

Pathogenic Effects of Antimyeloperoxidase Antibodies in Patients With Microscopic Polyangiitis

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Objective. Microscopic polyangiitis (MPA) is a small-vessel vasculitis associated with antimyeloperoxidase (MPO) antibodies in 70% of patients. Anti-MPO antibodies can trigger the release of MPO by neutrophils and monocytes, but their involvement in the pathogenesis of MPA is still questioned. The aim of this study was to investigate whether anti-MPO antibodies can activate MPO to generate an oxidative stress that is potentially deleterious to the endothelium.

Methods. MPA sera, purified IgG from MPA sera, normal control sera, and purified IgG from normal sera were incubated with MPO coated onto microtitration plates. The peroxidase activity of MPO was evaluated by adding *o*-phenylenediamine. Production of hypochlorous acid (HOCl) was determined by chemiluminescence. The cytotoxic properties of byproducts of MPO activation were tested on endothelial cells in culture.

Results. MPA sera with anti-MPO antibodies were found to activate MPO *in vitro* ($P < 0.0001$ versus normal sera) and to generate HOCl ($P < 0.001$), as did IgG purified from MPA sera ($P < 0.05$). MPA sera without anti-MPO antibodies and MPA IgG absorbed

on MPO did not show these activities. The byproducts of MPO activation by MPA sera exerted a strong cytolytic activity on endothelial cells in culture ($P < 0.01$). Both HOCl production and endothelial lysis were abrogated by *N*-acetylcysteine (NAC), an antioxidant molecule ($P < 0.05$ and $P < 0.0001$, respectively).

Conclusion. Anti-MPO antibodies could play a pathogenic role *in vivo* by triggering an oxidative burst, leading to severe endothelial damage. Treatment of MPA patients with NAC might be proposed in an attempt to abrogate these deleterious phenomena.

Microscopic polyangiitis (MPA) is a small-vessel vasculitis with frequent cutaneous, pulmonary, renal, and neurologic involvement. Antineutrophil cytoplasmic autoantibodies (ANCA) are detectable in the sera of ~70% of MPA patients and are mainly directed toward myeloperoxidase (MPO) (1,2). MPO is a 118-kd cationic protein that is present in primary azurophilic granules of polymorphonuclear neutrophils (PMNs) and monocytes. MPO uses hydrogen peroxide and chloride ions to generate hypochlorous acid (HOCl), a highly deleterious reactive oxygen species (ROS) that contributes to oxidative burst from PMNs in response to microbial infections (3).

The pathogenic role of anti-MPO IgG antibodies has recently been suggested by studies of the passive transfer of anti-MPO antibodies into Rag2^{-/-} mice, which was followed by the development of necrotizing crescentic glomerulonephritis and systemic vasculitis mimicking the disease in humans (4). However, the mechanism of the pathogenicity of anti-MPO antibodies is not completely understood. Some reports have suggested that anti-MPO antibodies can activate PMNs and monocytes and lead to the extracellular release of MPO and oxidative burst (5,6). We have hypothesized that, as already shown for proteinase 3 (PR3) (7,8), serum

Dr. Guilpain's work was supported by a grant from the Fondation pour la Recherche Médicale. Dr. Servettaz's work was supported by a grant from the Laboratoire Français du Fractionnement et des Biotechnologies.

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Submitted for publication July 11, 2006; accepted in revised form April 3, 2007.

Table 1. Clinical status of patients and serum sampling*

Patient group, clinical status	No. of sera (no. of patients)	No. of sera obtained before steroids and/or immunosuppressive drugs	BVAS, mean \pm SD (range)
MPA patients with anti-MPO (n = 19)			
Active disease	18 (13)	10 (10)	19.5 \pm 7.3 (9–36)
Remission	18 (10)	None (none)	0.61 \pm 0.9 (0–2)
MPA patients without anti-MPO (n = 13)			
Active disease	15 (13)	9 (9)	15.4 \pm 8.3 (6–36)

* There was no difference in the Birmingham Vasculitis Activity Scores (BVAS) between microscopic polyangiitis (MPA) patients with and those without antimyeloperoxidase (anti-MPO) antibodies.

anti-MPO antibodies can directly interact with MPO and alter its enzymatic activity.

In the present study, we investigated whether sera from MPA patients could increase the enzymatic activity of MPO, generate HOCl production, and lead to endothelial lesions and vasculitis. An experimental system was designed that allowed us to evaluate the modulation of exogenous MPO activity by sera from MPA patients and to correlate the data with biologic and clinical features of the disease. We found that anti-MPO antibodies present in sera from MPA patients can activate MPO, generate HOCl, and lead to endothelial cell death.

PATIENTS AND METHODS

Patients and controls. Thirty-two patients with MPA (14 men and 18 women) were included in the study. Their mean \pm SD age was 58.5 \pm 12.6 years (age range 35–77 years). Clinical manifestations at diagnosis were as follows: general symptoms (69% of patients), renal involvement (56%), peripheral neuropathy (56%), skin lesions (37%), alveolar hemorrhage (34%), and muscle (28%), ear/nose/throat (25%), bowel (22%), joint (22%), heart (9%), eye (6%), and central nervous system (3%) involvement. Histologic confirmation was obtained in 29 of the 32 patients. These proportions are similar to those observed in a previous series (2). Necrotizing vasculitis was confirmed on muscle specimens from 15 patients, kidney specimens from 11 patients, skin specimens from 4 patients, peripheral nerve specimens from 3 patients, and temporal artery specimens from 2 patients.

