

# Expression of myeloperoxidase (MPO) by neutrophils is necessary for their activation by anti-neutrophil cytoplasm autoantibodies (ANCA) against MPO

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**Abstract:** Anti-neutrophil cytoplasm autoantibodies (ANCA) directed against proteinase-3 and myeloperoxidase (MPO) activate tumor necrosis factor- $\alpha$ -primed neutrophils in vitro. We used neutrophils from one completely and one partially MPO-deficient donor to assess the requirement of MPO expression for neutrophil activation by anti-MPO antibodies. The MPO deficiencies were defined enzymatically, by immunocytochemistry and by immunoblotting. The mutations in the MPO genes of these donors were identified as a combination of a novel splice-site mutation at the 3' end of intron 11 (A-2→C), a deletion of 14 nucleotides in exon 9 (A1555–C1568), and a novel C1907 → T (636Thr→Met) substitution in exon 11 in the completely MPO-deficient donor and as the same splice-site mutation and a novel C995 → T (332Ala→Val) substitution in exon 7 in the partially MPO-deficient donor. Monoclonal antibody 4.15 against MPO and MPO-ANCA-immunoglobulin G induced no superoxide anion production in these MPO-deficient neutrophils despite a normal production induced by other stimuli. Thus, the presence of MPO is a *conditio sine qua non* for neutrophil activation by anti-MPO antibodies. Moreover, we demonstrated that by means of these MPO-deficient cells, hydrogen peroxide may diffuse from neutrophils to surrounding cells, which may contribute to the damage induced by oxygen radicals in the pathology of systemic vasculitides. *J. Leukoc. Biol.* 73: 841–849; 2003.

**Key Words:** myeloperoxidase deficiency · MPO mutations · neutrophil activation ·  $H_2O_2$  carryover

## INTRODUCTION

Anti-neutrophil cytoplasm autoantibodies (ANCA) are often present in sera from patients with inflammatory disorders, such as systemic vasculitides [1]. Proteinase-3 (PR3) and myeloperoxidase (MPO) are the two main target antigens of these antibodies [2–4], mainly located in the azurophil granules. ANCA are known to activate human neutrophils primed by tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in vitro [5]. It is assumed that

ANCA-immunoglobulin G (IgG) antibodies exert their effects by virtue of binding with their Fab regions to their target antigens expressed on the cell surface and binding with their Fc regions to Fc $\gamma$  receptors (Fc $\gamma$ R) on the same cell surface (Kurlander phenomenon) or on neighboring neutrophils [6]. Nevertheless, there is controversy regarding the involvement of the Fc $\gamma$ R: Some investigators report that at least part of the activation is non-Fc $\gamma$ R-mediated [5, 7, 8], whereas others dispute the idea that Fc $\gamma$ RIIIa is the sole receptor involved [9–11] or claim that Fc $\gamma$ RIIIb is also involved [12, 13]. Although there is general agreement on the initial binding of ANCA to their antigen on the cell surface, the requirement of the ANCA antigen for this neutrophil activation has never been experimentally verified. In the present study, we used neutrophils from two individuals with previously unreported genotypes of MPO deficiency to assess whether the presence of the MPO target antigen is a *conditio sine qua non* for the activating effect of anti-MPO antibodies in vitro. Anti-MPO antibodies did not induce superoxide anion ( $O_2^-$ ) production in these MPO-deficient neutrophils, despite a normal production induced by other stimuli. Hence, we demonstrated for the first time that MPO is absolutely required for activation of TNF-primed neutrophils with anti-MPO antibodies.

## MATERIALS AND METHODS

### Reagents and antibodies

Human fibronectin (FN), sodium azide, N-formyl-methionyl-leucyl-phenylalanine (fMLP), sodium barbital, Triton X-100, and EDTA were obtained from Sigma Chemical Co. (St. Louis, MO). Dihydro-rhodamine-1,2,3 (DHR) was purchased from Molecular Probes (Eugene, OR). Human recombinant TNF- $\alpha$  and *p*-nitrophenyl phosphate were from Roche Diagnostics (Mannheim, Germany). All other reagents were of analytical grade purity.

Monoclonal antibody (mAb) 12.8 against PR3 (mIgG<sub>1</sub>) and mAb 4.15 against MPO (mIgG<sub>1</sub>) were from the Central Laboratory of the Netherlands Blood Transfusion Service (CLB; Amsterdam). mAb MPO-7 against MPO was from Dako (Glostrup, Denmark). mAb 3G8 (CD16, mIgG<sub>1</sub>) against Fc $\gamma$ RIIIb and mAb IV.3 (CD32, mIgG<sub>2b</sub>) against Fc $\gamma$ RIIIa were from CLB. These

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antibodies were purified from hybridoma culture supernatant by precipitation with 50% saturated ammonium sulfate and subsequent protein-A affinity chromatography (Amersham BioSciences, Piscataway, NJ).

IgG from sera with PR3-ANCA or MPO-ANCA and from control sera was purified by passage over HiTrap protein-G sepharose (Amersham BioSciences). Purity of the IgG preparations as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was always greater than 95%.

## Isolation of neutrophils

Blood was obtained from control donors and from two unrelated, healthy MPO-deficient donors, CV and CP. Granulocytes were purified from blood anticoagulated with 0.4% (w/v) trisodium citrate (pH 7.4), as described [14]. In short, blood cells were separated by density gradient centrifugation over isotonic Percoll (Amersham BioSciences) with a specific gravity of 1.076 g/ml. The interphase, containing the mononuclear cells, was removed. The pellet fraction, containing erythrocytes and granulocytes, was treated for 10 min with ice-cold isotonic  $\text{NH}_4\text{Cl}$  solution (155 mM  $\text{NH}_4\text{Cl}$ , 10 mM  $\text{KHCO}_3$ , 0.1 mM EDTA, pH 7.4) to lyse the erythrocytes. The remaining granulocytes were washed twice in phosphate-buffered saline (PBS), were resuspended in incubation medium containing 132 mM NaCl, 6 mM KCl, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgSO}_4$ , 1.2 mM  $\text{KH}_2\text{PO}_4$ , 20 mM HEPES, 5.5 mM glucose, and 0.5% (w/v) human serum albumin (pH 7.4), and were kept at room temperature (RT) at a final concentration of  $2 \times 10^6$  cells/ml. Purity of neutrophils was more than 95% (the contaminating cells were mainly eosinophils), and viability was more than 98%.

