, Nikon AFX II A. The IIF
patterns were further confirmed on confocal laser scanning
microscope, LSM-510, Carl Zeiss, Germany, to reexamine the
slides under higher clarity and magnification, and image
rotation on X, Y, or Z axis, which gave very clear picture
showing the immunofluorescence staining patterns. ANCA
was also detected by a rapid ELISA using ultrasonicated
neutrophil cytoplasmic extract called as the ‘α granules’.

Anti-MPO and anti-PR3 antibodies were a gift by scientists
from Germany, Denmark and Hongkong and were used as
controls. The specificities of the antibodies were identified
by antigen binding ELISAs for anti-myeloperoxidase (MPO)
and anti-proteinase3 (PR3) using kits from Genesis (UK) as
per the manufacturer’s directions. A value < 3.0 u/ml was
negative, 3-5 u/ml was equivocal and >5 u/ml was positive.
Anti-lactoferrin (anti-LF) ELISA was developed in the
laboratory using purified lactoferrin from Sigma, USA. Anti-
cathepsin G (anti-CG) was detected by ELISA using purified
cathepsin G (Sigma, USA). ANCA was also qualitatively and quantitatively tested by IIF
method using HEP-2 cells obtained from Enterovirus Research
Center, ICMR, Mumbai. A cut off for positivity was 1:20
dilution for ANA testing. In a few cases where both ANA
and ANCA were present in the same sample, confocal
microscopy and ELISA tests were used for further
confirmation. Anti-dsDNA ELISA was standardized as per
the method described by Hatfield et al., Antibodies to single
stranded DNA (ssDNA), antibodies to ribonucleoprotein
(nRNP), and Smith antigen (Sm) and rheumatoid factor (RF)
were detected using commercial ELISA kits.

**RESULTS**

The pattern of clinical manifestations is depicted in Table
1. Involvement of CNS, GI tract and joints, along with serositis
and haematological manifestations was slightly higher in SLE
cases as compared to LN cases in which skin and renal
involvement was commonly seen. Other demographic features
are shown in Table 1. Patients had no history of taking any
drugs like hydralazine, propylthiouracil etc.

In all, twenty two patients (37.3%) were ANCA positives.
(table 2) with p-ANCA present in 20 patients (90.9%), while
two patients (9.1%) showed the X-ANCA pattern.

<table>
<thead>
<tr>
<th>Organ involvement</th>
<th>No. Positives</th>
<th>Lupus Nephritis (41)</th>
<th>SLE without renal involvement (18)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(%)</td>
<td>DPGN(21)</td>
<td>FPGN(14)</td>
</tr>
<tr>
<td>Skin</td>
<td>50 (84.7%)</td>
<td>21</td>
<td>13</td>
</tr>
<tr>
<td>Renal</td>
<td>41 (69.5%)</td>
<td>21</td>
<td>14</td>
</tr>
<tr>
<td>Joint</td>
<td>35 (59.3%)</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>Serositis</td>
<td>16 (27.1%)</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Hematological</td>
<td>8 (13.6%)</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>GI tract</td>
<td>8 (13.6%)</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>CNS</td>
<td>6 (10.2%)</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Age in years</td>
<td></td>
<td>15-58 years</td>
<td>10-50 years</td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td>31.2 ± 10.5 years</td>
<td>26.5 ± 12.1 years</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td>19.5 : 1</td>
<td>8 : 1</td>
</tr>
</tbody>
</table>

* Two cases had MPGN with crescents
Cytoplasmic pattern (c-ANCA) was absent among these patients. In LN group, p-ANCA titers varied from 1:80 to 1:160 and the higher titer was seen in patients with DPGN. The two X-ANCA positive cases had a titer of 1:160 each. In non-renal cases, comparatively lower ANCA titers with p-ANCA pattern, showing anti-MPO specificity. Both had cutaneous vasculitis. The ‘α granules ELISA’ picked up all 22 IIF ANCA positive cases, of which 12 patients (54.5%) showed anti-MPO antibodies, while two patients had X-ANCA pattern by IIF, showing specificities to both MPO and PR3 antigens. Antibodies to LF were seen in 10 cases (16.9%) and antibodies to CG in 8 cases (13.6%). (Table 2)

The ‘α granules ELISA’ showed a statistically significant difference (p< 0.005) when LN and SLE without nephritis groups were compared for ANCA positivity.