Nineteen patients had anti-MPO antibodies. All patients met the Chapel Hill Consensus Conference nomenclature for the diagnosis of microscopic polyangiitis (9), including histopathologic confirmation of the diagnosis in 29 of the patients. The 3 patients without an available histopathologic examination had anti-MPO antibodies. Serum sampling and clinical status of the study patients are presented in Table 1.

Eighteen serum samples from 13 of the MPA patients who had anti-MPO antibodies were obtained at the time of disease flare. Seven more samples from 4 of these patients and 11 samples from 6 other patients with anti-MPO antibodies were also obtained during clinical remission. Fifteen serum

samples from the 13 MPA patients without anti-MPO antibodies were obtained during disease flares.

Fifty healthy donors (22 men and 28 women) served as controls. Their mean \pm SD age was 53.3 \pm 10.5 years (age range 29–76 years). All study subjects gave their written informed consent.

Assessment of disease activity. Disease activity at the time of blood sampling was assessed by the Birmingham Vasculitis Activity Score (BVAS) (10). The BVAS is a clinical index of disease activity based on signs and symptoms in 9 separate organ categories (systemic signs, skin, mucous membranes and eyes, ear/nose/throat, chest, heart and vessels, gastrointestinal, kidney, and central nervous system). A pre-defined number of points are given for each category according to the number and severity of clinical and biologic symptoms. Then, the scores for the individual categories are added to yield the BVAS; the maximum possible score is 63. There was no difference in scores on the BVAS for patients with and those without anti-MPO antibodies.

ANCA and anti-MPO antibody assays. All sera were screened for ANCA by indirect immunofluorescence using ethanol-fixed normal fresh peripheral blood PMNs (11). Anti-MPO antibodies were determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Bio Advance, Emerainville, France). Results were expressed in arbitrary units (AU) per milliliter. Concentrations $<$ 20 AU/ml were considered negative.

In vitro study of the peroxidase activity of MPO in the presence of MPA sera. The assay for MPO peroxidase activity was adapted from the procedure described by Hassan et al (12). Purified MPO (Calbiochem, San Diego, CA) with a specific activity of 150–200 units/mg of protein was diluted at 2 μ g/ml in Dulbecco's phosphate buffered saline (PBS; 0.20 gm/liter of KCl, 0.20 gm/liter of KH₂PO₄, 8 gm/liter of NaCl, 1.15 gm/liter of Na₂HPO₄) and coated onto 96-well plates (Data Packaging Corporation, Cambridge, MA) overnight at 4°C. After 3 washes with PBS, 100 μ l of each serum diluted 1:10 was added in duplicate, and the plates were incubated for 60 minutes at room temperature. The 1:10 dilution was chosen because it provided a clear difference between MPA sera and control sera. After 5 washes, 0.4 mg/liter of *o*-phenylenediamine (Sigma Fast OPD; Sigma, St. Louis, MO) and 11.7 mM H₂O₂ (Sigma) in 0.05M citrate buffer (pH 5.0) were added.

After 60 minutes, optical densities (OD) were measured at 450 nm with a reference wavelength of 620 nm using a microplate reader (Dynatech, Mountain View, CA). Each

plate comprised wells containing positive sera, negative sera, and wells without MPO (blank controls). MPO peroxidase activity was expressed in AU.

In vitro generation of HOCl by MPO in the presence of MPA sera. The assay for HOCl generation by MPO in vitro was adapted from the procedure described by Paino et al (13). Aliquots of 100 μ l of each serum diluted 1:10 were deposited into wells of the microtitration plates (Black Optiplate; Packard, Warrenville, IL) coated with MPO as described above and incubated for 60 minutes at room temperature. After 5 washes, 36 μ M luminol and 400 μ M H₂O₂ diluted in PBS were added to start the reaction. After 5 minutes, HOCl production was measured by chemiluminescence using a spectrofluorometer/luminometer (Fusion; Packard) at 37°C. Controls were the same as described above. HOCl production was expressed in AU.

Serum MPO assay. Serum concentrations of MPO were determined by ELISA, according to the recommendations of the manufacturer (Sigma). Sera from all patients and healthy controls were analyzed. The threshold of sensitivity of the assay was >1.5 ng/ml.

In vitro generation of HOCl by MPO in the presence of anti-MPO antibodies. In the first set of experiments measuring the generation of HOCl by MPO in the presence of anti-MPO antibodies, total IgG antibodies were removed from serum samples by precipitation with G protein. Briefly, 20 μ l of G protein (Sigma) was added to the sera (diluted 1:10 in PBS) and incubated for 60 minutes at room temperature. Then, the samples were centrifuged at 30g for 10 minutes, and the supernatants were removed and used in chemiluminescence assays.

In the second set of experiments, IgG antibodies from the sera of 4 MPA patients with anti-MPO antibodies, 4 MPA patients without anti-MPO antibodies, and 13 healthy subjects were purified by affinity chromatography on protein G-Sepharose (Sigma). Purity of the purified IgG was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purified IgG antibodies were then tested for HOCl production by coated MPO.

In the third set of experiments, anti-MPO antibodies were removed from purified IgG antibodies by 2 successive absorptions on coated MPO for 1 hour at 4°C. After depletion of anti-MPO antibodies, purified IgG were tested on coated MPO for HOCl production.