## Characterization of the MPO deficiency

### Cytochemistry

Peroxidase cytochemistry was performed on cytopins made from purified neutrophils according to Kaplow, with 3-3'-diaminobenzidine as a substrate [15].

### Determination of peroxidase activity

Blood of each donor was analyzed for peroxidase activity with  $\text{H}_2\text{O}_2$  and 4-chloro-1-naphthol (Bayer Diagnostics, Tarrytown, NJ) in an ADVIA120 hematological analyzer. Moreover, peroxidase activity of solubilized neutrophils was quantitated by spectrophotometric determination of *o*-dianisidine and 4-amino-antipyrine oxidation, as described [16, 17].

### Immunocytochemistry

After centrifugation onto glass slides, neutrophils were fixed with 0.125% (w/v) glutaraldehyde in PBS and permeabilized with a methanol-acetone solution. Endogenous peroxidases were inactivated by incubation for 1 h at 37°C in PBS solution containing 10 mM glucose, 2 mM sodium azide, 1 U/ml glucose oxidase, and 5 mM resorcinol. Cytopins were then incubated for 1 h at RT with anti-MPO 4.15 or anti-MPO-7 mAbs, diluted in PBS. Immunoperoxidase vectastain kit (Vectastain ABC Elite system, Vector Laboratories, Burlingame, CA) was used to detect antibody binding. This technique uses unlabeled, primary antibody followed by biotinylated secondary antibody, biotinylated horse anti-mouse IgG, and then a preformed avidine-biotinylated horseradish peroxidase macromolecular complex. Thereafter, 3-3'-diaminobenzidine (Vector Laboratories) was used as a substrate.

### Immunoblot analysis of MPO-related peptides

Isolated neutrophils from one control donor and from the two MPO-deficient donors CV and CP were fractionated by sonication and sucrose-gradient centrifugation, as described [18]. Then, azurophil granules (25  $\mu\text{g}/\text{lane}$ ) were solubilized 1:1 with 4% reduced Laemmli sample buffer and electrophoresed onto a 10% (w/v) acrylamide gel under reducing conditions. Thereafter, separated proteins were electrotransferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) in transfer buffer (25 mM Tris, 0.192 M glycine, 20% methanol, pH 8.3) by means of a semidry blotting apparatus at a constant current of 1.0 mA/cm<sup>2</sup> for 1 h. Unoccupied sites on the membrane were blocked with 5% (w/v) skimmed milk powder in Tris-buffered saline Tween (TBST; 10 mM Tris-HCl, 150 mM NaCl, 0.5% Tween-20, pH 8.0) for 1 h at RT, and the blots were probed with a rabbit anti-human MPO (purified Ig fraction of rabbit antiserum; Dako), diluted at 1:500 in blocking buffer and TBST (v/v) for 2 h at RT. Afterwards, the blots

were washed extensively and incubated with an anti-rabbit IgG alkaline-phosphatase conjugate (Promega, Madison, WI), diluted at 1:7500. One hour later, the blots were thoroughly washed again several times, and bound antibodies were detected with a staining including 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium (Sigma Chemical Co.). Molecular weights were calculated by comparison with recombinant protein molecular weight markers, RPN 800 (Amersham BioSciences).

### Molecular analysis

Genomic DNA and RNA were isolated from circulating leukocytes [19]. Molecular analysis of MPO was performed by polymerase chain reaction (PCR) amplification of MPO exons from genomic DNA with primers annealing to intronic sequences. The PCR products were purified and sequenced by BigDye terminator cycle sequencing, analyzed on an ABI 377XL DNA sequencer (Perkin-Elmer Applied Biosystems, Warrington, UK). Preparation, PCR amplification, and sequencing of cDNA were performed as described [19], and primers are given in **Table 1**. Numbering of nucleotides within MPO cDNA starts with the A of the ATG translation start codon.

## Measurement of $\text{O}_2^-$ production by neutrophils

The production of  $\text{O}_2^-$  by neutrophils was measured with the ferricytochrome-*c* reduction assay [20]. In short, neutrophils ( $2 \times 10^6/\text{ml}$ ) were incubated in polystyrene tubes under gentle agitation for 5 min at 37°C in a shaking water bath before addition of ferricytochrome-*c* (60  $\mu\text{M}$ ) and sodium azide (2 mM). After another 5 min, TNF- $\alpha$  (2 ng/ml) was added to part of the cells, and the cell suspensions were divided in equal portions. After 10 min of priming with TNF- $\alpha$ , fMLP (1  $\mu\text{M}$ ), 3G8 mAb (5  $\mu\text{g}/\text{ml}$ ), anti-PR3 mAb (12.8, 5  $\mu\text{g}/\text{ml}$ ), anti-MPO mAb (4.15, 5  $\mu\text{g}/\text{ml}$ ), or purified IgG from sera with MPO-ANCA (75  $\mu\text{g}/\text{ml}$ ) was added. Control cells received PBS only. Reactions were stopped 10 min after the addition of fMLP and 40 min after the addition of the other stimuli. Then, the samples were microcentrifuged rapidly to remove the cells before analysis of the supernatants at 550 nm in a double-beam spectrophotometer (Model 24, Beckman Coulter, Fullerton, CA).

