THE ROLE AND CLINICAL IMPLICATIONS OF ANCA & ITS SUBSPECIFICITIES IN SOME RHEUMATIC DISORDERS

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ABSTRACT

Objective: Antineutrophil cytoplasmic antibodies (ANCA) and their sub specificity for target antigens: elastase, cathepsin G (CG) and lactoferrin (LF) have been detected in patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and systemic sclerosis (SSc). We aimed at finding out the prevalence of ANCA, their target antigens and their clinical implications in Egyptian patients.

Methods: Serum samples were collected from sixty-one patients. Thirty-two had rheumatoid arthritis (RA), sixteen had systemic lupus erythematosus (SLE) and thirteen had systemic sclerosis (SSc). Samples were examined for ANCA with the indirect immunofluorescence technique (IIF). Antibodies directed against elastase, CG and LF were determined using the solid phase enzyme immunometric assay (ELISA). Statistical studies were used to associate clinical findings to the presence of ANCA sub specificities.

Results: In our sample of Egyptian patients, the prevalence of ANCA in RA was 50%, in SLE 42.8% and in SSc it was 55%. The probability of occurrence of antibodies against elastase was 22.3%, CG 13.8% and LF 16.6% in RA patients. The most recognized antigen in SLE patients was anti-elastase 62.5%, CG 25% and LF 18.7%.
In RA patients, the presence of anti-elastase antibodies was associated with a long duration of the disease. The presence of CG was associated with the presence of renal affection and erosive changes in X-rays. Skin vasculitic lesions were associated with the presence of LF. In SLE patients, the presence of CG was associated with lupus nephropathy. Positivity for anti-elastase was associated with digital vasculitis and arthropathies. The presence of serositis in SLE patients was not associated with any of the target antigens. In RA patients, serum ANCA sub specificities (anti-elastase, LF) were significantly increased in cases than controls. In SLE patients serum anti-elastase level and anti-Cathepsin G were significantly higher in cases than controls. In SSc patients, only serum anti-elastase was significantly higher in cases than in controls.

Conclusion: The prevalence of ANCA in different rheumatic disorders indicates severe disease with increased inflammatory activity. The antibodies directed against target antigens may act as a marker for disease subsets.

INTRODUCTION

Rheumatic disorders can be complicated by vascular inflammation that mainly involves the small vessels such as venules, arterioles and capillaries (Skoloff and Bunim, 1957). A pathogenetic role for various autoantibodies in the development of vasculitis has been postulated.

Antibodies to neutrophil cytoplasmic antigens (ANCAs) have been extensively studied as markers for systemic vasculitis and crescentic glomerulonephritis (Kallenberg, 1994). Two major patterns of ANCAs can be identified using indirect immunofluorescence (IIF). The classic cytoplasmic pattern (c-ANCA) is seen mainly in Wagener’s disease and reflects the presence of anti-proteinase-3 antibody (Bini et al., 1992). The perinuclear ANCA (p-ANCA) staining pattern has been documented to occur in patients with necrotizing and crescentic glomerulonephritis, microscopic polyangiitis and Churg-Strauss syndrome (Cohen et al., 1990 and Cohen et al., 1991). However, (p-ANCAs) are far from any specific
strictly defined clinical disorder. They have been reported to occur in
many inflammatory disorders e.g. inflammatory bowel disease,
primary sclerosing cholangitis (Saxon et al., 1990) and rheumatic
disorders (Savige et al., 1991; Braun et al., 1993 and Waldender and
Schneider, 1993).

The target antigens for p-ANCAs in these disorders are
usually unclear although several were detected such as
myeloperoxidase (Falk and Jennette, 1988) lactoferrin (LF),
Cathepsin G (CG) (Hagen et al., 1996). Interestingly, different antigenic
specificities of ANCA have been associated with distinct clinical subsets i.e.
anti-elastase and/or anti-LF with central nervous system in SLE (Nassberger
et al., 1989). Also, a recent study by Zhoa et al. (1998) correlated anti-CG
with active renal lesions.

