Antineutrophil cytoplasm autoantibodies in patients with tuberculosis are directed against bactericidal/permeability increasing protein and are detected after treatment initiation

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ABSTRACT

Objective. To determine the prevalence of antineutrophil cytoplasm autoantibodies (ANCA) and its antigenic specificities in sera of patients with pulmonary tuberculosis (Tb) before and after treatment.

Patients and methods. Sixty-eight patients with culture-proven Tb were studied for the presence of ANCA, both by indirect immunofluorescence (IIF) and ELISA against proteinase-3 (PR3), myeloperoxidase (MPO) and bactericidal/permeability increasing protein (BPI).

They were sought before treatment and in 52 of them also after therapy for the infection. High sensitivity C-reactive protein (CRP) was also measured at both times.

Results. ANCA by IIF were found in 3/68 (4.4%) sera prior to treatment, one C-ANCA and two P-ANCA, all recognizing BPI. After treatment, this increased to 15/52 (28.8%), 3 C-ANCA and 12 P-ANCA, the majority directed against BPI (11/15, 73%). BPI-ANCA were positive in 6/68 (8.8%) and 15/52 (28.8%) before and after Tb after treatment initiation (p=0.003). PR3-ANCA and MPO-ANCA were negative in all Tb sera. A positive ANCA test correlated with CRP as inflammatory marker (p=0.001).

Conclusions. The prevalence of ANCA in culture positive Tb patients is modified by Tb chemotherapy. BPI is the main target antigen for ANCA in tuberculosis and BPI-ANCA increase after treatment.

Introduction

Antineutrophil cytoplasm autoantibodies (ANCA), though sensitive and specific for certain forms of small-vessel primary vasculitides (1), have also been reported in other diseases, among which, certain infections (2-6).

In tuberculosis (Tb), reports of ANCA prevalence are controversial (6-7). Their importance relates to the crucial issue of correct diagnosis and treatment of each condition, as clinical similarities between diseases like Wegener’s granulomatosis (WG) and Tb exist. Certain factors that may influence the results lie on the prevalence of Tb in different countries, its type, extent and stage, associated diseases, and especially, the time of serum sampling and ANCA search and its relationship to the prior or concomitant use of anti-tuberculous drugs, an effect which was not overcome in the largest studies (6-7). The association between ANCA and drugs used for Tb has not been formally studied. We have previously mentioned that based on reports from others, ANCA which recognise bactericidal/permeability increasing protein (BPI-ANCA) have been postulated as the more frequently found in Tb. Thereafter, and stimulated by our previous study (6), we decided to extend our observations of ANCA in Tb, launching a search for BPI-ANCA, and to determine if Tb chemotherapy against the infection influences the frequency of ANCA by IIF and ELISA against proteinase-3 (PR3-ANCA), myeloperoxidase (MPO-ANCA) and BPI (BPI-ANCA).

We took care that no prior Tb chemotherapy had been started before ANCA testing. Additionally, we retested after treatment.

Patients and methods

Patients

From September 2004 to November 2005, 76 consecutive patients, over 18 years of age, attending the Pneumology Service at University Hospital “Dr. Jose Eleuterio González” of the Universidad Autónoma de Nuevo León, Monterrey, Nuevo León, Mexico, with the suspicion of active pulmonary Tb were invited to participate. After approval of the study
by the Internal Ethical Committee and obtainment of patients’ written consent, blood was drawn and stored until testing -70°C before starting treatment for Tb with Rifater® (75 mg isoniazid, 150 mg rifampicin, 400 mg pyrazinamide) 4 tablets qd plus ethambutol 1200 mg qd for two months, and then Rifinah® (150 mg isoniazid, 200 mg rifampicin – both drugs from Aventis Pharma, Mexico) 4 capsules three times a week for four additional months, which is the usual scheme used for treatment in our institution. Once Tb was confirmed by culture, a second serum sample was obtained between 60-90 days of treatment and processed as described below. There were some patients initially suspected as having Tb, but in whom other mycobacterial or coccidiodial infections were diagnosed. We also tested their sera as another control group. Excluded were subjects without a bacteriological confirmation of Tb, younger than 18 years-old, pregnant, known to have cancer (past or present), those under treatment with immunosuppressive agents or Tb chemotherapy, or with prior intake of any of the following: procarcinamide, hydralazine, phenytoin, propylthiouracil, D-penicillamine and allopurinol, and those with inflammatory bowel disease.

There were some patients who, during the first serum sampling, were not known to be HIV positive. They were retained to determine the overall initial frequency of ANCA positivity in the group, as were those in whom the second serum sample was unavailable.