## Measurement of rhodamine-1,2,3 fluorescence in neutrophils

The assay to measure reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity in DHR-loaded neutrophils was performed essentially as described [21, 22]. Neutrophils ( $2 \times 10^6/\text{ml}$ ) were incubated in polystyrene tubes in a shaking water bath under gentle agitation for 5 min at 37°C. DHR (0.5  $\mu\text{M}$ ) and sodium azide (2 mM) were then added, and after another 10 min, TNF- $\alpha$  (2 ng/ml) was distributed to part of the cells. After 10 min of priming at 37°C, the cell suspensions were divided in equal portions (100  $\mu\text{l}$ ) and added to tubes containing fMLP (1  $\mu\text{M}$ ), 3G8 mAb (5  $\mu\text{g}/\text{ml}$ ), anti-PR3 mAb (12.8, 5  $\mu\text{g}/\text{ml}$ ), or anti-MPO mAb (4.15, 5  $\mu\text{g}/\text{ml}$ ). Control cells received PBS only. Unless indicated otherwise, the reactions were stopped after 30 min by addition of a 30-fold excess of ice-cold PBS containing 1% (w/v) bovine serum albumin (BSA). Thereafter, the tubes were centrifuged (400 *g*) for 5 min at 4°C, and the cells were resuspended in  $\sim 100 \mu\text{l}$  ice-cold PBS/BSA 1% and kept on ice in the dark until analysis in a flow cytometer (Epics profile, Coulter Corp., Miami, FL). Neutrophils were distinguished by forward-sideward scatter pattern, and data were collected from 5000 cells. The results are expressed as mean fluorescence intensity (MFI).

## Neutrophil adherence assay

Neutrophil adherence to FN was measured in flat-bottomed, 24-well (15.5 mm diameter) polystyrene plates (Nuncclon delta, Nunc, Roskilde, Denmark). The wells were pretreated for 1 h at 37°C with FN (10  $\mu\text{g}/\text{ml}$ ) dissolved in PBS and were then washed once with PBS and once with incubation medium at RT. Neutrophils ( $2 \times 10^6/\text{ml}$ ) were incubated in polypropylene tubes in a shaking water bath for 5 min at 37°C. Subsequently, these neutrophils were rapidly distributed at  $10^6$  cells per well over the coated wells. After another 10 min, TNF- $\alpha$  (2 ng/ml) was added to some of the wells. After 10 min of priming at 37°C, anti-PR3 mAb (12.8, 5  $\mu\text{g}/\text{ml}$ ) or anti-MPO mAb (4.15, 5  $\mu\text{g}/\text{ml}$ ) was added to the wells. Control

TABLE 1. Primers Used for PCR Amplification of DNA

## For genomic DNA

*Exon 1*

MPO-prom/S (−362 to −337 from transcription site) 5′-AAAGGCTGGGGACAATGCTGGCCCTC-3′  
 MPO-int1/AS (Intron 1, 62 to 39) 5′-AAGGGTCTCTGGAACACAACCAC-3′

*Exon 2*

MPO-int1/S (Intron 1, −70 to −47) 5′-CCTTCCTAGCTCTGGGGCCTGATA-3′  
 MPO-int2/AS (Intron 2, 63 to 41) 5′-CTGCTGCTTCTGTGAAAGGCCTG-3′

*Exon 3*

MPO-int2/S (Intron 2, −63 to −39) 5′-GCAGGTTGGCTCTGCTATCCCTTCT-3′  
 MPO-int3/AS (Intron 3, 59 to 35) 5′-GGTCCCTAGGAGGAGTCACTAGTGG-3′

*Exon 4*

MPO-int3/S (Intron 3, −63 to −38) 5′-TAGCCTAGGTTGCCTGGGATAGGAAG-3′  
 MPO-int4/AS (Intron 4, 61 to 38) 5′-TTGTGGCGTCCGGGACGCCTCTCT-3′

*Exon 5*

MPO-int4/S (Intron 4, −59 to −36) 5′-AGAGAGGCGTCCCGGACGCCACAA-3′  
 MPO-int5/AS (Intron 5, 37 to 16) 5′-TGGCCGGCCTCGCCCCCTCTGC-3′

*Exon 6*

MPO-int5/S (Intron 5, −40 to −18) 5′-TGC CGGACCCAGGCGCCACGTG-3′  
 MPO-int6/AS (Intron 6, 66 to 43) 5′-AGATGGCCCCCTCCAGAAACAATC-3′

*Exon 7*

MPO-int6/S (Intron 6, −64 to −40) 5′-CCAGTTCTGCCTGGGCACCTTCCTT-3′  
 MPO-int7/AS (Intron 7, 64 to 38) 5′-ACTCACTGTGGCTCCAACAGGGAACAT-3′

*Exon 8*

MPO-int7/S (Intron 7, −59 to −36) 5′-GGAGCAAATCTTTTCTGGGATGGA-3′  
 MPO-int8/AS (Intron 8, 60 to 38) 5′-CAGCTAGGATGTTGCAGGGACAC-3′

*Exon 9*

MPO-int8/S (Intron 8, −66 to −43) 5′-GTGCTGGGTCCGCCGTAAACGACA-3′  
 MPO-int9/AS (Intron 9, 58 to 34) 5′-TGATCCCTACCCACCTTTAGCTGT-3′

*Exon 10*

MPO-int9/S (Intron 9, −66 to −43) 5′-TGACTCCAATCTGAGCTCTGATAC-3′  
 MPO-int10/AS (Intron 10, 63 to 40) 5′-TGGGCTACCTAGGAGGCAGCTCAG-3′

*Exon 11*

MPO-int10/S (Intron 10, −60 to −36) 5′-GACCTCCCCACCTTAAGCAGAGAGA-3′  
 MPO-int11/AS (Intron 11, 70 to 46) 5′-TTTGGGCTCCAAGAGAGTCAAGGAT-3′

*Exon 12*

MPO-int11/S (Intron 11, −65 to −41) 5′-CCCATCGATGCCCTGCCAGCCGAGA-3′  
 MPO-3′UTR/AS (227 to 204 after stopcodon) 5′-ACATACACATAACCCATGAAACAC-3′

