Induction of neutrophil responsiveness to myeloperoxidase antibodies by their exposure to supernatant of degranulated autologous neutrophils

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Antibodies against myeloperoxidase (MPO) and proteinase 3 (PR3) are the predominant autoantibodies present in antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis. Their binding to the corresponding antigen on the surface of polymorphonuclear neutrophils (PMNs) is believed to trigger the disease process. Cytokines released during an inflammatory reaction are thought to prime resting PMNs, making them responsive to autoantibodies. In the present study we found that MPO but not PR3 could be detected on the cell surface of unstimulated PMNs after incubation with the supernatants of activated autologous PMNs. MPO was shown to be acquired from these supernatants, because PMNs did not express MPO when the supernatants were specifically MPO-depleted. In addition, purified soluble MPO bound to unstimulated PMNs. Unstimulated PMNs that had passively acquired MPO released oxygen radicals when incubated with monoclonal antibody anti-MPO or the immunoglobulin G fraction of a patient with MPO-ANCA. The data presented here suggest that, in ANCA-associated vasculitis, soluble MPO released by activated PMNs may bind to unstimulated PMNs, thereby making them reactive to anti-MPO antibodies. This mechanism of dispersing PMN activation would be specific for MPO-ANCA and may explain differences in the pathologic and clinical expression of MPO-ANCA versus PR3-ANCA vasculitis. (Blood. 2000;96:2822-2827)

Introduction

Antineutrophil cytoplasmic antibodies (ANCA) are associated with systemic vasculitis, especially Wegener's granulomatosis (WG) and microscopic polyangiitis (MPA).[1-5] Most of the identified ANCA target antigens are enzymes stored within polymorphonuclear neutrophil (PMN) primary granules. The assumed pathogenic role of ANCA remains controversial, because it is not readily apparent how extracellular ANCA can interact with intracellular primary granule constituents.

Myeloperoxidase (MPO) and proteinase 3 (PR3), the major target antigens of ANCA, have recently been shown to be exposed on the surface of apoptotic PMNs, leading to an increased reactivity of these PMNs with anti-MPO antibodies and both MPO-ANCA$^+$ and PR3-ANCA$^+$ sera.[6] This model suggests that apoptotic PMNs are recognized by MPO- and PR3-ANCA, secondarily activating nonapoptotic PMNs via cross-linking of FcγRIIa/IIIb.[6-12] In addition, expression of MPO and PR3 on apoptotic PMNs might also have implications for the induction of autoimmunity leading to ANCA-associated disorders, because antigens on apoptotic cells or apoptotic bodies are the natural targets for many autoantibodies.[13-15]

The second model of ANCA-mediated PMN activation relies on the priming of PMNs. Primed PMNs display no increased oxidative activity, but subsequent activation provokes a response that is larger than in nonprimed cells.[16] The intracellular signal transduction pathways that mediate priming are not fully elucidated, and crosstalks between different signaling pathways may occur.[16-20] Tumor necrosis factor (TNF)-α is frequently used to prime PMNs.[7,9,21-26]

The aim of this study was to analyze whether exposure of unstimulated PMNs to the supernatant of autologous degranulated PMNs may play a role in ANCA-associated vasculitis independent of both apoptosis and TNF-α priming.

Materials and methods

Isolation, priming, and stimulation of PMNs

PMNs (> 98% pure, > 99% trypan blue exclusion) were isolated from fresh buffy coats of normal donors according to the technique described.[27] Briefly, a fresh buffy coat obtained from approximately 400 mL of normal donor blood was diluted 1:1 in phosphate-buffered saline–ethylenediaminetetraacetic acid (PBS-EDTA) (2 mmol/L), mixed gently with 1:4 volume of 4% Dextran T500 (Amersham Pharmacia Biotech, Dübendorf, Switzerland) and left for 30 minutes for erythrocyte sedimentation. The leukocyte-rich supernatant was aspired and centrifuged for 10 minutes at 200*g*. The pellet was resuspended for 40 seconds in 9 mL of distilled water to lyse erythrocytes. Isotonicity was restored by addition of 3 mL of KCl (0.6 mol/L) and 40 mL of NaCl (0.15 mol/L). The cells were then centrifuged 10 minutes at 350*g* and resuspended in 20 mL of PBS-EDTA (2 mmol/L). This suspension was overlaid with 20 mL of Ficoll-Hypaque (Sigma Chemical, St Louis, MO) and centrifuged for 30 minutes at 350*g*. The PMN-rich pellet was recovered and washed twice in PBS-EDTA (2 mmol/L). All solutions used were tested to be lipopolysaccharide-free, and all manipulations were performed under sterile conditions at 4°C, thus minimizing PMN priming and stimulation. For priming, PMNs were resuspended (1×10^7 cells/mL) in Hank's balanced salt solution (HBSS) at 37°C in the presence of TNF-α (2 ng/mL) (Boehringer Mannheim Biochemica, Mannheim, Germany) for 10 minutes. For stimulation, PMNs

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were resuspended (1×10^7 cells/mL) in 37°C warm HBSS in the presence of 10 μmol/L N-formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma) for 15 minutes. Thereafter, cells and cell debris were removed by centrifugation (2×15 minutes at 4000*g* at 4°C).

