Anti-neutrophil cytoplasm autoantibodies (ANCA). Clinical and functional studies

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CHAPTER V

Pro-Myeloperoxidase: A Target Antigen for Anti-Neutrophil Cytoplasm Autoantibodies (ANCA)

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Pro-Myeloperoxidase: A Target Antigen for Anti-Neutrophil Cytoplasm Autoantibodies (ANCA)

To the Editor:

We read with interest the article by Russel *et al.* regarding the relationship between anti-neutrophil cytoplasm autoantibodies (ANCA) reacting with the pro-form of proteinase-3 (PR3) and disease activity in patients with Wegener's granulomatosis and microscopic polyangiitis [1]. The authors reported that PR3-ANCA subsets reactive with epitopes accessible on recombinant pro-PR3 are better correlated with disease activity than are subsets reactive with epitopes accessible only on recombinant mature PR3. Proteinase-3 and myeloperoxidase (MPO) are the two main target antigens of ANCA in systemic vasculitides [2-4]. Both proteins are located in the azurophil granules of neutrophils.

The aim of the current study was to assess the antigen specificity of MPO-ANCA by testing MPO-ANCA-positive sera in the standardized ANCA indirect immunofluorescence (IIF) assay on MPO-deficient neutrophils from three healthy, unrelated donors (CV, DC and JB). These neutrophils lacked the heavy and light subunits of the MPO molecule but contained the pro-MPO precursor protein, as previously described [5]. We now report that some MPO-ANCA-positive sera react with pro-MPO.

MPO deficiency was defined spectrally, enzymatically, and immunochemically. The donor subject DC has been previously described [6]. Azurophil granules from control neutrophils and MPO-deficient neutrophils (CV) were solubilized 1:1 with 4% reduced *Laemmli* sample buffer and electrophoresed into a 10% (w/v) acrylamide gel. Thereafter, separated proteins were transferred to nitrocellulose membranes, and the blots were probed with a rabbit anti-human MPO (Dako, Glostrup, Denmark). The immunoblot analysis of azurophil granules from control neutrophils showed the MPO subunits 59 kDa and 13.5 kDa, as well as the 39-kDa and 24-kDa proteins related to the 59-kDa and the 13.5-kDa proteins, respectively [7]. In addition, a ~50-kDa protein was found that may be a precursor form of the 59-kDa subunit. In contrast, the azurophil granules from the MPO-deficient neutrophils (CV) lacked the mature subunits (59 kDa and 13.5 kDa) and the related proteins (39 kDa and 24 kDa), but contained the pro-MPO form (90 kDa) and the ~50-kDa precursor protein (**Figure 1**).

The mutations in the MPO-deficient donor (CV) were characterized as a splice-site mutation at the 3' end of intron 11 (A-2→G) and a deletion of 14 nucleotides from A1555-C1568 in exon 9 of the MPO gene. Subject DC is a compound heterozygote for the R569W missense mutation in exon 10 causing MPO deficiency [8]. Subject JB has a normal exon 10, but the genetic basis for the MPO deficiency has not been defined.

Determination of ANCA by the standardized IIF assay [9] on control *versus* MPO-deficient neutrophils from CV, DC and JB was performed with 25 sera of high MPO-ANCA reactivity from patients with microscopic polyangiitis [10]. The 25 MPO-ANCA sera were perinuclear ANCA-positive in the IIF assay on control neutrophils. MPO-ANCA were detected by standardized enzyme-linked immunosorbent assay using human purified MPO from Calbiochem (La Jolla, CA, USA). When the fluorescence was positive (1:20 dilution), serum titration was carried out in two-fold dilutions to 1:1,280.