## For cDNA

*Primer in exon 7*

MPO-ex7/S (Exon 7, 906 to 930) 5′-CATCAAGAACCAAGCCGACTGCATC-3′

*Primer in exon 9*

MPO-ex9/S (Exon 9, 1461 to 1484) 5′-CCCACGCATCGCCAACGTCTTCAC-3′

*Primer in exon 10*

MPO-ex10/AS (Exon 10, 1672 to 1648) 5′-TCAGCTTGGCAGGGGTGGCCATGAG-3′

Amplification conditions for genomic DNA in Air Thermocycler 1605 (Idaho Technology, Idaho Falls): 50 rounds of 5 s at 95°C (denaturation), 10 s at 50°C (annealing), and 30 s at 72°C (extension), at full speed. Amplification conditions for cDNA in Air Thermocycler: 50 rounds of 5 s at 95°C, 30 s at 60°C, and 15 s at 72°C, at full speed.

cells received PBS only. Thirty minutes after addition of the stimulus, the supernatant of the coated wells, including the nonadherent cells, was removed by washing the plate twice with ice-cold incubation medium. Neutrophil adherence to FN was measured by alkaline phosphatase activity of adherent cells [23]. Briefly, 500  $\mu$ l buffer containing 50 mM sodium barbital (pH 10.5), 1 mM MgCl<sub>2</sub>, 0.1% Triton X-100, and 1 mg/ml *p*-nitrophenyl phosphate was added to the wells. After an incubation period of 30 min at 37°C, the supernatant was transferred to a microplate for determination of the optical density at 410 nm (Labsystems iEMS Reader MF, Helsinki, Finland). The percentage of adherent cells was calculated from appropriate standard curves, obtained by incubating known numbers of neutrophils with phosphatase activity substrate in the same microplate.

**Statistical analysis**

Results are expressed as mean  $\pm$  SEM of (n) independent experiments. Statistical comparisons were performed with the two-sided Student's *t*-test. A value of *P* < 0.05 was considered significant.

**RESULTS****Characterization of the MPO deficiency***Cytochemistry*

Peroxidase cytochemistry showed complete and partial absence of MPO activity in neutrophils from the MPO-deficient donors CV and CP, respectively (not shown).

*Peroxidase activity*

Both MPO-deficient donors were found to have MPO deficiency during routine blood examination in the analyzer ADVIA120 (Bayer Diagnostics). Biochemical analysis of MPO activity by spectrophotometric determination of *o*-dianisidine and 4-amino-antipyrine oxidation confirmed the complete absence

TABLE 2. Peroxidase Activity of Neutrophils from Control and MPO-Deficient Donors as Determined by the Spectrophotometric Determination of *o*-Dianisidine or 4-Amino-Antipyrine Oxidation

	Peroxidase activity	
	<i>o</i> -Dianisidine assay	4-Amino-antipyrine assay
	mIU/10 <sup>6</sup> cells	U/mg cell protein
Control neutrophils	536	264
MPO-deficient neutrophils (CV)	27	6
MPO-deficient neutrophils (CP)	169	69

(<5% of normal activity) and partial absence (<30% of normal activity) of peroxidase activity in neutrophils from donors CV and CP, respectively (Table 2).

#### Immunocytochemistry

Positive neutrophils appeared with bluish granules. Results obtained with anti-MPO 4.15 mAb or with anti-MPO-7 mAb were identical. Compared with control neutrophils (Fig. 1A), the reaction was completely negative with MPO-deficient neutrophils from donor CV (Fig. 1B) and weakly positive with MPO-deficient neutrophils from donor CP (Fig. 1C).

#### Immunoblot analysis of MPO-related peptides

The control neutrophils showed the MPO subunits of 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 [24], and showed a small amount of pro-MPO (90 kDa). In addition, a protein of ~50 kDa was found that might represent some partially but incorrectly processed product from the precursor form of the 59-kDa subunit. In contrast, the MPO-deficient neutrophils from donor 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 (90 kDa) and the ~50-kDa protein. The MPO-deficient neutrophils from donor CP showed less markedly the mature heavy and light subunits of MPO than did the control neutrophils, and weakly showed the pro-MPO of 90 kDa. The results are shown in Figure 2.

#### Molecular analysis

The mutations in the MPO genes of the completely MPO-deficient donor CV were identified as a combination of a splice-site mutation at the 3' end of intron 11 (A-2→C), a deletion of 14 nucleotides in exon 9 (A1555-C1568), and a C1907 → T (636Thr→Met) substitution in exon 11. In the partially MPO-deficient donor CP, we found the same splice-

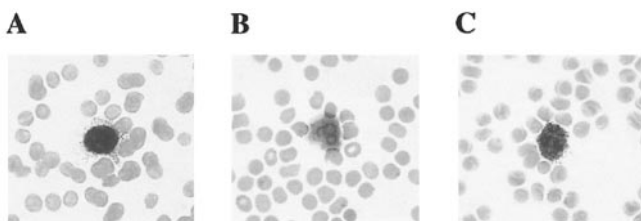


Fig. 1. Immunocytochemical reaction for MPO in neutrophils from (A) a control donor and from the MPO-deficient donors (B) CV and (C) CP.

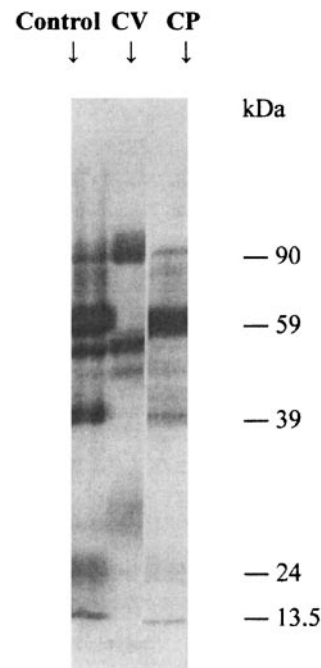
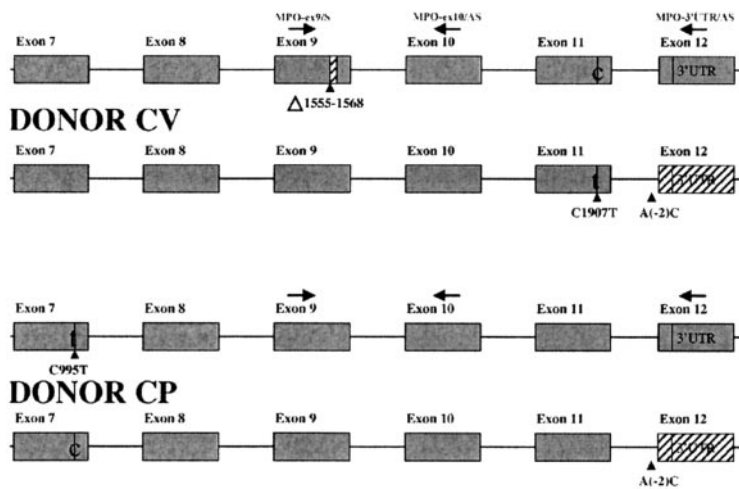


