Increased incidence of cytoplasmic ANCA (cANCA) and other autoantibodies in leprosy patients from Western India

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Summary The prevalence of various autoantibodies was studied in 75 leprosy patients comprising eight patients with lepromatous leprosy (LL), 36 patients with borderline lepromatous leprosy (BL) and 31 patients with borderline tuberculoid leprosy (BT), along with 100 normal controls. Certain autoantibodies such as antinuclear antibodies (ANA), anti-single stranded DNA (anti-ssDNA) and anti-neutrophil cytoplasmic antibodies (ANCA) were raised among leprosy patients. When ANCA specificities to anti-myeloperoxidase (anti-MPO), anti-proteinase3 (anti-PR3) and anti-lactoferrin (anti-LF) were studied, it was found that the patterns of immunofluorescence such as perinuclear (p-ANCA), cytoplasmic (c-ANCA) and atypical (X-ANCA) and specificity by ELISA to anti-MPO, anti-PR3 and anti-LF varied in the LL, BL and BT groups. However, a higher amount of c-ANCA was observed in 62.5% of leprosy cases, while the incidences of p-ANCA and X-ANCA were lower. The LL group showed a higher incidence of autoantibodies as compared with the BL and BT groups, along with a male preponderance for autoantibody development. Some unusual antibody profiles such as ‘X’-ANCA were also observed. The study suggests that autoantibody formation could be quite prevalent and also variable in the spectrum of leprosy cases, and there seems to be a serological overlap among leprosy and autoimmune disease, which could have pathogenetic importance in the leprosy patients developing complications.

Introduction

Leprosy is an infectious disease caused by Mycobacterium leprae, which presents as a spectrum of clinical responses. The disease has been classified on the basis of immunological, histopathological and microbiological parameters using a scale as described by Ridley and Jopling\(^1\) which groups the patients as lepromatous leprosy (LL), borderline lepromatous leprosy (BL), borderline tuberculoid (BT) and tuberculoid leprosy (TT). In the tuberculoid
forms, there is heightened cell mediated immunity while in the lepromatous form, there is an increased humoral immunity. The disease manifests in various forms depending on the immune status of the host.²

Autoantibodies of various types have been described in the sera of leprosy patients.³–⁶ Gouedès Barbosa et al. observed striking immunoserological abnormalities in patients with a relapse and observed a significant serological overlap between lepromatous leprosy and autoimmune disease.⁷ The purpose of this study was to investigate the prevalence of various autoantibodies such as anti-nuclear antibodies (ANA), anti-double stranded DNA (anti-dsDNA), anti-single stranded DNA (anti-ssDNA), autoantibodies to extractable nuclear antigens such as anti-ribonucleoprotein (anti-nRNP) and anti-Smith (anti-Sm), and anti-histone (AHA). Other autoantibodies such as anti-Scl 70 and rheumatoid factor were also tested in these patients, to identify the possible correlation of these autoantibodies with clinical manifestations in leprosy patients. The anti-neutrophil cytoplasmic antibodies (ANCA) with specificities to anti-myeloperoxidase (anti-MPO), anti-proteinase3 (anti-PR3) and anti-lactoferrin (anti-LF), which are directed against cytoplasmic granules of neutrophils and monocytes, and are known to be associated with small vessel vasculitis, were studied.

Materials and methods

Aliquots of 8 ml blood were collected from 75 leprosy patients from various dermatological and leprosy clinics in and around Mumbai. These patients were clinically diagnosed using World Health Organization guidelines. They comprised 36 cases of borderline lepromatous leprosy (BL) and 31 cases of borderline tuberculoid leprosy (BT) and eight cases with lepromatous leprosy (LL). The disease status was assessed by clinical and histopathological findings and classified according to Ridley and Jopling classification.¹ Serum was separated from all collected blood samples and stored in two aliquots, one at −80°C for future use and the other for current use. Serological investigations were completed within a month of blood collection. Normal controls consisted of 100 healthy blood donors and respective positive and negative control sera were run together with test sera. All patients were negative for HIV antibodies and also for hepatitis B (HBsAg) and anti-HCV antibodies.

