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Antineutrophil cytoplasmic antibodies in patients with systemic lupus erythematosus: prevalence, antigen specificity, and clinical associations

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Abstract Fifty-five patients with systemic lupus erythematosus (SLE) were examined for antineutrophil cytoplasmic antibodies (ANCA) by indirect immunofluorescence (IIF). Enzyme-linked immunosorbent assay (ELISA) for ANCA against myeloperoxidase (MPO), lactoferrin (LF), proteinase 3 (PR3), elastase (HLE), and bactericidal/permeability-increasing protein (BPI) was performed. The prevalence of ANCA by IIF was 29.1% (16/55 patients). MPO-ANCA were found in 10.9% (6/55), LF-ANCA in 18.2% (10/55), PR3-ANCA in 12.7% (7/55), BPI-ANCA in 23.6% (13/55), and HLE-ANCA in 1.8% (1/55). The levels of BPI-, LF-, and PR3-ANCA correlated with disease activity. A significant association between serositis and the presence of BPI-, LF-, and PR3-ANCA was observed, and PR3-ANCA were found to be associated with arthritis as well. Our results demonstrate that ANCA of various specificities occur in SLE, and BPI appears to be an important target antigen.

Keywords Antineutrophil cytoplasmic antibodies · Systemic lupus erythematosus

Introduction

Antineutrophil cytoplasmic antibodies (ANCA) are a system of autoantibodies targeting a variety of cytoplasmic constituents of neutrophils and monocytes. There are two main types of ANCA as determined by indirect immunofluorescence assay (IIF): C-ANCA that produce a cytoplasmic staining pattern and P-ANCA, producing a perinuclear staining pattern on ethanol-fixed neutrophils [1, 2]. Most C-ANCA react with proteinase 3 (PR3), whereas P-ANCA react with myeloperoxidase (MPO) but also with lactoferrin (LF), elastase (HLE), and other myeloid proteins [3, 4, 5]. C-ANCA with specificity to PR3 are considered important serological markers for Wegener’s granulomatosis (WG) [6]. P-ANCA with specificity to MPO have an established association with other necrotising small vessel vasculitides such as microscopic polyangiitis (MPA) and pauci-immune crescentic glomerulonephritis [4, 7]. In addition, a C-ANCA (atypical) pattern that produces homogeneous granular cytoplasmic staining of neutrophils without central accentuation was identified, but its corresponding antigen specificities are usually unknown [8].

The presence of circulating ANCA in serum is not confined to patients with systemic small vessel vasculitides. It can also be found in connective tissue diseases such as systemic lupus erythematosus (SLE) [9], but this occurrence has not received the same attention as in primary systemic vasculitides due to the presence of a wide array of autoantibodies, some of which are closely associated with specific clinical manifestations and disease activity. Vasculitis and glomerulonephritis are characteristic manifestations of SLE, which frequently appears in the differential diagnosis of primary systemic small vessel vasculitides. This fact necessitates the examination and characterisation of ANCA in SLE in order to establish their diagnostic value and the effective use of ANCA testing in clinical practice.

Recently, a number of articles concerning ANCA prevalence in SLE appeared. ANCA directed against
various neutrophil cytoplasmic constituents have been demonstrated by antigen-specific assays, but the antigens responsible for ANCA reactivity in SLE have still not been fully determined and the role of ANCA in SLE remains unclear. Moreover, reports are controversial concerning their clinical relevance [10, 11, 12, 13, 14, 15, 16].

The objectives of this study were to evaluate the prevalence of ANCA and their target antigens in Bulgarian patients with SLE, to correlate the presence of ANCA with disease activity, and to determine the possible association of ANCA with some of the clinical manifestations of SLE.

**Patients and methods**

**Patients**

From January 1996 to July 2000, serum samples were collected from 55 consecutive SLE patients (two male, 53 female, mean age 36.5 years, range 14–68 years). The samples included in this study were obtained for diagnostic purposes and routine testing from outpatients and inpatients of the Thracian University Hospital Department of Internal Medicine in Stara Zagora, Bulgaria. All patients fulfilled the 1982 revised criteria of the American Rheumatism Association for the diagnosis of SLE [17] and were enrolled in the study independently of disease status and medication. Patients with drug-induced lupus were excluded. Medical records of the study subjects were thoroughly reviewed retrospectively and all clinical and laboratory data relevant to SLE were registered. For each patient, the organ system involvement and disease activity in SLE at the time of the sample collection were assessed by a rheumatologist (M.D.) using the SLE disease activity index (SLEDAI) [18]. Any value above 0 was considered as indicating active disease, which was diagnosed in all 55 patients (median SLEDAI score 8, range 1–33). The frequencies of clinical manifestations as determined by SLEDAI were: central nervous system 5.5%, vascular 21.9%, renal 21.9%, musculoskeletal 52.7%, serosal 32.8%, dermal 72.7%, constitutional 41.8%, and hematologic 36.4%.