Conflicting evidence have been presented concerning the
spectrum of ANCA sub specificities, particularly autoantibodies
against elastase, cathepsin G and lactoferrin, in different rheumatic
disorders (rheumatoid arthritis, systemic lupus erythematosus and
systemic sclerosis) (Coccovo et al., 1999; Galeazzi et al., 1998 and
Brimnes et al., 1997).

Thus the aim of the present study was to determine the prevalence of
ANCAs and their sub specificities in RA, SLE, SSc patients and their
possible association with the development of clinical disease subsets.

MATERIAL AND METHODS

This study was conducted on thirty-two patients suffering of RA,
sixteen patients suffering of SLE and thirteen patients presenting with SSc,
al so diagnosed according to the American College of Rheumatology criteria
for these diseases (Arnett et al., 1988; Tan et al., 1982 and Masi et al.,
1980). All patients were selected from the Rheumatology and Rehabilitation
Out-patient Clinic of Ain Shams University Hospitals. Ten healthy subjects
matched for age and sex were also included in this study and served as
controls.

The disease activity index of RA patients was calculated according
to Mallya and Mace (1981). All RA patients were maintained on disease
modifying drugs (DMARDs) together with non-steroidal anti-inflammatory
drugs (NSAIDs). Disease severity was assessed according to Steinbroker et
The SLE activity was assessed according to the SLE disease activity index (SLE DAI) (Bombardier et al., 1992). All SLE patients were receiving steroids. Some patients with SSc were taking symptomatic treatment. Patients with recently diagnosed RA (<2 years) were considered as early RA and those with the disease for more than 2 years were considered long standing RA.

The Studied Cases Were Subjected To The Following:

- Full history taking.
- Thorough clinical examination (general, joints and other systems) presence of clinical vasculitis was diagnosed according to Coremans et al. (1992).
- Erythrocyte sedimentation rate (ESR), hemoglobin (Hb) and complete blood count (CBC).
- Rheumatoid factor.
- ANA, anti-double stranded DNA (anti ds-DNA).
- Anti centromere anti-Scl 70 (for suspected cases of SSc).
- Renal function (urine analysis, urea, creatinine).

Samples:

Sera from patients were studied for anti-elastase, anti-cathepsin G, and anti-lactoferrin using solid phase enzyme immunometric assay (ELISA). They were designed for quantitative determination of anti-neutrophil cytoplasmic antibodies (ANCA) directed against elastase, cathepsin G and lactoferrin, respectively. All assays recognize IgG class autoantibodies, the microplates are coated with one of the ANCA antigens elastase, cathepsin G or lactoferrin.

The binding of present autoantibodies as well as the formation of the sandwich complexes and enzymatic color reaction taking place in the reaction were estimated according to manufacturer instructions of ORGen Tec Diagnostika GmbH kupferbergterasse 17-19 Germany.

Phase 1:

Calibrators, controls and pre-diluted patients’ samples were pipetted into the wells of the microplate. Any present ANCA antibodies bind to the
inner surface of the wells. After 30 minutes incubation the microplate is washed with wash solution for removing non-reactive serum components.

**Phase 2:**

An anti-human-IgG horse raddish peroxidase conjugate solution was pipetted in to the wells of the microplate to recognize the autoantibodies bound to the immobilized antigens. After 15 minutes incubation, any excessive enzyme conjugate that was not specifically bound was washed away with wash solution.

**Phase 3:**

A chromogenic substrate solution containing TMB was dispersed into the wells. During the 15 minutes of incubation, the color of the solutions changed into blue. Color development was stopped through adding 1M hydrochloric acid. The solution color changes into yellow. The amount of color was directly proportional to the concentration of IgG in the original sample.