Two rheumatologists looked for rheumatic symptoms (JE and JR). Other data included: demography, acid-fast stains on sputum, prior history of exposure to Tb patients, of vaccination with Bacille Calmette-Guérin (BCG), time since first Tb symptoms and from that to the first serum sampling, and concurrent diseases. Controls were 39 healthy subjects (19 tuberculin skin test positive, 20 negative), all medical students who were volunteer blood donors from the same hospital.

ANCA and C-reactive protein (CRP) testing
Sera were tested for ANCA by indirect immunofluorescence (IIF) in commercially acquired slides (Immuno-Concepts, Sacramento, CA, USA). They were initially diluted 1:10. If positive, further dilutions were made until fluorescence was no longer detected. For those in whom a P-ANCA pattern was shown, confirmation followed with formalin-fixed neutrophils (same manufacturer). Antinuclear antibody testing in Hep-2 cells was also ran in such cases (Immuno-Concepts, Sacramento, CA, USA).

Capture ELISA testing for PR3-ANCA and direct ELISA for MPO-ANCA (Wieslab, Lund, Sweden) and direct ELISA for BPI-ANCA detection (Orgentec, Mainz, Germany) were made following each manufacturer’s instructions. Positive values for each specific autoantibody were: PR3-ANCA:>30 U/mL; MPO-ANCA:>25 U/mL and BPI-ANCA:>10 U/mL. Positive control sera were from three PR3-ANCA proven WG patients and one MPO-ANCA patient with microscopic polyangiitis.

High-sensitivity CRP in sample sera of patients before and after treatment was detected by immunoturbidimetric method with a kit from Randox Laboratories Ltd., Antrim, Great Britain (normal values 0-5 mg/L). All sera were tested for HIV with a kit from BioRad (Genscreen Plus HIV Ag-Ac, Raymond Poincaré, France). All stated assays were performed in duplicate.

Statistical analysis
Descriptive statistics was used for continuous variables. The Kolmogorov-Smirnov test was applied to determine if normal distribution was present. Student’s t-test was performed if the case, and for abnormal distribution, the Wilcoxon-U-Mann-Whitney test was used. For categorical variables, Pearson’s chi-square, and contingency tables with Fisher’s exact test were applied. P-values <0.05 were reported as significant.

Results
From the initial 76 suspected Tb patients, 68 were culture-positive for M. tuberculosis. Eight patients had other diseases: four coccidioidomycosis, three mycobacteriosis by atypical bacilli and one remained as probable Tb but was culture-negative. The bronchial investigations done to look for other infections did not report Pseudomonas. The presenting features of the 68 Tb patients are shown in Table I. Five of these patients proved to be HIV-positive when tested.

ANCA by IIF were positive in 3/68 patients (4.4%) before antituberculous therapy (one C-ANCA and two P-ANCA), all of them being also BPI-ANCA positive; the median (range) values of these three patients were 44.8 U/mL (25.4-78.3). PR3-ANCA and MPO-ANCA were negative in all sera.

Table I. Demographic, clinical and radiological characteristics of the 68 patients with pulmonary Tb (expressed in numbers and percentages unless stated).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females /Males</td>
<td>29/39 (43/47)</td>
</tr>
<tr>
<td>Mean age ± SD</td>
<td>39.2 ± 14.81</td>
</tr>
<tr>
<td>Acid-fast stain positive</td>
<td>52 (76.5)</td>
</tr>
<tr>
<td>History of BCG vaccination</td>
<td>51 (75)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>22 (32.4)</td>
</tr>
<tr>
<td>Suspected positive prior exposure to M. tuberculosis infected subjects</td>
<td>19 (28)</td>
</tr>
<tr>
<td>Alcohol abuse</td>
<td>18 (26.5)</td>
</tr>
<tr>
<td>Tobacooism</td>
<td>18 (26.5)</td>
</tr>
<tr>
<td>Marihuana use</td>
<td>11 (16)</td>
</tr>
<tr>
<td>HIV-positive</td>
<td>5 (7.4)</td>
</tr>
<tr>
<td>Cocaine abuse</td>
<td>2 (3)</td>
</tr>
<tr>
<td>Days with symptoms before first sample obtainment (mean ±SD)</td>
<td>64.6 ± 55.5</td>
</tr>
<tr>
<td>Fibrocutaneous infiltrate</td>
<td>50 (73.5)</td>
</tr>
<tr>
<td>Reticulonodular infiltrate</td>
<td>40 (58.8)</td>
</tr>
<tr>
<td>Consolidation</td>
<td>9 (13.2)</td>
</tr>
<tr>
<td>Atelectasis</td>
<td>7 (10.3)</td>
</tr>
<tr>
<td>Pleural effusion</td>
<td>4 (5.9)</td>
</tr>
<tr>
<td>Pneumothorax</td>
<td>1 (1.5)</td>
</tr>
</tbody>
</table>
BPI-ANCA positivity was found in 6/68 patients (8.8%), values are shown in Table II. Three of them were ANCA negative by IIF.