In the fourth set of experiments, a polyclonal rabbit anti-MPO antibody (Rockland, Gilbertsville, PA) was added at concentrations ranging from 1.6 μ g/ml to 200 μ g/ml, and HOCl production was measured. In another set of experiments, 3 aliquots consisting of 1 ml of IgG antibodies purified from 3 patients with anti-MPO antibodies and 1 aliquot from a healthy subject were lyophilized (Freezemobile 12SL; VirTis, Gardiner, NY). Each sample of lyophilized IgG antibodies was then added at increasing concentrations to a normal serum sample that was devoid of anti-MPO antibodies. Samples of normal serum with increasing amounts of IgG antibodies were then incubated with coated MPO for 1 hour, and HOCl production was determined as described above.

In vitro effect of ceruloplasmin on HOCl production by MPO. MPO-coated plates were incubated with sera from patients with anti-MPO antibodies or with sera from healthy subjects, in the presence and absence of 2 μ g of ceruloplasmin

per well. In another experiment, MPO-coated wells were incubated with PBS or with a 1:10 dilution of polyclonal rabbit anti-MPO in the presence of increasing concentrations of ceruloplasmin (0–50 μ g/ml), an inhibitor of MPO enzymatic activity.

Cultures of endothelial cells. Human umbilical vein endothelial cells were obtained by digestion of freshly obtained umbilical cords with 1.5 mg/ml of collagenase (Sigma) (14). Cells were cultured at 37°C in an atmosphere of 5% CO₂ in 75-cm² flasks (Costar; Corning, Corning, NY) containing 12 ml of endothelial cell growth medium (PromoCell, Heidelberg, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS; BioWest, Nuaille, France), 100 units/ml of penicillin, 100 μ g/ml of streptomycin (Gibco, Paisley, UK). The endothelial phenotype of these cultured cells was confirmed by their typical growth in cobblestone monolayers and by labeling of von Willebrand factor antigen by a specific polyclonal rabbit antibody (Dako, Glostrup, Denmark). After the second passage, cells were detached using 0.05% trypsin, 0.53 mM EDTA. Thus, 10⁴ cells per well were seeded onto a 96-well microtitration plate (Nunclon; Nunc, Roskilde, Denmark) and incubated for 10 hours at 37°C.

In vitro effect of HOCl generation on endothelial cell viability. Sera from 10 MPA patients and 10 healthy subjects were incubated with coated MPO as described above. H₂O₂ (400 μ M) in PBS was added to each microwell for 1 hour at 37°C in the presence of coated MPO or in the presence of coated MPO plus 5 \times 10⁴ PMNs/well. The PMNs had been isolated by gradient centrifugation (15) from whole blood obtained from a healthy blood donor and were exposed or were not exposed to 10 μ g/ml of lipopolysaccharide (LPS) for 4 hours. After 1 hour, plates with PMNs were centrifuged at 1,500 revolutions per minute for 5 minutes. Finally, 75 μ l of each supernatant was distributed into a 96-well microtitration plate (Nunclon) coated with 10⁴ endothelial cells/well in the presence of 125 μ l of endothelial cell growth medium (PromoCell) without additional FCS. The plate was further incubated for 12 hours, and then cell viability was evaluated by mitochondrial-dependent reduction of MTT (16). Briefly, 100 μ l of MTT (0.2 mg/ml) was added for 4 hours at 37°C. The medium was discarded and the OD read at 600 nm after addition of 100 μ l of DMSO per well (Sigma).

In vitro effect of antioxidant drugs on the activation of MPO by MPA sera. Coated MPO was incubated with a 1:10 dilution of sera from 3 MPA patients with anti-MPO antibodies and 3 healthy subjects, in the presence and absence of the antioxidant drugs deferoxamine (500 μ M; Sigma), D-mannitol (500 μ M; Sigma), mangafodipir (200 μ M) (Teslascan; GE Healthcare, UK), and N-acetylcysteine (NAC; 500 μ M) (Sigma). After 1 hour at room temperature, HOCl production was measured as described above.

Effect of NAC on endothelial cell lysis induced by HOCl. Ten MPA sera were incubated with coated MPO as described above. H₂O₂ (400 μ M) in PBS was added to each microwell for 1 hour at 37°C. After 1 hour, 75 μ l of each supernatant was distributed into a 96-well microtitration plate (Nunclon) coated with 10⁴ endothelial cells per well that had been pretreated or not pretreated with NAC (1.2 mM) for 12 hours. The plate was further incubated for 12 hours, and cell viability was evaluated by MTT as described above.