To read the optical density, a microplate reader with a 450 nm titter was required. Bi-Chromatic measurement with a 600-690 nm reference was recommended. The optical density for each calibrator may be graphically plotted against the concentration of IgG and unknowns extrapolated from the culture.

The normal range for studied serum samples form healthy blood donors has been established to be less than 10µ/ml. It is considered elevated if > or = 10 µ/ml.

Indirect immunofluorescence testing for ANCA was done according to the method described by Wilk et al. (1988), adopted at the first international workshop.

Serial dilutions of patients or control sera (1:16 to 1:512) in phosphate buffered saline were tested. A titer of > or =1:32 was considered positive. Fluorescence patterns were classified as classic cytoplasmic (c-ANCA), perinuclear (p-ANCA) or atypical. To investigate whether the antigens recognized by p-ANCA were artifactually redistributed during ethanol fixation, phosphate buffered paraformaldehyde (0.5%) at pH 8.5 was additionally used to fix granulocyte. After which testing for ANCA was performed as described above.
Statistical methods:

Statistical analysis was done using SPSS software version 7.4. The logistic t test was used for comparison between 2 mean groups for skewed data. The Spearman’s rank correlation test was used to study the association between all studied variables in each group. The probability of error at <0.05 was selected as significant level.

RESULTS

The prevalence of ANCA was 50% in RA patients, 42.86% in SLE patients and 55% in SSc patients. The rate of occurrence of ANCA in long standing RA was higher than in cases with early RA (54% versus 16%). The occurrence of anti-elastase in RA patients was 22.3%, anti-lactoferrin 16.6% and anti-Cathepsin G 13.8%. The most recognized antigen in SLE patients was anti-elastase 62.5% then anti-Cathepsin G 25% and least was anti-lactoferrin 18.7%.

The descriptive data of the 3 studied groups are presented in table (1) including age, duration of disease, ESR, Hb, RBCs, total leucocytic count, platelet, serum creatinine, anti-elastase, anti-Cathepsin G and anti-lactoferrin.

Comparing the mean serum levels of ANCA’s sub specificities, there was a significant difference between serum anti-elastase and anti-lactoferrin in RA patients as compared to controls. Also in SLE, serum anti-elastase and anti-Cathepsin G showed a highly significant difference in cases as compared to controls (Figure 1).

SSc patients showed a highly significant increase in serum anti-elastase as compared to controls, which was not the same for anti-Cathepsin G and anti-lactoferrin (Figure 1). Comparing the 3 studied groups together there was no significant difference regarding anti-elastase. SLE cases showed a significant increase in anti-Cathepsin G as compared to other groups and RA patients showed a significant increase in anti-LF level as compared to other groups.
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Correlation matrix between different quantitative parameters in relation to ANCA and their target antigens:

Serum anti-elastase correlated with the duration of the disease ($r=0.446$). Disease activity indices in RA patients did not correlate with the presence or absence of ANCA or target antigens. In SLE patients both anti-Cathepsin G and anti-lactoferrin seemed to be related to the duration of the disease ($r=0.5135$, $r=0.5901$ respectively). Also, anti-Cathepsin G was related to disease activity indices.

Correlation of the clinical manifestation to the presence of specific target antigens:

In RA, there was a significant increase in anti-elastase in relation to the presence of subcutaneous nodules. The presence of progressive X-ray erosions was associated with an increase of anti-Cathepsin G. Moreover, cases with renal affection showed a highly significant increase of anti-Cathepsin G as compared to other cases. The presence or absence of RF was not related to ANCA positivity or presence of any of its target antigens. Skin vasculitic lesions were associated with an increase in serum anti-lactoferrin (table 2).
In SLE patients, it is noteworthy that skin manifestations (malar rash, photosensitivity) were associated with the presence of anti-Cathepsin G. Moreover, digital vasculitis was associated with the presence of anti- elastase. SLE nephropathy was associated with the presence of anti- Cathepsin G. Cases with arthropathies were associated with the presence of anti-elastase. The presence of serositis was not associated with the presence of any of the target antigens (table 3).