IIF titers of these 25 MPO-ANCA sera on control *versus* MPO-deficient neutrophils are shown in **Figure 1**. ANCA were positive in 32%, 88%, and 60% of sera tested on neutrophils from

subjects CV, DC, and JB, respectively. These sera exhibited either a diffuse cytoplasmic or a perinuclear fluorescence pattern. IIF titers obtained with the MPO-deficient neutrophils were somewhat lower than IIF titers obtained with control neutrophils. Therefore, the sera that react with the MPO-deficient neutrophils might contain not only anti-pro-MPO but also anti-mature MPO. Three of 8, 12 of 22, and 6 of 15 ANCA-positive sera with MPO-deficient neutrophils from subjects CV, DC, and JB, respectively, displayed similar IIF titers as with control neutrophils. This suggests that these sera contain mainly reactivity with pro-MPO.

In conclusion, the polyclonality of MPO-ANCA described in systemic vasculitides [11] may be relevant to the disparity of the ANCA-IIF results on these MPO-deficient neutrophils. The pro-MPO, which resides in the endoplasmic reticulum, is hereby described for the first time as a potential target antigen for MPO-ANCA-positive sera. It is conceivable that some MPO-ANCA gain access to pro-MPO *in vivo*, as was recently suggested for PR3-ANCA toward pro-PR3, for instance at times of accelerated neutrophil apoptosis, *i.e.*, during active inflammation [1].

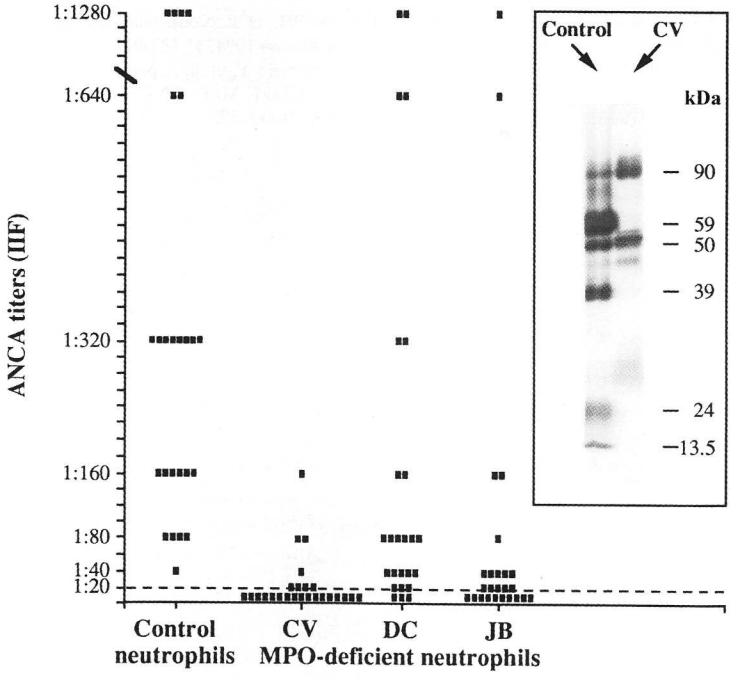

Figure 1. Titers of anti-neutrophil cytoplasm autoantibodies (ANCA) by the standardized indirect immunofluorescence (IIF) assay on control neutrophils *versus* myeloperoxidase (MPO)-deficient neutrophils from CV, DC and JB, performed with 25 MPO-ANCA-positive sera. Boxed area shows immunoblot analysis of azurophil granules from control neutrophils *versus* MPO-deficient neutrophils from subject CV.

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ADDENDUM: Assessment of Myeloperoxidase (MPO)-ANCA-Positive Sera Reacting with the Pro-MPO

ANCA patterns with myeloperoxidase-deficient neutrophils

MPO-ANCA-positive sera were obtained from three selected patients (1, 2, and 3) among the aforementioned 25 patients with microscopic polyangiitis, and were tested by the standardized IIF assay on MPO-deficient neutrophils from donor CV (**Figure 2**). The serum from patient 1 gave a negative ANCA pattern, and thus does not react with pro-MPO. In contrast, the two other sera from patients 2 and 3, gave a perinuclear ANCA (P-ANCA) pattern and an atypical ANCA patttern, respectively. The atypical pattern exhibits some weak perinuclear fluorescence and a diffuse cytoplasmic fluorescence without any granular fluorescence or staining of the central part of the cells. The sera did not react with PR3, azurocidin, bactericidal/permeability-increasing protein, cathepsin G, lactoferrin, lysozyme or elastase (not shown). Hence, the two sera from patients 1 and 2 might react with pro-MPO.