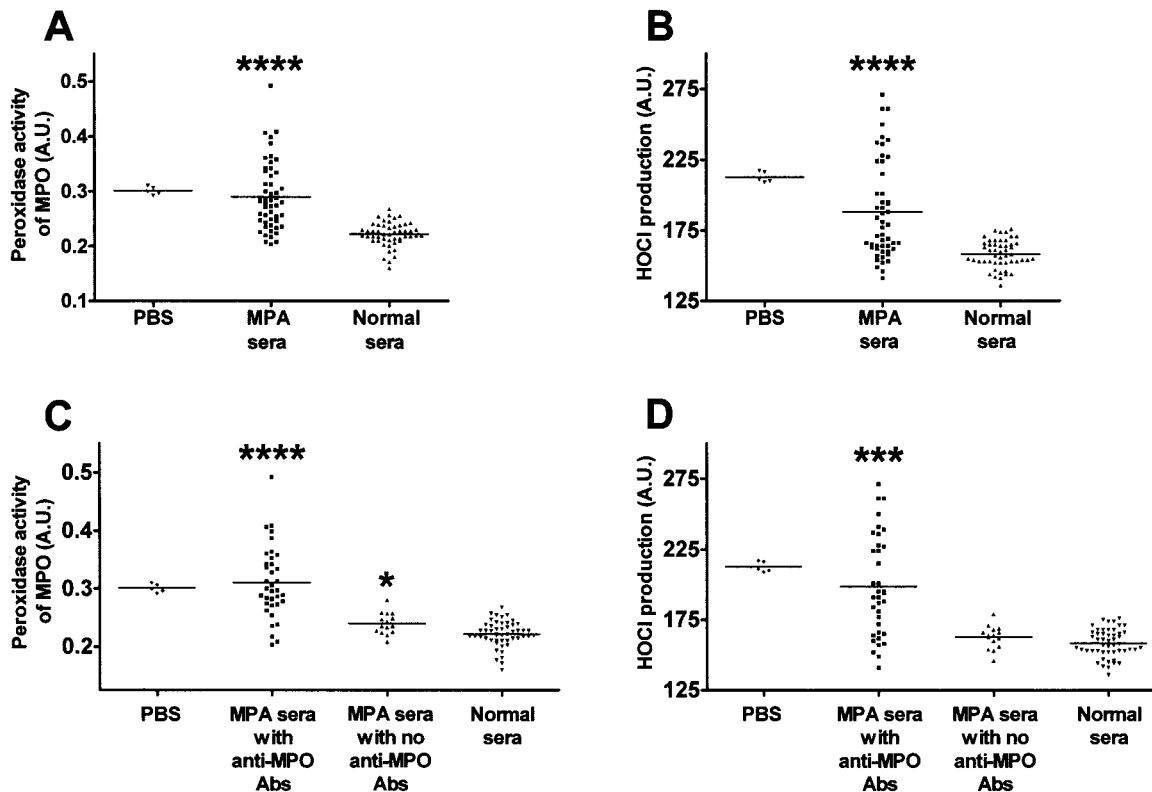


Figure 1. In vitro activation of coated myeloperoxidase (MPO) and generation of HOCl in the presence of sera from patients with microscopic polyangiitis (MPA). **A**, Peroxidase activity and **B**, HOCl production by MPO in the presence of MPA sera (51 samples) or normal sera (50 samples). **C**, Peroxidase activity and **D**, HOCl production by MPO in the presence of MPA sera with and without anti-MPO antibodies (Abs) (36 samples and 15 samples, respectively). Horizontal lines show the mean. **** = $P < 0.0001$; * = $P < 0.05$; *** = $P < 0.001$ versus normal sera. AU = arbitrary units; PBS = phosphate buffered saline.

Quantification of serum ceruloplasmin. Serum concentrations of ceruloplasmin were measured by immunonephelometry, according to the manufacturer's instructions (BN II nephelometer; Dade-Behring, Paris, France). Normal values were between 0.17 mg/liter and 0.70 mg/liter.

Statistical analysis. Data are expressed as the mean \pm SD. Statistical analysis was performed using the nonparametric Mann-Whitney U test for unpaired data, Wilcoxon's matched pairs test, or regression analysis according to Spearman's test for paired data. P values less than 0.05 were considered significant.

RESULTS

Activation of peroxidase and generation of HOCl in the presence of MPA sera in vitro. First, the specificity of the measurement of MPO activity was assessed by showing a dose-dependent inhibition of MPO activity by ceruloplasmin (data not shown). The mean peroxidase activity of coated MPO was higher after incubation with sera from MPA patients than after incubation with sera from healthy subjects ($P < 0.0001$) (Figure 1A). The

mean peroxidase activity of MPO was also higher when sera contained anti-MPO antibodies than when they were devoid of anti-MPO antibodies ($P < 0.0001$) (Figure 1C).

The mean HOCl production by coated MPO was higher with sera from MPA patients than with sera from healthy subjects ($P < 0.0001$) (Figure 1B). Likewise, sera from MPA patients who had anti-MPO antibodies induced higher rates of HOCl production by MPO than did sera from MPA patients who did not have anti-MPO antibodies ($P < 0.001$) (Figure 1D). Compared with normal sera, MPA sera devoid of anti-MPO antibodies slightly, but significantly, increased the peroxidase activity of coated MPO ($P < 0.05$) (Figure 1C), but not HOCl production (P not significant) (Figure 1D). In addition, a strong correlation was observed between the peroxidase activity of MPO and the production of HOCl ($r = 0.7486$, $P < 0.0001$). The inhibition of MPO activity by normal sera is explained by the presence of natural MPO inhibitors.