None of ANCA target antigens was associated with any of the clinical manifestations in cases of SSc except anti-Cathepsin G with renal affection and anti-elastase with vasculitic lesion.

**DISCUSSION**

p-ANCA has been observed in a wide range of rheumatic disorders such as rheumatoid arthritis, systemic lupus erythematosus and systemic sclerosis (Savige et al., 1991). p- ANCA react with different target antigen, elastase, cathepsin G and anti-lactoferrin and others. The frequency of ANCA in RA patients and its clinical significance are not well documented.

In the present study, the prevalence of ANCA with IIF was 50% in RA patients. The rate of occurrence of ANCA in long-standing RA was higher than in those with early RA (54% versus 16%). My results were comparable with Mulder et al. (1993) who found p-ANCA in 70% of RA patients. The prevalence of p- ANCA in early RA patients was 65% and 85% in long-standing RA. De Bandt et al. (1996) suggested that the incidence of ANCA is 48% in long-standing RA and 28% in early RA. Anu Mustalia et al. (1997) found almost comparable result.

It was clear that the disease activity indices of RA patients did not correlate with the positivity of p-ANCA. This was in accordance with the study of Mulder et al. (1993). On the other hand Anu Mustila et al. (1996) showed that positivity of p-ANCA was associated with clinical and laboratory findings indicating increased inflammatory activity. The presence of ANCA was associated with the duration of the disease.

The occurrence of anti-elastase 22.3%, anti-lactoferrin 16.6% and anti-Cathepsin G 13.8 was found in the study performed by Mulder et al. (1993). Other values were found in the study of De Bandet et al. (1996).
The percentage of occurrence of anti-elastase was (14%) followed by anti-lactoferrin (45%) of RAV. A noticeably higher incidence of anti-lactoferrin (32%) in RA patients, was found by Braun et al. (1993).

In our trial, the correlation of the presence of extra-articular manifestations with the presence of specific target antigens, showed a significant increase in anti-elastase in relation to the presence of subcutaneous nodules and the duration of the disease. Progressive X-ray erosive changes were associated with an increase in anti-Cathepsin G. Moreover, cases with renal affection showed a significant increase of anti-Cathepsin G. The presence or absence of RF was not related to ANCA positivity or the presence of any of its subsets.

Rheumatoid vasculitis was associated with anti-lactoferrin antibodies. This was in accordance with Coreman et al. (1992). On the other hand, Coccovo et al. (1999) showed no significant difference between serum anti-lactoferrin from RA patients as compared to controls. Also, serum levels did not correlate with disease activity.

ANCA have been found in patients with SLE. However, the prevalence of ANCA and their target antigens is still not certain. Also, the association between the clinical findings and the specific antigens is not fully verified. In the present study the prevalence of ANCA in SLE patients was 42.86%, which was mainly of the p-ANCA pattern. None of the c-ANCA was detected. The most recognized antigen was anti-elastase, which presented in 62.5%, then anti-Cathepsin G 25% and anti-lactoferrin 18.76%. It was evident that the presence or absence of ANCA and its subsets were not predictive of disease exacerbation. In addition, none of the target antigens correlated with disease activity except anti-Cathepsin G.

In previous studies, the prevalence of ANCA ranged from 16.4 to 45% (De Bandt et al., 1996; Sponk et al., 1996; Zhoa et al., 1998 and Galeazzi, 1998). The difference in incidence may be attributed to differences in patients’ populations or difference in methods of assessment. Most of former studies support the idea that the presence of ANCA or its subsets was not related to clinical activity markers (Sponks et al., 1996). On the other hand some authors declared that patients positive for ANCA had higher scores of SLE disease activity index than those without ANCA (Nishiya et al., 1997).
Regarding the duration of the disease, it was suggested that there was a positive correlation between the duration of the disease and anti-Cathepsin G as well as anti-lactoferrin serum levels. Sponk et al. (1996) proved that the prevalence of ANCA of defined specificity tends to be higher in patients with disease history more than five years.