Incubation of PMNs with supernatant of autologous activated PMNs

Freshly isolated PMNs were incubated for 10 minutes at 4°C with supernatant of autologous PMNs stimulated as described above. PMNs were then washed and resuspended in HBSS. If indicated, autologous serum was added to the supernatant before incubation with PMNs.

PMNs were assessed for their priming status before and after incubation with supernatant from degranulated autologous PMNs. Both freshly isolated PMNs as well as PMNs previously incubated with supernatant from autologous cells released background amounts of O_2^- radicals when incubated with fMLP (10^{-7} mol/L) (freshly isolated PMNs: 2.1 ± 0.4 nmol O_2^-/60 min/10^6 PMNs; incubated PMNs: 2.3 ± 0.3 nmol O_2^-/60 min/10^6 PMNs). TNF-α priming similarly enhanced responsiveness of both native and incubated cells (native PMNs: 17.8 ± 1.6 nmol O_2^-/60 min/10^6 PMNs; incubated PMNs: 16.3 ± 1.1 nmol O_2^-/60 min/10^6 PMNs). (Results are given ± SEM of 4 independent experiments.) These data indicated that isolation as well as subsequent incubation with supernatant from autologous PMNs did not significantly prime the cells.

Determination of surface antigen expression

The expression of surface antigens on PMNs was analyzed by FACScan (Becton Dickinson, Mountain View, CA). PMNs were activated with fMLP, primed with TNF-α, or preincubated with supernatant of activated autologous PMNs as indicated above. Cells were then resuspended in PBS, 1% bovine serum albumin (BSA), and 10 mmol/L NaN_3 (FACS buffer) and incubated for 30 minutes with monoclonal antibody (mAb) anti-MPO (MPO7, immunoglobulin [Ig] G1; Dako Diagnostics AG, Zug, Switzerland), mAb anti-PR3 (CLB-12.8, IgG1; Research Diagnostics, Flanders, NJ), or mAb anti-CD3 (UCHT1, IgG1; Serotec, Oxford, England) with 1 μg mAb per 10^6 PMNs, respectively. The anti-MPO mAb was directly fluorescein isothiocynate (FITC)-labeled, while the anti-PR3 and anti-CD3 mAbs were detected with 1:250 FITC-labeled polyclonal goat-antimouse antibody (Sigma). Flow cytometry was performed on the same day, and 10 000 events per sample were collected on a FACScan with the Cell Quest program (Becton Dickinson).

Measurement of superoxide anion production

The production of superoxide anions (O_2^-) was measured with the ferricytochrome c reduction assay.[28] When indicated, PMNs were primed for 10 minutes at 37°C with 2 ng/mL TNF-α or preincubated for 10 minutes at 4°C with supernatant of autologous PMNs stimulated as described above. Cells were then incubated (10^6 PMNs/mL) in the presence or absence of anti-MPO mAb (MPO7, IgG1), anti-PR3 mAb (CLB-12.8, IgG1), anti-CD3 mAb (UCHT1, IgG1) (each 10 μg/mL), or control IgG and perinuclear $ANCA^+$ and cytoplasmic $ANCA^+$ IgG (each 100 μg/mL) as well as 60 μmol/L of ferricytochrome c (Sigma). The assay was repeated with constantly shaken or unshaken microtiter plates. Incubation time was 60 and 90 minutes for TNF-α–primed (lag phase approximately 20 minutes) and unstimulated (lag phase approximately 50 minutes) PMNs, respectively. All samples were tested in triplicates, including controls, with 75 U/mL of superoxide dismutase (Sigma). Optical density (OD) at 550 nm was measured every 5 minutes, and released O_2^- was calculated from the ΔOD baseline versus end point of the ΔOD curve. Control experiments indicated that observed increases in absorbance were completely abolished in the presence of superoxide dismutase.

Immunodepletion of MPO in the supernatant of activated PMNs

For immunodepletion, tosyl-activated dynabeads (Dynal, Milan Analytica, LaRoche, Switzerland) were covalently coated with mouse mAb. Antibodies used for coating were anti-MPO (MPO-7, IgG1) using anti-CD3 (UCHT1, IgG1) as control. Coating was performed as suggested by Dynal. Briefly, 2×10^8 tosyl-activated dynabeads were washed twice with PBS–0.1% BSA and resuspended in borate buffer, pH 9.5. Antibodies were added at 5 μg mAb per 10^7 dynabeads. After 10 minutes, BSA was added to a final concentration of 0.1 g/100 mL for optimal orientation of mAb. After 24 hours at 37°C incubation, coated dynabeads were washed in PBS–0.1% BSA before blocking free binding sites in Tris buffer (pH 8.5) for 4 hours. For immunodepletion, 5×10^7 coated beads were incubated with 1 mL of supernatant from stimulated PMNs for 30 minutes at 4°C. To obtain complete depletion, a second round of immunodepletion under the same conditions was necessary.