**Controls**

Twenty blood donors and 20 patients with reactive arthritis served as controls (four male, 36 female, mean age 32.6 years, range 11–63 years). All serum samples were stored frozen at −20°C until assayed.

**Indirect immunofluorescence assay**

Indirect immunofluorescence assay (IFA) for ANCA was performed by a standard method delineated at the First International Workshop on ANCA, Copenhagen 1988 [19]. Briefly, human peripheral blood neutrophils were deposited on glass slides by cytopsin, fixed in 96% ethanol at 4°C for 5 min, and air-dried. The slides were incubated with patient sera in a dilution of 1:20 in phosphate-buffered saline (PBS); titration of the sera was not performed. Antibody binding was detected with fluorescein isothiocyanate (FITC)-labelled sheep antihuman IgG (Binding Site, Birmingham, UK). The slides were examined with an epifluorescence microscope at ×400 magnification by two independent investigators. Positive and negative control sera were included for all assays. Fluorescence patterns of ANCA were classified as cytoplasmic (C-ANCA), perinuclear (P-ANCA), or C-ANCA (atypical) [20]. All samples with perinuclear or combined perinuclear and nuclear staining patterns were additionally tested on formaldehyde-fixed granulocytes (Binding Site) for differentiation of P-ANCA from antinuclear antibodies (ANA). If there was no cytoplasmic fluorescence on formaldehyde-fixed granulocytes, the result was considered negative.

*Antinuclear antibodies were detected on human epithelioma type 2 (HEp-2) cells using a commercial kit system (Binding Site). Serum samples positive for ANA at the screening dilution of 1:40 were subsequently tested at twofold dilution to determine a titre. The fluorescence patterns were assessed and recorded as homogeneous, rim, speckled, nucleolar, or centromeric.*

**ELISA for ANCA specificities**

Commercial quantitative ELISA kits (Binding Site) were used for detection of anti-PR3, anti-BPI, and anti-MPO. Serum dilution of 1:50 was used in all assays. The test samples were run in duplicates along with six standards (3.1 U/ml, 6.2 U/ml, 12.5 U/ml, 25 U/ml, 50 U/ml, and 100 U/ml). Peroxidase-labelled antihuman IgG was used as a second antibody. The reaction was visualised with 3,3′,5′-tetramethylbenzidine (TMB) as a substrate and read at 450 nm. Following the manufacturer’s instructions, values exceeding 3.5 U/ml for anti-PR3, 15 U/ml for anti-BPI, and 9 U/ml for anti-MPO were regarded as positive.

The use of ELISA for detecting anti-HLE and anti-LF has been previously described [21]. Briefly, ELISA microtitre plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with elastase from human leukocytes (Sigma, St. Louis, Mo., USA) and lactoferrin purified from human milk (Sigma) at concentrations of 1 μg/ml and 3 μg/ml, respectively, in 0.05 M carbonate buffer (pH 9.6). After washing with PBS containing 0.05% polysorbate (Tween) 20 (PBS/T20), the wells were incubated with blocking buffer (1% bovine serum albumin in PBS) for 1 h at room temperature. Test and control sera were applied to duplicate wells at a dilution of 1:50 in dilution buffer (1% bovine serum albumin in PBS/T20) for 1 h at room temperature. Bound antibodies were detected by incubation for 1 h at room temperature with alkaline phosphatase-conjugated goat antihuman IgG (anti-chain specific antibody) (Sigma) diluted to 1:20,000 in dilution buffer. After a final wash, the substrate p-nitrophenyl phosphate at a concentration of 1 mg/ml in 0.05 M carbonate buffer (pH 9.8) containing 1.0 mM MgCl₂ was added and incubated for 30 min at room temperature. The optical density (OD) was measured at 405 nm. Results were expressed as OD index (ODI), which is the ratio of OD in the patient serum to the mean of normal control sera. The positive cutoff values (2.41 ODI for anti-HLE and 2.00 ODI for anti-LF) were determined as the mean value of 40 normal sera ± 3 SD.

**Statistics**

To establish the statistical significance of the observed differences, Fisher’s exact test (frequencies) and the nonparametric Mann-Whitney U test (means) were used. Optical density values are expressed as mean ± SD. Possible correlations between levels of autoantibodies and SLEDAI score were examined using Spearman’s correlation test. For all statistical tests, P < 0.05 was considered significant.

**Results**

The results of ANCA testing by IIF and antigen-specific ELISA in SLE patients are summarised in Table 1.

**Prevalence of ANCA by IIF**

Perinuclear/nuclear staining patterns on ethanol-fixed granulocytes occurred in 41 of 55 patient sera, and one