It is noteworthy that skin manifestations, mainly malar rash and photosensitivity, are associated with increased serum levels of anti-Cathepsin G. Also, cases with digital vasculitis seem to be related to the presence of anti-elastase antibodies. To the contrary Schnabel et al. (1995) did not find any association of p-ANCA or any sub specificity with any clinical manifestation of SLE, notably lupus vasculitis.

In the present study, there was a significant difference in the serum level of anti-Cathepsin G in patients with SLE nephropathy compared to others. In support to our findings Zhao et al. (1998) found a highly striking incidence of anti-Cathepsin G that reached 62%. The higher incidence of anti-Cathepsin G in the latter study may be attributed to the selection of patients (all patients had lupus nephritis). Those authors found that anti-lactoferrin antibodies had no correlation with active renal disease.

Nishiya et al. (1997) found that the prevalence of anti-Cathepsin G and anti-lactoferrin was quite low as compared to our results. None of the sera recognized anti-elastase. Also there was no correlation with ANCA subsets and organic involvement (the incidence of anti-lactoferrin was 36%, 64% for anti-Cathepsin G and none for HLA.

Nassberger et al. (1989) suggested that 6 out of 104 SLE patients had anti-elastase reactivity and 4 had neurological manifestations. Those authors concluded that anti-elastase might be a marker of neurological involvement in SLE. This was different to our findings. Anti-elastase seemed to be associated with the presence of arthritis in SLE patients. In a former study, they suggested that anti-Cathepsin G tends to be associated with arthritis (Sponk et al., 1996). Those authors found 9% of their patients positive for anti- cathepsin G and 15% for anti-lactoferrin.

In our studied cases, arthropathies seemed to have a high level of anti-elastase. The presence of serositis was not associated with the presence of high titer of anti-lactoferrin in contrast to the study performed by Niles et al. (1989).
Regarding systemic sclerosis 55% of the patients had +ve ANCA and anti-elastase was detected in 66% of patients’ sera. Anti-cathepsin G was recognized in only 2 patients and anti-lactoferrin in one patient. There was a significant increase in serum levels of anti-elastase in SSc patients when compared with controls. Moreover, there was an association between the positivity of ANCA and the presence of anti-elastase. The presence of skin vasculitic lesion was associated with the presence of anti-elastase. Patients in whom anti-cathepsin G was detected had renal affection. Thus, the presence of anti-Cathepsin G may be related to renal affection but the patients’ number was quite small to verify this finding that needs further investigation. None of the clinical findings seems to be related to the presence of anti-lactoferrin.

In accordance with our results, Hiroko et al. (1998) found the presence of p-ANCA 55% and the prevalence of anti-Cathepsin G and anti-lactoferrin (1 each out of 5). The incidence of the subset target antigens was not verified in other data. None of the previous studies correlated the clinical findings to the presence of the antigens.

The exact mechanism by which ANCA and its subsets may contribute to granulocyte mediated vascular damage is multiple. Perinuclear ANCA has been shown to induce an increased release of reactive oxygen species and granule contents by granulocytes (Falk et al., 1990 and Mulder et al., 1993). Another pathogenetic mechanism may be cross-reactivity between epitopes on the granulocyte and endothelial cell surface. Such cross reactivity has been suggested by demonstration of shared antigen between granulocytes and endothelial cells (Hogg et al., 1984). This hypothesis is supported by the finding that the granulocyte stimulated with p-ANCA in combination with tumor necrosis factor α (TNF-α) and lipopolysaccharide are capable of inducing damage to cultured endothelial cells (Ewert et al., 1990).