Standard methods were used for the detection of autoantibodies in patient’s sera. An indirect immunofluorescence test was used for the detection of anti-nuclear antibodies (ANA) using cultured HEp-2 cells as a substrate⁸–¹⁰ and various patterns were noted by using indirect immunofluorescence microscopy (IIF) technique. The cutoff for ANA positivity was at a 1:20 dilution positivity. Anti-dsDNA ELISA was standardized using the method described by Hatfield et al.,¹¹ Anti-histone ELISA was standardized using the method described by Krippner et al.¹² Antibodies to single stranded DNA(ssDNA), antibodies to ribonucleoprotein (nRNP) and Smith antigen (Sm), Scl-70 and rheumatoid factor (RF) were detected by using commercially available ELISA kits (Clark’s diagnostics, USA).

The initial identification of ANCA was carried out by IIF test, which is considered as the ‘gold standard’ for ANCA screening,¹³,¹⁴ using human neutrophils (PMN) as well as a human promyelocytic leukaemic cell line (HL-60) obtained from the National Centre for Cell Sciences (NCCS, Pune, India) and maintained in minimal essential medium (MEM) as a continuous culture and harvested at log phase of growth. The cells were used to prepare a cytospin substrate using a cytocentrifuge (Hettich Universal 16A, Germany) and one set of the slides was fixed with chilled 96% ethanol and the other with formalin, before treating with
patient sera. Slides were probed using FITC tagged polyvalent anti-human globulin serum. The cutoff for positivity was at 1:20 dilution and the fluorescence patterns were noted using a fluorescent microscope, (Nikon, Optiphot II, Japan). Various ANCA immunofluorescence patterns such as perinuclear (p-ANCA), cytoplasmic (c-ANCA) and atypical (X-ANCA) were evaluated.

Commercially available ELISA kits were used to detect ANCA specificities like anti-myeloperoxidase (MPO) and anti-proteinase3 (PR3) antibodies and anti-lactoferrin ELISA as described by Esnault et al.\textsuperscript{15} A standardized broad spectrum ELISA was performed for the detection of many ANCA specificities other than anti-MPO and anti-PR3, using an α-granule preparation as described by Rasmussen et al.\textsuperscript{16,17} A cutoff of normal human serum (NHS) + 2 SD was taken and the optical density readings taken at 405 nm; values above this range were considered as positives. The control group comprised of 100 age and sex matched normal healthy blood donors. The data of clinical and laboratory parameters were analysed using Microsoft Excel (5.0) and FoxPro (2.6) programs.

Results

In this study, 75 samples of leprosy patients comprising BL (n = 36) and BT (n = 31) and LL (n = 8) were investigated for presence of various autoantibodies. The age and sex distribution of these patients are shown in Table 1. In the LL group, six patients were smear positive and two patients were smear negative, in the BL group, eight patients were smear positive and 28 were smear negative, whereas in the BT group, only three patients were smear positive and remaining 28 patients were smear negative.

Various autoantibodies are found to be present in leprosy patients. In the LL group, 75% males showed presence of autoantibodies, whereas in BL and BT groups the distribution was in 45-2% and 58-3%, respectively, while the overall combined positivity was 50% in LL, 44-4% in BL and 54-8% in BT. The overall incidence of ANA was 34-7%, anti-dsDNA was 17-3%. There were 13 cases having both ANA and anti-dsDNA. Titres of ANA ranged from 1:40 to 1:160 of ANA and anti-dsDNA ranged from 54 IU/ml to 204 IU/ml by ELISA. Three cases had both ANA and RF, where the ANA titres ranged from 1:40 to 1:80 and the strength of RF was 48 IU/ml and 320 IU/ml, respectively. The overall incidence of anti-ssDNA was 30-7%, while anti-nRNP and RF was found to be present in 6-7% of patients. Anti-Scl-70 could be detected in 12% cases, while anti-histone and anti-Sm were found to be totally absent. The presence of antibodies among the three groups is shown in Table 2.