MPO-ANCA antibodies normally produce a P-ANCA pattern on ethanol-fixed neutrophils with the IIF assay [1]. Baslund *et al.* have described sera from some patients that are MPO-positive in ELISA but produce a cytoplasmic pattern, which is normally seen in conjunction with antibodies to PR3 [2]. These authors concluded that not all MPO relocates after ethanol fixation. The current study suggests that the diffuse cytoplasmic pattern observed with some MPO-ANCA-positive sera may be due to reactivity with pro-MPO, which resides in the endoplasmic reticulum.

Immunoblot analysis of myeloperoxidase-related peptides

We assessed whether MPO-ANCA-positive sera truly react with the pro-MPO protein, by means of immunoblotting (**Figure 3**). We purified immunoglobulin G (IgG) from sera 1, 2, and 3 with MPO-ANCA by passage over HiTrap protein-G sepharose (Amersham Biosciences, Pistacaway, NJ, USA). Purity of the IgG preparations as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was always greater than 95%. Azurophil granules from MPO-deficient neutrophils from donor CV were solubilized 1:1 with 4% reduced *Laemmli* sample buffer and electrophoresed onto a 10% (w/v) acrylamide gel. Thereafter, separated proteins were transferred to nitrocellulose membranes, and the blots were probed with purified IgG from the three MPO-ANCA-positive sera. The immunoblot in **Figure 3** shows no band in lane 1, but it does show a reaction in lanes 2 and 3 at 90 kDa, the molecular mass of pro-MPO. Hence, we directly prove that MPO-ANCA-positive sera from patients 2 and 3, which gave a P-ANCA and an atypical ANCA pattern by the IIF assay on MPO-deficient neutrophils, do react with the pro-MPO protein.

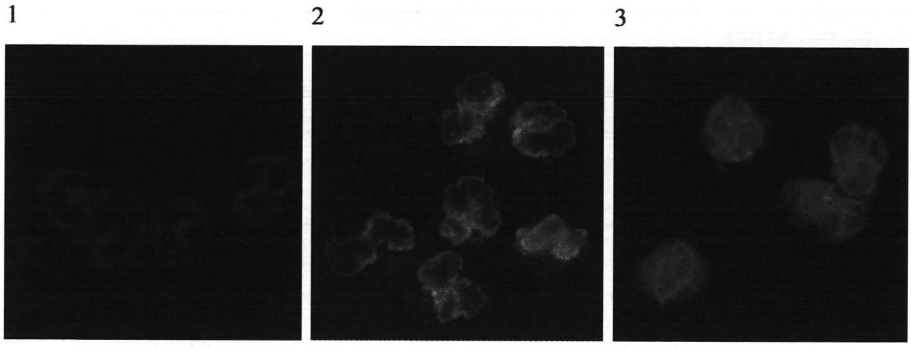

Figure 2. ANCA patterns of myeloperoxidase (MPO)-ANCA-positive sera from three different patients with microscopic polyangiitis tested with the indirect immunofluorescence assay on MPO-deficient neutrophils from donor CV.
(**1**) Negative ANCA pattern, (**2**) P-ANCA pattern, and (**3**) an atypical ANCA pattern.

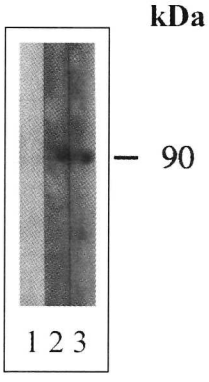

Figure 3. Immunoblot analysis of myeloperoxidase (MPO)-related peptides from MPO-deficient neutrophils (donor CV) probed with purified immunoglobulin G from the three MPO-ANCA-positive sera 1, 2, and 3.

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