In conclusion, the prevalence of ANCA in different rheumatic disorders (RA, SLE and SSc) indicates severe disease with increased inflammatory activity. The antibodies directed against target antigens may act as a marker for disease subsets.
REFERENCES


Anti-Elastase, Anti-Cathepsin G & Anti-Lactoferrin in Rheumatic Disorders

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دور و التأثير الإكلينيكي لمشتقات الأنكا في بعض الأمراض الروماتيزمية

أما مصطلفى الجنسي:

طريقة إجراء البحث: تم جمع عينات المصل من 32 مريض مصابون بمرض الربان المفصلي و16 مريضا بذلية الحمراء بالإضافة إلى 13 مريض مصاب بمرض التصلب الجلدي. تم فحص العينات لكشف عن وجود مستضدات سيتوبلازم الخلاء المتعادلة باستخدام تقنية استشعار الفلوروسين المناعي، كما تم قياس مستضدات الالاستاز والكالتينين (جي) واللاكتوฟيرين بطريقة الإليزاء. استخدمت الدراسات الإحصائية للتلازم بين الظواهر الإكلينيكي للأمراض ووجود مستضدات سيتوبلازم الخلاء المتعادلة.

النتائج: أُسَرَت نتائج البحث عن انتشار مستضدات سيتوبلازم الخلاء المتعادلة في مرض الربان المفصلي بنسبة 50%، و في مرض الذبابة الحمراء بنسبة 42.8، وأما في مرض التصلب الجلدي فقد كانت سائدة بنسبة 55%، و قد وجد أن معدل وجود مستضدات انزيم الإلئاز هو 22.3%، و الكالتينين (جي) 13.8% أما اللاكتوفرن فين كان 16.6% في مرض الربان المفصلي.

و بالنسبة لمرض الذبابة الحمراء، فقد كان مولد المضاد الأكثر تميزا هو الإلئاز بنسبة 62.5%، و الكالتينين (جي) بنسبة 25%، أما اللاكتوفرن بنسبة 18.7. أيضا أُسَرَت نتائج البحث (الدراسة) عن وجود علاقة بين وجود مستضدات الالاستاز و طول مدة المرض في المرضى المصابين بالربان المفصلي. أما وجود الكالتينين (جي) فقد كان مرتبطة بإصابات الكلي بينما ارتبطت الالتهابات الأوعية الدموية الجلدية بوجود اللاكتوفرن. أما بالنسبة لمرضى الذبابة الحمراء فإن وجود الكالتينين (جي) فقد كان مرتبطة بإصابات الكلي و الالتهابات المفصلية بينما ارتبط وجود مستضدات الإلئاز بالتهاب الأوعية الدموية الإصبعية.

كما وجد أن وجود الالتهابات بالغشية المنكوبة في مرضى الذبابة الحمراء كان مصاحبا لوجود عباراً عيانيا من مستضدات اللاكتوفرن. لاحظ وجود زيادة ذات دلاله إحصائية بين مرضى الربان المفصلي والأصحاء في مستوى مستضدات سيتوبلازم الخلاء المتعادلة كما لاحظ أيضا أن هناك ارتفاع ذو دلالة إحصائية في مستوى مستضدات الالاستاز في مرضى الذبابة الحمراء. هذا إلى جانب ارتفاع نسبة الكالتينين (جي) و اللاكتوفرن و لكنه لم يصل إلى حد الدالة الإحصائية. أما في مرض التصلب الجلدي فقد كان الفارق ذو الدلالة الإحصائية بين المرضى والأصحاء في مستوى مستضدات الإلئاز فقط.

الاستنتاج (النتيجة): يعد انتشار مستضدات سيتوبلازم الخلاء المتعادلة في الأمراض الروماتيزمية المختلفة مؤشرًا لدرجة حدة ونشاط المرض. أيضا مستضدات الموجهة ضد مولادات المضاد المستهدفة يمكن استخدامها كمؤشر للأمراض المختلفة للمريض الواحد.