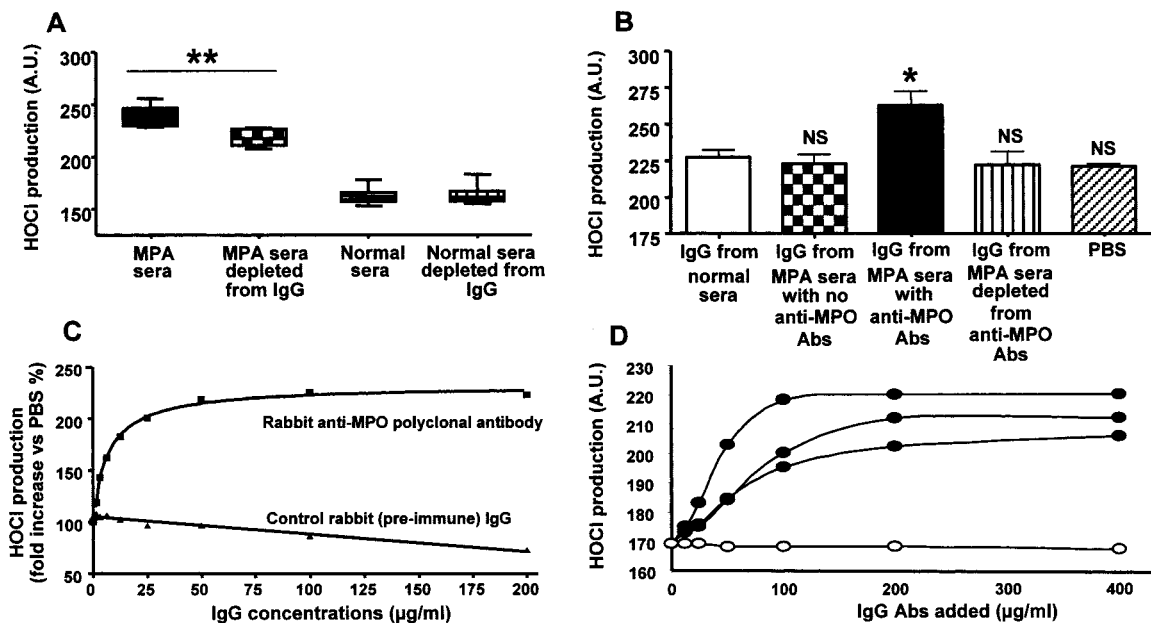


Figure 2. In vitro effect of antimyeloperoxidase (anti-MPO) antibodies on the generation of HOCl by MPO. **A**, HOCl production before and after removal of serum immunoglobulins by immunoprecipitation with G protein. Values are the mean \pm SD. ** = $P < 0.01$ versus sera depleted of IgG antibodies. AU = arbitrary units. **B**, HOCl production in the presence of IgG purified from normal sera, from microscopic polyangiitis (MPA) patient sera without anti-MPO antibodies (Abs), and from anti-MPO-positive MPA patient sera depleted of anti-MPO antibodies by absorption. Values are the mean and SD. * = $P < 0.05$ versus IgG from normal sera. NS = not significant; PBS = phosphate buffered saline. **C**, Effect of rabbit anti-MPO polyclonal antibody and control rabbit (pre-immune) IgG on HOCl production. **D**, Dose-dependent increase in HOCl production following addition of increasing amounts of lyophilized IgG antibodies from 3 patients with anti-MPO IgG antibodies (\bullet) and from 1 healthy subject (\circ) to a normal serum devoid of anti-MPO antibodies.

Effect of anti-MPO antibodies on the generation of HOCl. The correlation between the titers of anti-MPO antibodies and the activity of coated MPO ($r = 0.4182$, $P < 0.05$) or the production of HOCl ($r = 0.5789$, $P < 0.001$) prompted us to investigate whether anti-MPO antibodies were responsible for MPO activation and HOCl production under the experimental conditions we used.

In the first set of experiments, the removal of anti-MPO antibodies by immunoprecipitation of serum immunoglobulins with G protein resulted in a significant decrease in HOCl production ($P < 0.01$) (Figure 2A).

In the second set of experiments, purified IgG antibodies from MPA patients with anti-MPO antibodies induced the production of higher levels of HOCl than did IgG antibodies purified from sera obtained from healthy subjects or patients without anti-MPO antibodies ($P < 0.05$ for each comparison) (Figure 2B).

In the third set of experiments, the removal of anti-MPO antibodies absorbed on MPO-coated plates resulted in a decrease in HOCl production ($P < 0.05$) (Figure 2B).

In the fourth set of experiments, rabbit anti-MPO polyclonal antibody caused a dose-dependent increase in HOCl production by human MPO (Figure 2C). Finally, adding IgG anti-MPO antibodies from 3 patients at increasing concentrations to a normal serum devoid of anti-MPO antibodies, resulted in a dose-dependent increase in HOCl production in each case (Figure 2D).

Serum myeloperoxidase levels. Serum MPO levels did not differ between the following study groups: patients with anti-MPO antibodies versus patients without anti-MPO antibodies (mean \pm SD 4.05 ± 3.60 ng/ml versus 3.99 ± 4.30 ng/ml; $P = 0.72$), patients with anti-MPO antibodies versus healthy controls (4.05 ± 3.60 ng/ml versus 5.83 ± 5.68 ng/ml; $P = 0.41$), and patients without anti-MPO antibodies versus healthy controls (3.99 ± 4.30 ng/ml versus 5.83 ± 5.68 ng/ml; $P = 0.42$).