Of the 68 patients, 52 were able to be tested after antituberculous treatment. Seven were lost for follow up, six did not accept a second blood sample being drawn, and three died; all were HIV-positive.

After Tb chemotherapy, there were 15/52 (29%) positive by IIF, 3 C-ANCA and 12 P-ANCA, for a significant difference when compared to the results prior to treatment (p=0.003). PR3-ANCA and MPO-ANCA were negative, but BPI-ANCA were detected in 11/15 (73.3%) patients who were IIF positive, for these sera the median (range) values were 16.4 U/mL (10.24-64). Three of the fifteen positive ANCA by IIF had also tested positive before anti-tuberculosis therapy and remained so after treatment. Only one of these 3 BPI-ANCA positive subjects had a negative seroconversion after therapy, while the other two remained BPI-ANCA positive.

BPI-ANCA was positive in 15/52 patients (28.8%), but only 8/15 were ANCA-positive by IIF. Four of these 15 sera were positive to BPI-ANCA before Tb chemotherapy.

The results of ANCA by each method and for every group are shown in Table II. All HIV-positive patients were ANCA negative prior or after treatment. Three of these patients died during follow-up and two of them had already started treatment for Tb when HIV infection was demonstrated. There were 11 patients with history of marihuana and drug abuse, two of them had positive BPI-ANCA before and after anti-tuberculosis therapy but P-ANCA by IIF was detected only in these two patients after Tb chemotherapy. Two of 11 these patients had cocaine-abuse history and both were ANCA negative by any method.

There were 5 Tb chemotherapy-resistant patients. None of them was ANCA positive by any method before Tb chemotherapy was instituted. However, one of these patients developed P-ANCA (at a 1:40 dilution) associated with positive BPI-ANCA (23.7 U/mL) and other positive BPI-ANCA (10.72 U/mL) with negative ANCA by IIF, all of them after treatment. We could not obtain the second sample from one patient, and the remaining stayed ANCA negative. All these patients were HIV negative. The mean values of ANCA by ELISA and CRP in these patients were not different to those treatment-responsive.

A difference in the average value of CRP was found for the whole group before and after treatment. In the first sample it was of 1.91±1.23 mg/L, while after therapy it increased to 3.05±1.91 (p=0.005). Patients who were ANCA positive after treatment had absolute values of CRP higher than those ANCA negative, 4.35±1.67 mg/L versus 2.51±1.75, respectively (p=0.0001).

No differences regarding age, gender, time of symptoms evolution, acid-fast bacilli stain sputum results, radiographic findings, HIV status nor concomitant diseases were found for the following groups: between ANCA positive and negative patients prior to treatment, between those patients who remained negative and to those who developed ANCA after treatment, between HIV-positive and negative patients and between those therapy-sensitive and resistant. No characteristic seemed to predict which patient would be ANCA positive after starting Tb treatment.

**Discussion**

In this study we found that ANCA in Tb patients are mainly directed against BPI and mostly appear after treatment. In contrast to the largest studies published, (6-7) we were able to obtain samples prior to treatment initiation, having therefore, overcome this caveat. In the study by Flores-Suárez et al., (6) though the patients were prospectively...
studied, the majority were already under therapy; besides, only a small proportion of patients had culture-proven *Tb*. In contrast to that article, we could not confirm reactivity against PR3, a fact that had been stated as unexplained by the authors. In the one by Teixeira et al., (7) although all patients had positive cultures for *M. tuberculosis*, the samples came from a serum bank and 20% had already started treatment. ANCA determination was transversal and no serial serum sampling was done. A question raised in both studies relates to the possibility that ANCA positivity could be associated with treatment. Several drugs have been reported to promote ANCA production and at least theoretically, some of them like isoniazid could be transformed by the products of activated neutrophils and lead to ANCA induction (8-14).