<table>
<thead>
<tr>
<th>Table 1. Clinical characteristics of the study population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>Number tested</td>
</tr>
<tr>
<td>(% of total no. tested)</td>
</tr>
<tr>
<td>Age range in years (mean SD)</td>
</tr>
<tr>
<td>1:1</td>
</tr>
<tr>
<td>3:4:1</td>
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</table>
Table 2. Autoantibody profile in leprosy patients

<table>
<thead>
<tr>
<th>Type</th>
<th>ANF</th>
<th>Anti-dsDNA</th>
<th>Anti-ssDNA</th>
<th>ANCA</th>
<th>Anti-nRNP</th>
<th>RF</th>
<th>SCL-70</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL (8)</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>BL (36)</td>
<td>10</td>
<td>6</td>
<td>12</td>
<td>12</td>
<td>10</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>BT (31)</td>
<td>12</td>
<td>5</td>
<td>8</td>
<td>9</td>
<td>7</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Controls (100)</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total (75) % positives</td>
<td>26 (34.7%)</td>
<td>13 (17.3%)</td>
<td>23 (30.7%)</td>
<td>24 (32%)</td>
<td>20 (26.7%)</td>
<td>5 (6.7%)</td>
<td>5 (6.7%)</td>
</tr>
</tbody>
</table>

* Anti-histone antibodies and anti-Sm were absent in these patients.

It was observed that ANCA were present in 32% leprosy patients when compared with 3% in controls using PMN and HL-60 cells. PMN is a better substrate than HL-60 in detecting ANCA, and the X-ANCA specific for LF could not be detected using HL-60 cells. A slightly higher incidence of ANCA, 37.5% was seen in the LL group as compared with 33.3% and 29% in BL and BT, respectively. When ANCA patterns by IIF such as p-ANCA, c-ANCA and X-ANCA were noted along with their individual specificities to anti-MPO, anti-PR3 and anti-LF, it was seen that in the LL group, all three patients showed c-ANCA by IIF, and anti-PR3 by ELISA. In the BL group, out of 12 ANCA positive patients, five showing the p-ANCA pattern had anti-MPO and four showing the c-ANCA pattern had anti-PR3. Interestingly, in the BT group, none of the patients showed the p-ANCA pattern, while five patients showing c-ANCA had anti-PR3. All the IIF positive samples were also identified by the broad-spectrum α-granule ELISA (Table 3) and X-ANCA was seen in four cases, two each in BL and BT groups. All four cases had anti-LF, while a further two anti-LF positive patients showed the p-ANCA pattern. Smear positivity was high in the LL group (75%), while it was low in BL and BT groups, when the data were analysed on the basis of smear positivity, a higher incidence of ANF, anti-ssDNA was observed in the LL and BL smear positive cases, while in the BT group the incidence of all antibodies was higher in the smear negative groups.

Discussion

Striking immunoserological abnormalities in patients with leprosy were observed by Kroumpouzos et al.,18 who predicted a significant serological overlap between leprosy and classical autoimmune disease, where they observed a link between autoimmunity and leprosy and reported that leprosy is associated with a variety of serological autoimmune phenomenon and suspected that leprosy could promote the development of autoimmune disease.

In this study on 75 cases, 34.7% of patients had ANA, and this incidence was increased among the LL group, with high titre values, while earlier studies by Miller et al.17 showed an incidence of 16% ANA positives with low titre values, none of them showing the presence of anti-dsDNA antibodies. Bonafő20 observed a lower frequency of ANA in LL patients in contrast to earlier reports and noted differences in frequency of autoantibodies among various racial groups, including Caucasians, Africans and Thai leprosy patients. However,
Table 3. NCA detected by immunofluorescence and ELISA

<table>
<thead>
<tr>
<th>Type, no. positive, percentage</th>
<th>p-ANCA</th>
<th>c-ANCA</th>
<th>X-ANCA</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PMN</td>
<td>HL-60</td>
<td>PMN</td>
<td>HL-60</td>
</tr>
<tr>
<td>LL, 3, (37.5%)</td>
<td>0</td>
<td>0</td>
<td>3 (1:160)</td>
<td>3 (1:160)</td>
</tr>
<tr>
<td>BL*, 12, (33%)</td>
<td>5 (1:80)</td>
<td>5 (1:80)</td>
<td>5 (1:80)</td>
<td>5 (1:80)</td>
</tr>
<tr>
<td>BT*, 9, (29%)</td>
<td>0</td>
<td>0</td>
<td>7 (1:80)</td>
<td>7 (1:80)</td>
</tr>
<tr>
<td>Total, 24, (32%)</td>
<td>5</td>
<td>5</td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>