Effect of ceruloplasmin on HOCl production by MPO in vitro. Adding increasing concentrations of ceruloplasmin, an inhibitor of MPO, to 1 MPA serum and 1 healthy serum resulted in a linear decrease in HOCl production (data not shown). When tested in all sera with anti-MPO antibodies, the addition of $2 \mu\text{g}$ of

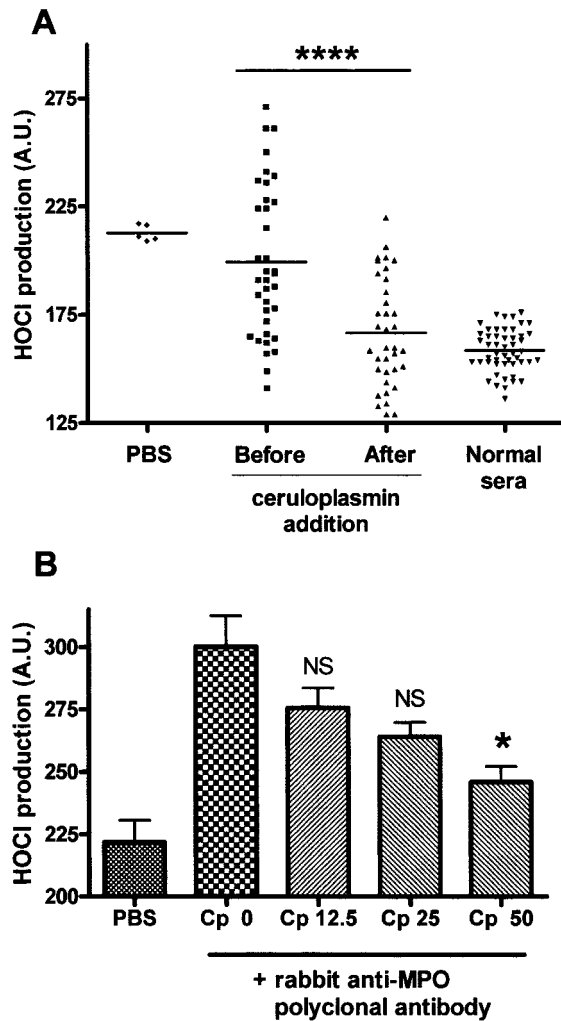


Figure 3. In vitro effect of ceruloplasmin (Cp) on HOCl production by myeloperoxidase (MPO). **A**, HOCl production by MPO in the presence of microscopic polyangiitis (MPA) patient sera before and after incubation with ceruloplasmin or in the presence of normal sera. Horizontal lines show the mean. **** = $P < 0.0001$ versus addition of ceruloplasmin. AU = arbitrary units; PBS = phosphate buffered saline. **B**, Dose-dependent decrease in HOCl production in the presence of rabbit anti-MPO polyclonal antibody after addition of ceruloplasmin (0–50 $\mu\text{g/ml}$). Values are the mean and SD. * = $P < 0.05$ versus PBS. NS = not significant.

ceruloplasmin per well decreased HOCl production by coated MPO ($P = 0.0001$) (Figure 3A). The levels of HOCl production by these sera after the addition of ceruloplasmin were not significantly different from the levels of HOCl production by sera from healthy subjects (Figure 3A). The addition of ceruloplasmin was also able to dose-dependently reverse the overproduction of HOCl induced by the addition of rabbit anti-MPO polyclonal antibodies ($P < 0.05$) (Figure 3B).

Effect of the byproducts of MPO activation on endothelial cell viability. The byproducts of MPO activation exerted a higher cytolytic effect on endothelial cells when MPO was incubated with MPA sera containing anti-MPO antibodies than when MPO was incubated with sera from healthy subjects ($P < 0.01$) (Figure 4A). The byproducts obtained in the presence of LPS-stimulated or unstimulated PMNs exerted a cytolytic effect on endothelial cells that was not significantly different from that obtained in the absence of PMNs.

Effect of antioxidant drugs on the activation of MPO by MPA sera and on cell death in vitro. The antioxidant molecules deferoxamine, D-mannitol, and mangafodipir did not reduce the activation of coated

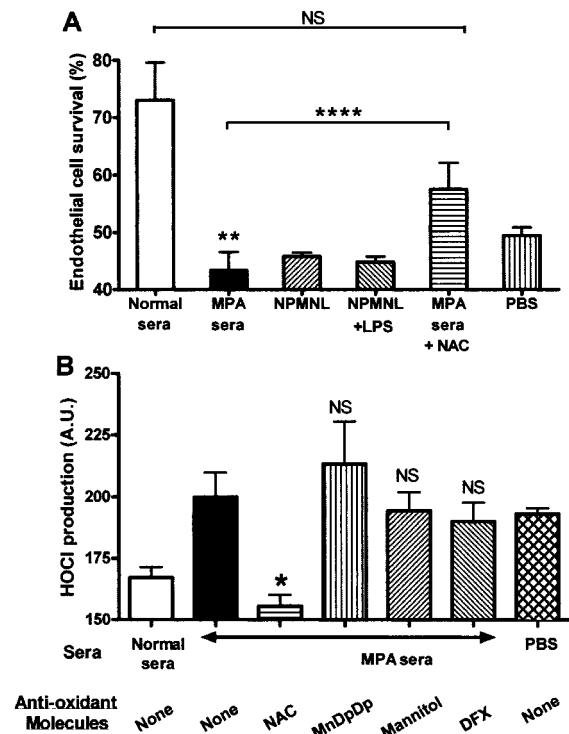


Figure 4. In vitro effect of the byproducts of myeloperoxidase (MPO) activation on endothelial cell viability. **A**, Endothelial cells in culture were incubated with the byproducts of MPO activation in the presence and absence of polymorphonuclear neutrophils (NPMNL) or *N*-acetylcysteine (NAC; 1.2 mM), as described in Patients and Methods. **** = $P < 0.0001$. ** = $P < 0.01$ versus normal sera. **B**, HOCl production by MPO was studied in the presence and absence of the antioxidant molecules NAC (500 μM), mangafodipir (MnDpDp; 200 μM), D-mannitol (500 μM), or deferoxamine (DFX; 500 μM). Values are the mean and SD. * = $P < 0.05$ versus phosphate buffered saline (PBS). NS = not significant; MPA = microscopic polyangiitis; LPS = lipopolysaccharide; AU = arbitrary units.