Table 3 gives the details of organ involvement with ANCA serology and other autoantibodies. It was observed that anti-MPO and also the two cases of dual specificities (anti-MPO + anti-PR3) were associated with renal and skin manifestations, while anti-LF along with anti-MPO were seen in renal and joint involvement. Also four cases that had anti-LF alone had serositis. Patients having only anti-CG had a varied clinical presentation, renal (6), joint and CNS (3), serositis and haematological (2) and GI (1) while patients with anti-CG along with anti-MPO had renal and GI tract involvement only. The SLEDAI and titers of ANCA were observed to be increased with a rise in the SLEDAI score values (Table 4). In the LN group, SLEDAI scores were higher, ranging from 16-30, while SLE cases without nephritis, two cases that were ANCA positive had SLEDAI scores of 20 and 24 respectively and each had ANCA titers of 1:80. Also cases with low SLEDAI scores were ANCA negative.

**DISCUSSION**

A variety of autoantibodies are present in SLE patients. ANCA have been reported in 3-69% of SLE cases. Chin et al. have reported 37.3 % of patients with LN, having p-ANCA which was mainly associated with DPGN, with a lower incidence in patients without renal involvement. Nishiya et al have also reported 42 % SLE patients having only the p-ANCA pattern.

In our study, the LN group had 48.8 % positivity for ANCA as compared to 11.1 % in SLE patients without nephritis. The predominant ANCA pattern observed was p-ANCA, of which 54.5% had anti-MPO antibodies. Manolova et al. had observed an incidence of 29.1 % p-ANCA positivity with 10.9 % anti-MPO. Our study has also identified two rare cases of ‘X-ANCA’ pattern in the LN group having antibodies with dual specificities, i.e., both anti-MPO and anti-PR3. It is an unusual finding. There are few reports on dual specificities.

Normally ANCA’s show two distinct and separate

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### Table 2: ANCA serology by IIF and ELISA in SLE (n=59)

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Immunofluorescence</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p-ANCA</td>
<td>X-ANCA*</td>
</tr>
<tr>
<td>Lupus nephritis (41)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPGN with crescents (21)</td>
<td>8 (1: 80-1:160)</td>
<td>0</td>
</tr>
<tr>
<td>FPGN with crescents (14)</td>
<td>5 (1: 80-1:160)</td>
<td>2 (1:160)</td>
</tr>
<tr>
<td>RPGN with crescents (4)</td>
<td>4 (1:80)</td>
<td>0</td>
</tr>
<tr>
<td>MPGN with crescents (2)</td>
<td>1 (1:80)</td>
<td>0</td>
</tr>
<tr>
<td>SLE without renal involvement (18)</td>
<td>2 (1:80)</td>
<td>0</td>
</tr>
<tr>
<td>Total (59)</td>
<td>20 (1:80-1:160)</td>
<td>2 (1:160)</td>
</tr>
</tbody>
</table>

* Two patients showing X-ANCA by IIF had dual specificity to anti-MPO and anti-PR3; ** ‘p’ value <0.05 was considered significant by ‘X2 test’.

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### Table 3: Correlation of organ involvement with ANCA serology and other autoantibodies.

<table>
<thead>
<tr>
<th>Organ involvement</th>
<th>ANCA serology</th>
<th>Other autoantibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>anti-MPO</td>
<td>anti-LF</td>
</tr>
<tr>
<td></td>
<td>(12/59)</td>
<td>(10/59)</td>
</tr>
<tr>
<td>Skin (50)</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Renal (41)</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>DPGN with crescents (21)</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>FPGN with crescents (14)</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>RPGN with crescents (4)</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>MPGN with crescents (2)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Joint (35) **</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Serositis (16)</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Haematological (8)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GI tract (8)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CNS (8)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Two cases of FPGN with crescents had dual specificity; ** Four cases with joint involvement were RF positive.
immunofluorescence patterns like p-ANCA and c-ANCA. The mixed pattern of immunofluorescence which is a combination of p-ANCA and c-ANCA and also the ‘atypical’ X-ANCA immunofluorescence pattern is rarely seen and are difficult to interpret on immunofluorescence microscopy, though the Confocal Laser Scanning microscopy shows good clarity of the mixed immunofluorescence patterns.23 One drawback of IIF testing is that it is a screening assay which is not antigen specific, and it is known that, other than anti-MPO and anti-PR3 ANCA specificities do exist, which can only be detected by specific ELISAs which we had carried out. A common problem in SLE is that nearly all the patients are ANA positive by IIF and this might vitiate the IIF readings for ANCA, so the ELISA test is absolutely essential as it detects the presence of specific antibodies to individual ANCA specificities.

