Anti-neutrophil cytoplasm antibodies (ANCA) in rheumatoid arthritis: relationship to HLA-DR phenotypes, rheumatoid factor, anti-nuclear antibodies and disease severity

Abstract To investigate a possible relationship between the presence of anti-neutrophil cytoplasm antibodies (ANCA), rheumatoid factors (RF), anti-nuclear antibodies (ANA), disease severity and HLA-DR phenotypes, 46 consecutive ANCA+ and 48 ANCA-, clinically well-documented RA patients were studied for RF, ANA and HLA-DR phenotypes. The 46 ANCA+ patients showed predominantly an atypical perinuclear staining pattern (89%). ANCA positivity was associated with higher RF titres (P < 0.005) and advanced functional Steinbrocker grades III/IV (P < 0.015). ANCA+ patients were also more often positive for ANA than ANCA- patients (P < 0.008). There was no correlation between ANCA positivity and certain HLA-DR phenotypes although the frequency of DR4+ (67% vs 52%) and, in particular, of DR4+DR1- RA patients were twice as frequent in the ANCA+ than in the ANCA- group (22.9% vs 8.7%). Correspondingly, the DR4+DR1- phenotype was increased among ANCA+ RA patients. Regarding functional Steinbrocker grades, the DR4+ phenotypes were slightly but not significantly increased in grades III and IV whereas ANCA positivity was significantly associated with severe functional Steinbrocker grades III/IV (66% ANCA+ vs 39% ANCA-, P < 0.015). ANCA positivity identified a population of RA patients with a long-standing and severe clinical course of the disease. There was no correlation between ANCA positivity and certain HLA-DR phenotypes.

Key words Anti-neutrophil cytoplasmic antibodies (ANCA) · Rheumatoid arthritis · HLA-DR antigens

Introduction

Anti-neutrophil cytoplasm antibodies (ANCA) are predominantly directed against lysosomal enzymes of human neutrophils and monocytes. On ethanol-fixed granulocytes two major immunofluorescent staining patterns can be distinguished: the diffuse, granular cytoplasmic staining (cANCA) with specificity in ELISAs for the neutral proteinase 3 (PR3) [1] is highly specific for Wegener’s granulomatosis (WG) [2-4], while the perinuclear staining pattern (pANCA) with specificity for myeloperoxidase (MPO) is more common in microscopic polyarteritis (mPAN) and rapidly progressive segmental necrotizing and crescentic glomerulonephritis (RPGN) [5-7].

More recently, ANCAs have also been described in a variety of diseases other than primary systemic vasculitis [8-14]. Thus, in ulcerative colitis (UC) a fine granular fluorescent pattern with perinuclear accentuation (“snow-drift pattern”) is often observed [9, 10, 15] and correlates probably to the previously described granulocyte-specific ANAs [16, 17]. Similarly, in RA, fine granular, atypical pANCAAs have been reported in 17-40% of the patients and are likely to fit into the same category of granulocyte-specific ANAs [18-21]. The ANCA target antigens are not clearly defined for either UC or RA. In ELISAs, the patient sera often react with multiple lysosomal enzymes such as cathepsin G, lactoferrin, lysozyme, elastase, β-glucuronidase and others [21-29].

RA is genetically associated with specific alleles of the human major histocompatibility complex (MHC), in particular with HLA-DR4 and to a lesser extent with DR1 and DRw6 [30-41]. Moreover, DR4+ RA has been associated with a more severe course of the disease [42-49]. The purpose of this study was to examine whether ANCA+ RA patients differ from ANCA- patients with regard to rheumatoid factor (RF), anti-nuclear anti-
bodies (ANA), clinical course of RA and distribution of HLA-DR antigens. In view of recent reports of an increased mortality in certain subtypes of RA [49], ANCA status may gain a certain importance.

**Patients and methods**

**Patients**

Forty-six consecutive ANCA-positive (ANCA +) and 48 ANCA-negative (ANCA -) Caucasian RA patients fulfilling four or more of the 1987 revised American Rheumatism Association (ARA) criteria [50] were examined for serological markers (RF, ANA), HLA-DR phenotypes and functional Steinbrocker grades [51]. Patients were electively admitted to the Department of Rheumatology at the University of Freiburg Medical Centre for diagnosis and treatment. They originated from a geographically limited and homogeneous area, the upper Rhine valley. Altogether, the patients groups comprised 69 females and 25 males with a mean age of 58.5 years (range 26–90) and a mean duration of disease of 10.5 years (range 1–42).

**Methods**

**ANCA testing by immunofluorescence and ELISA**

ANCA were detected by the standard method of the First International Workshop on ANCA [52]. Briefly, human neutrophil granulocytes from healthy donors (blood group 0 Rh) were separated by Percoll gradient centrifugation and washed twice in medium RPMI 1640. By means of a Shandon Elliot cytocentrifuge, \(2 \times 10^6\) cells were centrifuged onto precleaned slides. The cells were then fixed in fresh 96% ethanol for 5 min at \(-20^\circ\)C and incubated with 1:10 diluted patients' sera for 30 min at room temperature. Following extensive washing in phosphate-buffered saline (PBS), pH 7.2, the cells were stained with fluorescein-conjugated rabbit-anti-human IgG antibodies (Dakopatts Lab.), washed again and viewed under a fluorescent microscope (Zeiss Axioskop) using oil immersion and a 100 x 100 magnification. Two fluorescent staining patterns were distinguished. Most sera produced a fine granular, atypical pANCA, while a few sera yielded a cANCA pattern similar to that seen in Wegener's granulomatosis (Fig. 1a, b). Microphotographs were taken by means of the Zeiss MC 100 microscope camera. All sera were tested as anonymous coded samples and evaluated independently by two investigators. Sera were considered ANCA - if their titre exceeded 1:40. The positive titres ranged between 1:40 and 1:400.

To exclude unspecific binding of RF to ethanol-fixed granulocytes, two RF + sera were extensively absorbed with RF-latex beads (Behring Werke AG) until the RF titres became negative. Subsequent staining for ANCA still gave positive results (Fig. 1c, d) suggesting that RF is not the cause of ANCA positivity in RA patients' sera.

All ANCA + sera were tested for anti-MPO reactivity in a MPO-specific ELISA. The five cANCA-positive sera were also tested for anti-PR3 reactivity in a PR3-specific ELISA (ELISA Diagnostic, Freiburg, Germany).

**Indirect immunofluorescence on HEp-2 cells**

Commercially available slides with fixed HEp-2 cells (Kallestad Lab.) were used to screen the patients' sera (starting dilution: 1:50) for ANAs of the IgG class. The staining and washing procedure was the same as described for the ANCA test.

**Rheumatoid factor (RF)**

The presence of RF was ascertained by the Waaler-Rose test (Behring Werke AG). RF + individuals were defined as having Waaler-Rose titres of equal or greater than 1:16.

**HLA typing**

HLA typing of DR antigens was performed using a standard complement-dependent microcytotoxicity assay [53].

Fig. 1a–d  Fine granular, atypical pANCA fluorescence, seen with most sera from rheumatoid arthritis (RA) patients (a). For comparison: typical diffuse cytoplasmic staining pattern (cANCA) seen in Wegener's granulomatosis (b). The cANCA fluorescence seen with few RA sera was much weaker. Fluorescence pattern of a strongly pANCA + serum (titre >1:400) before absorption of rheumatoid factor (c) and after absorption of rheumatoid factor (d). All sera were tested at 1:40 dilution and viewed under a Zeiss Axioskop fluorescent microscope using oil immersion and a 10 x 100 magnification.