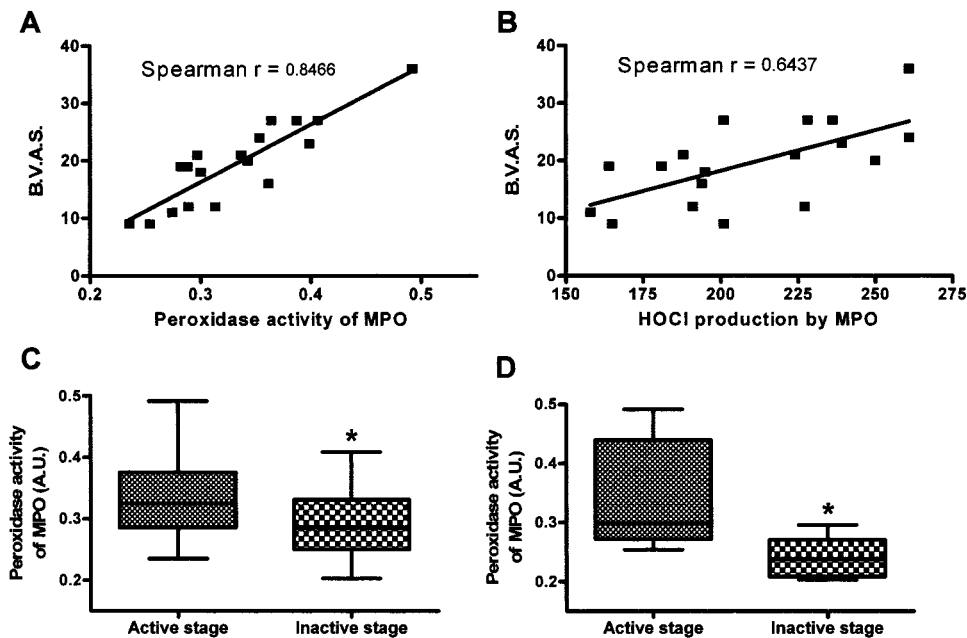


Figure 5. Correlation between the in vitro myeloperoxidase (MPO) activity or HOCl production and the clinical activity of microscopic polyangiitis (MPA). **A**, Correlation between scores on the Birmingham Vasculitis Activity Score (BVAS) and peroxidase activity of MPO. **B**, Correlation between scores on the BVAS and the production of HOCl. **C**, MPO activity in the presence of sera from patients with active (18 samples) and inactive (18 samples) MPA. **D**, Peroxidase activity of MPO in the presence of sera from 4 MPA patients collected during periods of active disease and remission. Values in **C** and **D** are the mean \pm SD. * = $P < 0.05$ versus normal sera. AU = arbitrary units.

MPO (Figure 4B). Only NAC induced a significant decrease (22.1%) in MPO activation ($P < 0.05$). Cells exposed to NAC exhibited a higher survival rate than did cells not exposed to NAC ($P < 0.0001$) (Figure 4A). The survival rate of cells incubated with the byproducts of MPO activated by MPA sera was not different from the survival rate of cells incubated with the byproducts of MPO activated by sera from healthy subjects (P not significant).

Correlation between the in vitro peroxidase activity or HOCl production and the clinical activity of MPA. During flares of MPA, a strong correlation was observed between MPO peroxidase activity as well as HOCl production and the clinical activity of the disease as assessed by the BVAS (Spearman's $r = 0.8466$, $P < 0.0001$ and $r = 0.6437$, $P < 0.01$, respectively) (Figures 5A and B). The correlation between anti-MPO antibody titers and the BVAS score was weaker, but remained significant ($r = 0.4738$, $P < 0.05$). In 4 patients with remittent MPA, anti-MPO antibodies and in vitro MPO activity remained high. Ceruloplasmin levels were not correlated with the peroxidase activity of MPO, HOCl

production, or ANCA titers (P not significant for each comparison) (data not shown).

During periods of remission, in vitro peroxidase activity and HOCl production were still observed in 8 sera with persistent anti-MPO antibodies, but the levels were both significantly lower than during active phases of the disease (mean \pm SD 0.289 ± 0.054 AU versus 0.332 ± 0.064 AU [$P < 0.05$] and 187.8 ± 36.39 AU versus 209.1 ± 32.82 AU [$P < 0.05$]) (Figure 5C). Sera collected during active and remittent periods in the same patient were available from 4 MPA patients. In these 4 patients, peroxidase activity and HOCl production of MPO were increased during flares and decreased to basal levels during remissions (mean \pm SD 0.3365 ± 0.088 AU versus 0.2421 ± 0.035 AU [$P < 0.05$] and 212.0 ± 29.68 AU versus 167.6 ± 11.39 AU [$P < 0.01$]) (Figure 5D).