Though correlation between p-ANCA (mainly anti-MPO) and microscopic polyarteritis and c-ANCA (anti-PR3) and Wegener’s granulomatosis have high diagnostic potential, the role of anti-LF and anti-CG and their clinical significance is slowly being realized. Our results show a statistically significant correlation (p<0.005) for ‘α granule’ positivity between LN and SLE without nephritis groups. The highly purified ‘α granule’ extract, contains most of the cytoplasmic antigenic granules, indicate ANCA are also directed towards antigens other than MPO and PR3 like CG, LF and HLE in these cases. These could have immunodiagnostic potential and further studies need to be done to elucidate their role in ANCA associated ‘pauci-immune’ vasculitis.

Savige et al, have also reported occurrence of ‘atypical’ or ‘X-ANCA’ among SLE patients showing specificities to cathepsin G and lactoferrin.24 Zhao et al, in their study on 95 renal biopsy proven LN cases, have observed 62.1 % having anti-CG while 8.4% sera had anti-LF by ELISA.6 In our study 13.6 % patients had anti-CG while 16.9 % had anti-LF, seen in only DPNG, FPGN and RPGN groups all having crescentic glomerulonephritis while they were totally absent in SLE cases without nephritis. In a study by Lee et al, 25 an incidence of 39.2 % was reported for anti-LF antibodies which was further correlated with a clinical flare and crescentic glomerulonephritis. Manolova et al, 21 observed 18.2 % having anti-LF and found a significant association with serositis. Our study too has shown 66.6 % anti-LF positivity in patients with serositis.

ANCA have been often used as diagnostic and prognostic markers of vasculitis and the present study of ANCA in SLE with and without renal involvement shows that ANCA detection both by IIF and ELISA and its correlation with SLEDAI could be helpful to clinicians, because of the possibility of detecting and differentiating various types of SLE, and more studies addressing this issue are required as it is also possible that the presence of other ANCA specificities could also contribute to the heterogeneity of clinical manifestations in SLE patients.

### References

1. Rose NR, Mackay IR. The autoimmune diseases Vol II., 1992; Academic press , INC, 279-300.

| Table 4 : Comparison of SLEDAI in ANCA positive and ANCA negatives in SLE. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| SLEDAIScores   | DPNG (21)       | FPGN (14)       | RPGN (4)        | MPGN (2)        | SLE without renal |
| ANCA ANCA      | ANCA ANCA      | ANCA ANCA      | ANCA ANCA      | ANCA ANCA      | ANCA ANCA      |
| Pos Neg        | Pos Neg        | Pos Neg        | Pos Neg        | Pos Neg        | Pos Neg        |
| (8) (13)       | (7) (7)        | (4) (0)        | (1) (1)        | (2) (16)       |
| 0-10           | 0              | 0              | 2              | 0              | 0              |
| SLEDAI range   | 8              | 6-8            | 6-8            | 4              | 0              |
| ANCA titers    | nil            | nil            | 6-8            | 0              | 0              |
| 10-20          | 1              | 1              | 1              | 2              | 2              |
| SLEDAI range   | 1-2            | 1-2            | 1-2            | 0              | 0              |
| ANCA titers    | nil            | nil            | nil            | 0              | 0              |
| 20-30          | 4              | 5              | 1              | 0              | 0              |
| SLEDAI range   | 22-28          | 22-30          | 24             | 24             | 24             |
| ANCA titers    | 1: 160         | nil            | 1: 80          | nil            | 1: 80          |

* Two cases had dual specificities to anti-MPO + anti-PR3.


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