Fig. 2. Immunoblot analysis of MPO-related peptides from one control donor and from the MPO-deficient donors CV and CP.

site mutation and a C995 → T (332Ala→Val) substitution in exon 7 (Fig. 3). No other alterations in base sequences were detected. By testing DNA from 47 healthy donors (94 alleles), the two missense mutations were excluded as common polymorphisms; in none of these did we find the C1907T mutation, and in one of these, we found the C995T mutation (on one allele). The splice-site mutation was checked for its effect on the mRNA constitution by PCR amplification of cDNA from donor CV with a sense primer in exon 9 and an antisense primer in exon 10 or in exon 12 (Table 1; Fig. 3). With the antisense primer in exon 10, a PCR product was generated that contained the 14-bp deletion in heterozygous form, similar to the product generated from genomic DNA with primers in the flanking introns of exon 9. However, when cDNA was PCR-amplified with a sense primer in exon 9 and an antisense primer in exon 12, the product contained the 14-bp deletion in apparently homozygous form, indicating that the product was derived from only one allele. Thus, the splice-site mutation caused skipping of exon 12 from the mRNA, and this is the reason that the antisense primer in exon 12 can bind only to the cDNA from the allele that carries the 14-bp deletion and not to the other allele. This same allele with the splice-site mutation in donor CV was also found to carry the C1907T mutation, in accordance with the finding that donor CV's mother was a carrier of the 14-bp deletion only. The father of this donor was not available for this study. The mother of donor CP was found to be a carrier of the splice-site mutation, as was one control donor. The father of donor CP is deceased.

#### O<sub>2</sub><sup>-</sup> production by neutrophils

Activation of the neutrophils from the MPO-deficient donors with fMLP, mAb 3G8 anti-FcγRIIIb (known to activate neutrophils through binding with their Fc region to FcγRIIa; ref.



**Fig. 3.** Schematic representation of the mutations found in the MPO gene of the two MPO-deficient donors. In donor CV (completely MPO-deficient), a 14-bp deletion in exon 9 was found on one allele, as well as a C1907T substitution in exon 11 and an A(-)C splice-site mutation at the 3' end of intron 11 on the other allele. In donor CP (partially MPO-deficient), a C995T substitution in exon 7 was found on one allele, as well as an A(-)C splice-site mutation at the 3' end of intron 11 on the other allele. The position of the primers used for cDNA analysis (Table 1) is also shown. 3' UTR, 3'-Untranslated region.

[25]), or mAb 12.8 anti-PR3 induced a normal  $O_2^-$  production. In contrast, mAb 4.15 anti-MPO induced no reaction in the neutrophils from the two MPO-deficient donors (Table 3). In one experiment performed with IgG purified from an MPO-ANCA-positive serum, we found a similar failure to induce  $O_2^-$  release from the completely MPO-deficient neutrophils (0.9 nmoles/ $10^6$  cells vs. 4.2 nmoles/ $10^6$  control cells) and also from the partially MPO-deficient neutrophils (0.8 nmoles/ $10^6$  cells). Activation was not detected in TNF-treated neutrophils incubated with an irrelevant antibody (CD2, mIgG<sub>1</sub>; not shown). Both MPO-deficient neutrophils primed by TNF- $\alpha$  expressed PR3 on their surface membrane, similar to control neutrophils (not shown), as determined by indirect immunofluorescence with flow cytometry.

### Rhodamine-1,2,3 fluorescence in neutrophils

The DHR assay, as an MPO-mediated reaction [22], did not yield a positive result with any stimulus in completely MPO-

deficient neutrophils primed by TNF- $\alpha$ . In contrast, the rhodamine-1,2,3 fluorescence was normal or slightly depressed in partially MPO-deficient neutrophils primed by TNF- $\alpha$  and exposed to fMLP, mAb 3G8, mAb 12.8 anti-PR3, or mAb 4.15 anti-MPO (Table 4).

### H<sub>2</sub>O<sub>2</sub> diffusion from completely MPO-deficient neutrophils to control neutrophils

Although mAb 12.8 anti-PR3 induced a normal, respiratory burst in the completely MPO-deficient neutrophils, this agent did not yield a positive result in the DHR assay with these neutrophils (Tables 3 and 4, respectively, and Fig. 4A). Rhodamine-1,2,3 formation was only detected in control neutrophils exposed to mAb 12.8 but was not observed in neutrophils pretreated with the anti-Fc $\gamma$ RIIa antibody (mAb IV.3), as previously reported [10] (Fig. 4B). H<sub>2</sub>O<sub>2</sub> diffusion from the completely MPO-deficient neutrophils to control neutrophils

TABLE 3.  $O_2^-$  Production in Control and MPO-Deficient Neutrophils Primed by TNF- $\alpha$  and Exposed to fMLP, 3G8 mAb, Anti-PR3, or Anti-MPO mAbs

	$O_2^-$ production (nmoles/ $10^6$ cells)		
	Control neutrophils	Completely MPO-deficient neutrophils	Partially MPO-deficient neutrophils
(+) fMLP	7.7 $\pm$ 1.3	9.1 $\pm$ 2.1 <sup>a</sup>	3.9 $\pm$ 1.1 <sup>a</sup>
(+) 3G8 mAb	1.5 $\pm$ 0.3	1.4 $\pm$ 0.1 <sup>a</sup>	0.9 $\pm$ 0.3 <sup>a</sup>
(+) Anti-PR3 mAb	1.5 $\pm$ 0.6	1.2 $\pm$ 0.5 <sup>a</sup>	1.4 $\pm$ 0.4 <sup>a</sup>
(+) Anti-MPO mAb	1.3 $\pm$ 0.5	0.2 $\pm$ 0.2 <sup>b</sup>	0.2 $\pm$ 0.1 <sup>b</sup>