DISCUSSION

In this study, we found that anti-MPO antibodies in the sera of patients with MPA trigger MPO activation in vitro and generate HOCl, a ROS that is highly

deleterious to endothelial cells. Three sets of data suggest that the *in vitro* MPO-activating effect of MPA sera is linked to the presence of anti-MPO antibodies: first, the depletion of IgG antibodies abrogated the proactivating properties of sera; second, IgG antibodies isolated from MPA sera activated MPO *in vitro*; and third, the addition of increasing amounts of anti-MPO IgG antibodies to normal serum resulted in a dose-dependent increase in MPO activation.

The modulation of enzymatic functions by autoantibodies has already been shown in Wegener's granulomatosis patients with anti-PR3 antibodies, which interfered with the proteolytic activity of PR3 (7,8). Anti-MPO antibodies may be able to trigger MPO activation through 2 nonexclusive mechanisms. First, these autoantibodies could directly increase the enzymatic activity of MPO, as was observed with anti-C1s autoantibodies, which stimulated the enzymatic activity of C1s *in vitro* (17). Second, anti-MPO antibodies could enter into competition with ceruloplasmin, the physiologic inhibitor of MPO, as anti-PR3 antibodies do with α_1 -antitrypsin, the natural inhibitor of PR3 (18,19). On the other hand, the increased MPO activity observed *in vitro* after incubation with MPA sera is not linked to serum soluble MPO, since the MPO concentration was similar in sera from normal subjects and patients with MPA.

Taken together, our data suggest that the proactivating effect of anti-MPO antibodies can play a role in the development of MPA. Indeed, the activation of MPO generates HOCl, a potent cellular oxidant formed by the conversion of hydrogen peroxide. Since high amounts of ROS, including H₂O₂, are produced by PMNs activated by ANCA (5,20–22), H₂O₂ could serve as a substrate for MPO to produce HOCl, which, in our experience, is deleterious to endothelial cells independently of PMNs.

Since endothelial injuries are key features of small-vessel vasculitides (23), we investigated whether the oxidative stress produced by anti-MPO antibodies on MPO could be deleterious to endothelial cells. We found that the byproducts of antibody-activated MPO inflicted lethal injuries to endothelial cells. These findings suggest that MPA sera convey signals that could possibly be deleterious to endothelial cells *in vivo*, resulting in necrosis and detachment from the basement membrane, as observed in biopsy tissues from the MPA patients. This hypothesis is also consistent with the high numbers of circulating necrotic endothelial cells detected in MPA (24). Thus, MPO activation could be crucial for the development of MPA, and its abrogation could improve the clinical manifestations of the disease.

During disease flares, the *in vitro* activation of MPO is strongly correlated with the activity of MPA, as assessed by scores on the BVAS. This index, which classically, is used to determine the activity of small-vessel vasculitis, has never been found to be so strongly correlated with any other biologic marker. Indeed, plasma concentrations of C-reactive protein lack specificity, and the correlation of anti-MPO antibody titers with the activity of MPA is still a subject of controversy. Some reports have suggested that ANCA levels correlate with clinical status (25–27), whereas anti-MPO antibodies sometimes remain elevated during clinical remission (28). In our experience, the determination of the proactivating effect of total MPA sera on MPO better reflects the *in vivo* condition than do titers of anti-MPO antibodies. Indeed, we cannot rule out the possibility that soluble mediators other than anti-MPO antibodies might be involved in triggering the oxidative burst. This could explain the dissociation between anti-MPO antibody levels and/or *in vitro* MPO activation and the activity of MPA in a few cases. An alternative view is that some antibodies may be enhancing while others could be neutral or inhibitory. Thus, the diversity of anti-MPO antibodies could play a key role in the activation of MPO.

The *in vitro* proactivating effect of sera on MPO could be used to monitor the activity of MPA, in particular, to assess the intensity of flares at their onset. The strong correlation between MPO activation and the intensity of flares has prompted us to consider the MPO/H₂O₂/chloride system as an experimental model by which to assess the effectiveness of various molecules for the treatment of MPA. Several antioxidant molecules have been tested, including NAC, deferoxamine, D-mannitol, and a manganese superoxide dismutase mimic, mangafodipir. Only NAC significantly reduced the activation of MPO. Furthermore, NAC improved the survival of endothelial cells exposed to the byproducts of MPO activation. These data are consistent with the known properties of NAC as a ROS scavenger and as an effective treatment of experimental systemic vasculitis with anti-MPO antibodies in brown Norway rats injected with mercuric chloride (29). This observation suggests that NAC could be effective in patients with MPA, and prospective clinical trials should be designed to evaluate NAC as a potential therapy for MPA.

In conclusion, this study is the first to suggest that anti-MPO antibodies play a key role in the pathogenesis of MPA in humans through the activation of MPO, the production of HOCl, and the aggression of endothelial cells. The *in vitro* assay of MPO activation provides

information on the intensity of flares and could be used to evaluate new therapies aimed at modulating in vitro the activity of MPO and the production of ROS by sera from patients with MPA.

ACKNOWLEDGMENTS

We thank the study patients and the staff of the Department of Internal Medicine, Hôpital Cochin, for their involvement in this study.

AUTHOR CONTRIBUTIONS

Dr. Batteux had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Guilpain, Mouthon, Batteux.

Acquisition of data. Guilpain, Servettaz, Goulvestre, Barrieu, Borde-rie, Chéreau, Kavian, Pagnoux, Guillevin, Batteux.

Analysis and interpretation of data. Guilpain, Servettaz, Goulvestre, Weill, Batteux.

Manuscript preparation. Guilpain, Weill, Batteux.

Statistical analysis. Guilpain, Servettaz.

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