* ENL one each in BL and BT were encountered and had multiple autoantibodies.  
** One patient with BT had both anti-PR3+ anti-LF.
differences in positivity rates due to variations in methodologies and in age, gender, polar forms, therapy and other elements need to be considered.21

Our findings are the first report showing the prevalence and the titres of autoantibodies among leprosy patients from Western India. Some earlier studies19,20 have shown that autoantibodies to dsDNA are not present in leprosy sera; however, we encountered 13 patients who had ANA and anti-dsDNA autoantibodies. Guedes Barbosa et al.7 have also observed an incidence of 7.6% anti-dsDNA in LL cases while McAdams et al.22 observed a high percentage of anti-dsDNA in LL cases. Although earlier Furukawa et al.23 had observed anti-dsDNA in LL cases, in the present study the anti-dsDNA autoantibodies were seen in 17.3% of the leprosy cases and were observed in LL, BL and BT groups, although in LL, the percentage was higher. However, it was difficult to see these cases exhibiting somewhat typical SLE type manifestations. Also, 23 cases (30.7%) had anti-ssDNA antibodies, which unlike anti-dsDNA, are seen in other disorders as well. Low levels of anti-ssDNA were found in 23% of leprosy sera by Bonfa et al.20

In leprosy, musculoskeletal symptoms are seen quite frequently after skin and neurological involvement, therefore research has been focused on inflammatory changes resembling rheumatological diseases. Chogle23 has encountered five cases in which leprosy was diagnosed when the patients presented with rheumatic complaints along with the occurrence of organ as well as non-organ-specific autoantibodies. There is an emphasis on identifying the importance of rheumatic manifestations in LL. As early as 1973 in Thai LL cases, an incidence of rheumatic manifestations in 3.4% was reported,24 though a higher frequency and titres of RF in multibacillary forms of leprosy were observed.6

ANCA directed to cytoplasmic constituents of neutrophil granules are considered as diagnostic serological markers for some forms of vasculitis like the ‘pauci-immune’ type, especially Wegener’s granulomatosis, which also shows an immune response and anatomic-pathological features similar to leprosy, as both are chronic granulomatous diseases. Our study showed 32% ANCA in leprosy, with a overall preponderance of c-ANCA as compared to p-ANCA and X-ANCA; a slightly higher incidence of autoantibodies was also seen in the LL group as compared to BL and BT groups. Medina et al.25 had observed 21% and 16% p-ANCA in LL and BL groups, respectively, while c-ANCA was seen in 5% of LL cases and X-ANCA were absent. The HL-60 cell line is known to exhibit only the primary granules. Therefore, they may not be a good substrate to detect X-ANCA, which may be present in secondary cytoplasmic granules specific for LF.26 Freire et al.27 found X-ANCA in 28.8% in the LL group and did not find anti-MPO and anti-PR3 by ELISA among them. However, in our four cases of X-ANCA, we detected anti-LF, where one patient in the BT group had both anti-PR3 and anti-LF. Also, it has been proposed by Luqmani et al.28 that the clinical features of an ANCA related disease, Wegener’s granulomatosis, reflect a spectrum of immune reactivity which is often seen in mycobacterial diseases as mentioned by Turk et al. nearly 30 years ago whereby the chronic local inflammation in the upper respiratory tract and lungs reflects the tuberculoid end of the disease, while kidney vasculitis would reflect the lepromatous form.

The recent findings suggest that autoantibodies to various autoantigens seem to be quite variable and common in the leprosy spectrum probably due to an adaptive immune response or polyclonal B cell activation or cross reactivity between M. leprae antigens and host immunogenetics which could differ among patients. The serological autoimmune profile seen in leprosy could be significant, and these autoantibodies could have some pathogenetic importance in leprosy patients developing complications.
References

1. Ridley DS, Jopling WH. Classification of leprosy according to immunity, a five group system. *Int J Lepr*, 1996; **34**: 255–273.