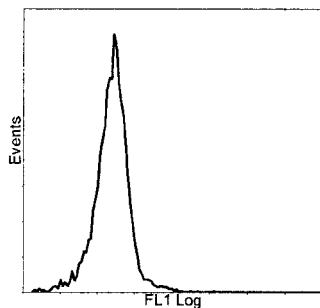
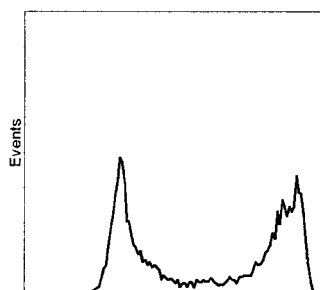
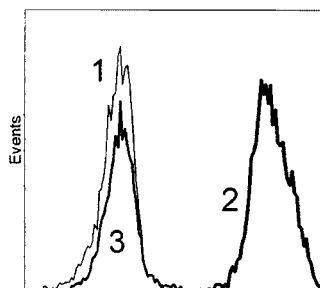
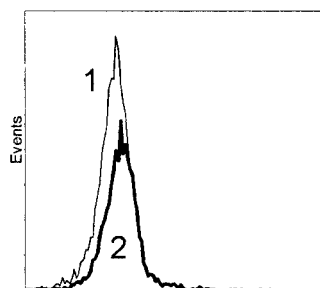
Statistical differences between cells from control donors and MPO-deficient donors: <sup>a</sup> N.S.; <sup>b</sup>  $P < 0.05$ . Neutrophils ( $2 \times 10^6$ /ml) from control donors and from the completely and partially MPO-deficient donors (CV and CP, respectively) were incubated at 37°C with ferricytochrome-*c* in polystyrene tubes under gentle agitation, as described in Materials and Methods, in the presence of TNF- $\alpha$  (2 ng/ml). After 10 min of priming, the cells were stimulated for 10 min with fMLP (1  $\mu$ M) and for 40 min with 3G8 mAb (5  $\mu$ g/ml), anti-PR3 mAb (12.8, 5  $\mu$ g/ml), or anti-MPO mAb (4.15, 5  $\mu$ g/ml). Control cells received PBS only. The value of unstimulated cells was subtracted from each value of stimulated cells for each individual experiment. Rates of  $O_2^-$  production (nmoles/ $10^6$  cells; mean  $\pm$  SEM of four to six experiments) were calculated over the 40-min time interval after the stimulus addition.

TABLE 4. Measurement of Rhodamine-1,2,3 Fluorescence in Control and MPO-Deficient Neutrophils Primed by TNF- $\alpha$  and Exposed to fMLP, 3G8 mAb, anti-PR3, or anti-MPO mAbs

	Rhodamine-1,2,3 fluorescence (MFI)		
	Control neutrophils	Completely MPO-deficient neutrophils	Partially MPO-deficient neutrophils
(+) fMLP	635 $\pm$ 157	0 $\pm$ 0 <sup>a</sup>	288 $\pm$ 80 <sup>b</sup>
(+) 3G8 mAb	698 $\pm$ 82	2 $\pm$ 2 <sup>c</sup>	415 $\pm$ 98 <sup>c</sup>
(+) Anti-PR3 mAb	569 $\pm$ 112	6 $\pm$ 4 <sup>c</sup>	497 $\pm$ 139 <sup>d</sup>
(+) Anti-MPO mAb	833 $\pm$ 12	5 $\pm$ 2 <sup>a</sup>	488 $\pm$ 182 <sup>d</sup>

Statistical differences between cells from control donors and MPO-deficient donors: <sup>a</sup>  $P < 0.001$ ; <sup>b</sup>  $P < 0.05$ ; <sup>c</sup>  $P < 0.01$ ; <sup>d</sup> N.S. Neutrophils ( $2 \times 10^6$ /ml) from control donors and from the completely and partially MPO-deficient donors (CV and CP, respectively) were incubated at 37°C with DHR-1,2,3 in polystyrene tubes under gentle agitation, as described in Materials and Methods, in the presence of TNF- $\alpha$  (2 ng/ml). After 10 min of priming, the cells were stimulated for 15 min with fMLP (1  $\mu$ M) and for 30 min with 3G8 mAb (5  $\mu$ g/ml), and anti-PR3 mAb (12.8, 5  $\mu$ g/ml), or anti-MPO mAb (4.15, 5  $\mu$ g/ml). Control cells received PBS only. The value of unstimulated cells was subtracted from each value of stimulated cells for each individual experiment. Samples were processed for flow cytometry to measure rhodamine-1,2,3 fluorescence. Results (MFI) are the mean  $\pm$  SEM of three to four experiments.

**Fig. 4.** Measurement of respiratory burst in DHR-1,2,3-loaded neutrophils by flow cytometry. H<sub>2</sub>O<sub>2</sub> carryover from completely MPO-deficient to control neutrophils. (A) Completely MPO-deficient neutrophils (donor CV; 2×10<sup>6</sup>/ml) were incubated at 37°C with DHR in polystyrene tubes under gentle agitation, as described in Materials and Methods, in the presence of TNF-α (2 ng/ml). After 10 min of priming, (1.) part of these cells was left unstimulated, and (2.) the other part was stimulated with anti-PR3 mAb (5 μg/ml) for 30 min. Unstimulated cells received PBS only. Samples were processed for flow cytometry to measure rhodamine-1,2,3 fluorescence. (B) Control neutrophils (2×10<sup>6</sup>/ml) incubated at 37°C with DHR in polystyrene tubes under gentle agitation, were (1. and 2.) untreated or (3.) treated with mAb IV.3 (anti-FcγRIIa; 10 μg/ml) before addition of TNF-α (2 ng/ml). After 10 min of priming, (1.) part of the cells was left unstimulated, and (2. and 3.) the other part was stimulated with anti-PR3 mAb (5 μg/ml) for 30 min. Unstimulated cells received PBS only. Samples were processed for flow cytometry to measure rhodamine-1,2,3 fluorescence. (C) Demonstration of a H<sub>2</sub>O<sub>2</sub> carryover from completely MPO-deficient neutrophils to control neutrophils. Completely MPO-deficient neutrophils (DHR-loaded, TNF-treated, and exposed to anti-PR3 mAb for 30 min) were mixed 1:1 with control neutrophils (DHR-loaded, TNF-treated, and blocked with the anti-FcγRIIa antibody) and incubated at 37°C in polystyrene tubes under gentle agitation for another 30 min. Samples were processed for flow cytometry to measure rhodamine-1,2,3 fluorescence. (D) Addition of catalase (13 U/ml) to the aforementioned (C) mixed neutrophil suspensions. Samples were processed for flow cytometry to measure rhodamine-1,2,3 fluorescence. The results are representative of three independent experiments.