Previous information exist on BPI-ANCA on other conditions besides vasculitides, among them, infections. In an article from the Lübeck group, the authors found that BPI-ANCA were present in approximately 5% of patients with infections, mainly HIV, but also though less frequently, in others. Included were 10 patients with *Tb*, but detailed information about this subgroup was not presented (15). After this and our previous findings we decided to launch this search with a longitudinal approach in where all patients had proved lung *Tb* and were later subject to the same therapy, thereby eliminating the possibility that other drugs were prescribed at the time of the second sample.

It is tempting to propose that BPI-ANCA production is at least partially triggered by treatment. Anti-Tb therapy may lead to mycobacterial death and release of cell wall constituents (see below) that could react with BPI. However, further studies are necessary to prove this and to explain why at least in certain subjects, such a response happens. Also, the role of BPI-ANCA development in *Tb* patients is uncertain. We did not observe that the course of the infection was altered by BPI-ANCA. Nonetheless, those with these antibodies had higher values of CRP. We decided to measure CRP to evaluate response to *Tb* therapy. Decrease in CRP levels during *Tb* treatment could be indicative of response (16). It was interesting that some patients had an increase, suggesting that the inflammatory response is greater in the presence of ANCA, or lead to speculation that these antibodies could fulfill some pathogenic role in the lung inflammatory response as has been described in WG or other pulmonary inflammatory processes (17, 19).

BPI is a cationic protein with high affinity for lipopolysaccharide (LPS), present in Gram-negative bacteria. In bacterial infections it participates in the opsonisation, phagocytosis and killing of microorganisms (20); contributes to the presentation of bacterial antigens to immunocompetent cells, as shown in the case of *Neisseria meningitidis* outer membrane vesicles, which were efficiently internalized by monocyte-derived dendritic (MDDC) cells by BPI in a dose-dependent fashion, suggesting a novel, efficient antigenic delivery to MDDC and the enhancement of these cells antigen-presenting functions (21). There is also, evidence of other properties by BPI, such as endothelium damage protection (22). However, although it increases in active *Tb* patients, (23, 24) its role in this disease is unclear. It is tempting to speculate it may operate in a similar fashion as in bacillary infections, interacting with molecules such as TLR4, which are known to be operative in *M. tuberculosis* infection (reviewed in 25). In this sense, LPS and lipoarabinomannan (LAM), a lipidic glycoprotein in the cell wall of *M. tuberculosis* hold similar characteristics. LAM shares affinity to other proteins which interact with LPS, like LPS binding protein (LBP) and CD14 (23). It increases in active *Tb* patients too. Therefore, it is possible that BPI could interact with LAM. Indeed, different studies have described increased expression of BPI by peripheral blood mononuclear cells from *Tb* patients; a recent one used gene profiling to show the increased expression at this level too (23, 24, 26).

As for BPI-ANCA, it has been shown that they inhibit BPI function and could, therefore, interfere with the protecting properties of the latter. In this particular scenario, antibodies against BPI could favour the presence of mycobacteria in the extracellular milieu, leading to enhanced inflammation. This might explain the higher CRP levels observed in positive BPI-ANCA patients after treatment. On the other hand, BPI-ANCA presence after therapy may also be the consequence of higher levels of BPI which may operate in a similar way as in Gram-negative bacterial infections, trying to enhance antigenic presentation to immunocompetent cells. They can also induce reactive oxygen species release from neutrophils, an effect also induced by MPO and PR3-ANCA. As such, they could elicit or amplify tissue damage when present in different diseases. All this, though, remains speculative.

BPI-ANCA recognise mainly conformational epitopes on the BPI molecule, the majority directed to the C-terminal portion, although N-terminal directed IgG-BPI-ANCA isolated from a Wegener’s granulomatosis patient were shown to interfere with the antimicrobial activity of BPI in vitro (19, 27) Consequently, distinct effects could be induced by each BPI-ANCA subtype, and it remains to see which one is present in different diseases where they have been detected. These properties need to be examined in BPI-ANCA from *Tb* patients, either those present prior to treatment, or as it seems from our results, possibly induced by antituberculosis chemotherapy.

Undoubtedly, certain ANCA have a precise diagnostic role in vasculitides. However, as these diseases have a lower prevalence worldwide in comparison to *Tb*, they can present with clinical features which simulate infections (4, 5). Therefore, their judicious search and better characterisation in certain countries with high *Tb* prevalence is important. In fact, not only the report from our country, but also from others raised this issue (6, 28).

In conclusion, ANCA found in *Tb* mainly develop after treatment for the disease and are directed against BPI. The effects of these antibodies and of its target antigen in *Tb*, as well as the influence of BPI-ANCA in the course of the infection need evaluation. Knowledge of the mechanisms that trigger these autoantibodies production is desirable.
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