### A. Completely MPO-deficient neutrophils

DHR loaded and TNF-treated

1. unstimulated
2. + anti-PR3 mAb

### B. Control neutrophils

DHR loaded and TNF-treated

1. unstimulated
2. + anti-PR3 mAb
3. + anti-FcγRIIa/+ anti-PR3 mAbs

### C. H<sub>2</sub>O<sub>2</sub> carry-over

#### Completely MPO-deficient neutrophils

DHR loaded and TNF-treated,

+ anti-PR3 mAb

*mixed 1:1 with control neutrophils*

DHR loaded and TNF-treated, + anti-FcγRIIa mAb

### D. Effect of catalase on the H<sub>2</sub>O<sub>2</sub> carryover

#### Completely MPO-deficient neutrophils

DHR loaded and TNF-treated,

+ anti-PR3 mAb, + catalase

*mixed 1:1 with control neutrophils*

DHR loaded and TNF-treated, + anti-FcγRIIa mAb

was followed by the MPO-mediated conversion of DHR into rhodamine-1,2,3 in the control neutrophils (Fig. 4C). Addition of catalase to the mixed neutrophil suspensions inhibited this H<sub>2</sub>O<sub>2</sub> carryover (Fig. 4D).

### Neutrophil adherence assay

To evaluate whether NADPH-oxidase activity was the only neutrophil function that needs MPO expression for its activation by mAb or ANCA against MPO, we also measured neutrophil adhesion. The TNF-induced adherence of neutrophils to FN observed in neutrophils from control donors was enhanced by addition of anti-PR3 or anti-MPO mAbs. In contrast, no additional adhesion was induced by mAb 4.15 anti-MPO in the neutrophils from the completely MPO-deficient donor CV (Table 5). Thus, MPO expression is an absolute requirement for neutrophil activation in general by antibodies against MPO.

### DISCUSSION

In this study, we used neutrophils from two donors with MPO deficiency to investigate the requirement of this antigen for activation of neutrophils by anti-MPO mAb or ANCA IgG anti-MPO. In the first part of our study, we defined the genetic basis of the MPO deficiencies in these donors. Mature MPO is a heterotetramer composed of two identical heavy chains and two identical light chains [26]. The mRNA is translated into a single protein of ~90 kDa, which displays enzymatic activity [27] and undergoes proteolytic maturation into a heavy chain of ~59 kDa and a light chain of ~13.5 kDa; these subunits then dimerize into the mature tetramer [28–30]. Hereditary MPO deficiency, the most common biochemical defect in neutrophils, is defined as a lack of MPO activity and of detectable, mature MPO protein, but the neutrophils may contain small

TABLE 5. Adherence to FN-Coated Wells of TNF-Untreated Versus Treated Neutrophils from Control Donors and from the Completely MPO-Deficient Donors Exposed to Anti-PR3 or Anti-MPO mAbs

	Adherence to FN (%)	
	Control neutrophils	Completely MPO-deficient neutrophils
TNF-untreated	3 ± 1	6 ± 1
TNF-treated	30 ± 3	28 ± 2
(+) Anti-PR3 mAb	43 ± 3*	36 ± 5*
(+) Anti-MPO mAb	43 ± 8*	29 ± 4

\* Significantly different from TNF-treated unstimulated cells ( $P < 0.05$ ). Neutrophils ( $1 \times 10^6$ /well) from control donors and from the completely MPO-deficient donor (CV) were incubated at 37°C in polystyrene wells coated with FN (10 µg/ml) for 5 min prior to addition of TNF-α (2 ng/ml) to part of the cells. After 10 min of priming, the cells were stimulated with anti-PR3 mAb (12.8, 5 µg/ml) or anti-MPO mAb (4.15, 5 µg/ml). Part of the cells was left untreated, as indicated. Control cells received PBS only. After 30 min of stimulation, neutrophil adherence to FN (%) was measured as described in Materials and Methods. Results are the mean ± SEM of three to six experiments.

amounts of the 90-kDa MPO precursor protein. This last observation together with normal amounts of MPO mRNA in neutrophil precursors indicate that a modified pro-MPO is synthesized with a defect in post-translational processing [31, 32]. However, a pretranslational defect in MPO synthesis with a total lack of MPO mRNA has also been reported [33]. Most people with MPO deficiency are healthy, although an increased susceptibility to infections with *Staphylococcus aureus* and *Candida albicans* has been reported, especially in patients also affected with diabetes [31].

In the literature, only four mutations in the MPO gene of MPO-deficient donors have been described [34–37]. The mutation initially found in several North American MPO-deficient subjects is a missense mutation at position 1705 (renumbered) in exon 10, changing the CCG codon for Arg569 into the TGG codon for Trp [34, 35]. DeLeo et al. [36] have characterized a genotypically distinct form of MPO deficiency in another American donor as a G → A substitution at position 518 (renumbered) in exon 4, whereby the TAC codon for Tyr173 is replaced by the TGC codon for Cys. The impact of these two missense mutations on the biosynthesis and maturation of MPO has been investigated [36, 38]. Additionally, two other mutations have been described in an Italian family, namely a 752T→C transition (renumbered) in exon 6, changing the ATG codon for Met251 into the ACG codon for Thr, and a deletion of 14 bases (C1552–A1565, renumbered) within exon 9, resulting in a frameshift and predicting a premature termination of protein synthesis at codon 539 [37].

We now report several additional mutations in two French donors. The 14-bp deletion found in the completely MPO-deficient donor CV (A1555–C1568) is the same as that described in the Italian family, although the nucleotide numbering slightly differs. This difference is a result of a CCC sequence in front and at the end of a deleted 11-bp stretch and can thus be included at the beginning or at the end of the deleted sequence. We prefer to start counting at the first

nucleotide that affects the coding sequence. This deletion may be expected to completely abrogate MPO synthesis from this mutant gene. The other three mutations reported by us are novel. Surprisingly, the completely MPO-deficient donor CV carried two additional, distinct mutations on the other allele of her MPO gene. One of these, the splice-site mutation at the 3' end of intron 11 (A-2→C), was also detected in the partially MPO-deficient donor CP. Splice-site mutations are known to often allow some formation of correctly spliced mRNA and hence do not always completely silence correct protein synthesis. However, the completely MPO-deficient donor CV also carried a C1907T mutation on this allele, predicting substitution of Thr636 by Met. The replacement of the relatively small threonine by the much larger methionine most probably affects the local 3-D structure of the protein (the MPO heavy chain), which is indicated by analysis of this replacement (WebLab Viewerlite 4.0 program, Accelrys, San Diego, CA) in the structure of MPO, as determined by Fiedler et al. [39]. The partially MPO-deficient donor CP carried only the splice-site mutation on this allele but had an additional C995T mutation on the other allele, predicting replacement of Ala332 by Val, also in the MPO heavy chain. This amino acid replacement probably has less dramatic effects on the protein stability and/or function, compatible with substantially higher MPO protein level and enzymatic activity in this donor (Fig. 2; Table 2). However, the exact effects of the mutations on MPO stability and activity require further studies. The presence of pro-MPO detected in donor CV is consistent with the hypothesis of synthesis of a modified pro-MPO that undergoes defective post-translational processing, resulting in a failure to package the enzyme correctly into the azurophil granules. This too needs further studies for complete characterization.

In the second part of this study, we investigated whether any neutrophil activation can be achieved with ANCA in the absence of the ANCA antigen. It is not obvious when, where, or under which conditions ANCA interact with their target antigens in vivo, as the antigens are mostly compartmentalized intracellularly. The generally accepted idea related to neutrophil activation by ANCA involves expression or “up-regulation” of ANCA antigens on the surface of neutrophils, by priming with proinflammatory cytokines or by passive binding of these antigens to the cell surface. Also during neutrophil apoptosis, subcellular particles are formed that are rich in ANCA antigens and may serve as binding sites for ANCA. Thus, ANCA have also been described to bind to antigens exposed on apoptotic neutrophils and to activate neighboring, viable neutrophils with their Fc regions in the absence of neutrophil priming [40]. Hess et al. [41] have recently shown that soluble MPO released by activated neutrophils can bind to nonactivated neutrophils and thus render these cells reactive to anti-MPO antibodies, even in the absence of priming agents. This mechanism of spreading-neutrophil activation was specifically observed with MPO–ANCA but not with PR3–ANCA. However, in our opinion, the absence of cell priming will preclude cell activation, as we have indications that cell priming is necessary for adequate neutrophil activation by ANCA, at least in vitro [11].

We have used MPO-deficient neutrophils to assess the MPO requirement for activation of TNF-primed neutrophils by anti-

MPO antibodies *in vitro*. Table 3 clearly shows that O<sub>2</sub><sup>-</sup> was not produced in the completely or partially MPO-deficient neutrophils exposed to mAb 4.15 anti-MPO, despite a normal NADPH-oxidase enzyme in these cells, as evidenced by the respiratory burst induced by fMLP, mAb 3G8 anti-FcγRIIIb, and mAb 12.8 anti-PR3. Purified IgG from MPO-ANCA-positive serum also failed to activate the MPO-deficient cells. In addition, adherence to FN-coated wells of TNF-treated neutrophils from the completely MPO-deficient donor CV was also not enhanced by mAb 4.15 anti-MPO (Table 5). In conclusion, our results show the absolute requirement of an ANCA antigen on neutrophils for the activation of these cells by ANCA, with regard to NADPH-oxidase activation and adhesion.

In the highly sensitive DHR assay for measuring the NADPH-oxidase activity, the DHR is converted into the fluorescent rhodamine-1,2,3, which remains cell-associated and hence can be detected by flow cytometry. Rhodamine-1,2,3 fluorescence was not detected in the completely MPO-deficient cells (Table 4), despite a normal respiratory burst. This confirms that the DHR oxidation by H<sub>2</sub>O<sub>2</sub> is MPO-dependent [22]. The difference between the values of cytochrome-*c* reduction and DHR oxidation by partially MPO-deficient neutrophils exposed to anti-MPO mAb may be a result of the fact that the first assay only measures extracellularly generated superoxide, whereas the second assay also measures intracellularly generated reactive oxygen species. Moreover, the DHR assay enabled us to demonstrate that H<sub>2</sub>O<sub>2</sub> generated by one cell type (MPO-deficient cells) can be carried over to another cell type (control cells blocked by anti-FcγRIIIa; Fig. 4). Therefore, H<sub>2</sub>O<sub>2</sub> released extracellularly from activated neutrophils can diffuse into other cells [42]. Hence, the H<sub>2</sub>O<sub>2</sub> carryover from neutrophils to tissue cells may contribute to the damage induced by oxygen radicals in the pathology of systemic vasculitides.

In conclusion, this work presents two sets of novel findings, one dealing with previously unreported genotypes of MPO deficiency and another dealing with the important debate about the pathophysiological role of ANCA in the inflammatory syndrome associated with vasculitis and the mechanisms of neutrophil activation by ANCA. For the first time, the absolute requirement of MPO expression for activation of TNF-primed neutrophils with anti-MPO antibodies has been demonstrated.

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