MPO activity assay

Enzymatic activity of MPO was measured in a colorimetric assay where 100 μL of substrate buffer (50 mL of citrate-phosphate buffer, pH 5; 20 μL of 30% H_2O_2; 20 mg of orthophenylenediamine) was added to 20 μL of supernatant of activated PMNs. Purified MPO (Calbiochem-Novabiochem, La Jolla, CA) was used as standard.[29,30] The reaction was stopped with H_2SO_4 and the absorbance measured with a microplate reader (Thermo Max, Molecular Device, Munich, Germany) at 490 nm.

Detection of PR3 by Western blot

Supernatants from primed and degranulated PMNs were denatured, but not reduced, and separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were blotted onto nitrocellulose membranes, and blots were blocked overnight at 4°C with 7.5% milk powder before incubation with mAb anti-PR3 (CLB-12.8, IgG1). Control experiments were performed with isotype-matched mAb. Blots were then washed, and bound mAb was revealed with a biotinylated sheep IgG against mouse IgG, used at a dilution of 1:1000. Streptavidin–horseradish peroxidase at a dilution of 1:2000 was added, and the blots were exposed with enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech).

Isolation of IgG from $ANCA^+$ patients

Sera from MPO- and PR3-$ANCA^+$ patients were applied to a protein G-Sepharose 4B column (Sigma) and were IgG eluted using 0.1-mol/L glycine HCl, pH 2.8. The pH of the fractions was immediately neutralized with 1.0 mol/L Tris buffer, pH 9.0. The IgG fractions were pooled, dialyzed against PBS, and concentrated with Microsep 30 K filters (Pall Filtron; Skan AG, Allschwil, Switzerland). Immediately prior to use, pooled IgG was ultracentrifuged for 60 minutes at 200 000*g* at 4°C to remove aggregated IgG.

Results

Expression of MPO and PR3 on the surface of unstimulated, stimulated, and primed PMNs

By FACScan analysis, we first confirmed that activation of PMNs increased expression of both MPO and PR3 on their cell surface (Figure 1). Because supernatant of degranulated PMNs is known to contain both MPO and PR3, we then performed experiments to see whether exposing unstimulated PMNs to supernatant of degranulated PMNs has an effect on their cell surface expression of MPO and PR3. Incubation of unstimulated PMNs with the supernatant of degranulated autologous PMNs for 10 minutes at 4°C resulted in a dose-dependent acquisition of MPO on these cells (Figure 2A-B), suggesting that soluble MPO was able to bind to the cell surface of unstimulated cells. Purified MPO showed a similar binding affinity for unstimulated PMNs (Figure 2C). Addition of autologous serum (up to 50%) to the supernatant of degranulated PMNs only slightly reduced the amount of MPO expressed on PMNs after exposure (Figure 2D). Degranulated PMNs contained 53% ± 8% of the MPO activity as compared with unstimulated cells, and 86.4% ± 4.1% of the MPO activity lost from the intracellular pool

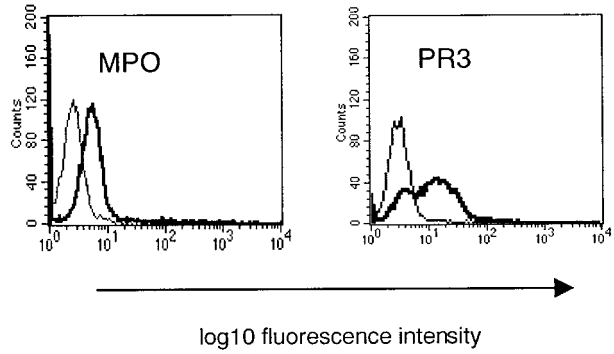

Figure 1. Cell surface expression of MPO and PR3 on unstimulated PMNs and on PMNs stimulated with 10 μmol/L fMLP (FACScan analysis). Typical shifts observed after stimulation are shown (bold curve), indicating expression of MPO and PR3. Similar results were obtained for 4 individual experiments.

could be detected in the supernatant of degranulated PMNs. (Results given ± SEM of 4 independent experiments.)

A small percentage of the MPO activity detected in the supernatant of degranulated PMNs was lost after incubation with autologous PMNs as described in "Materials and methods" (percentage loss 4.8% ± 1.3%, SEM of 4 independent experiments). This loss probably reflects the fraction of MPO binding to the cell surface of bystander PMNs.

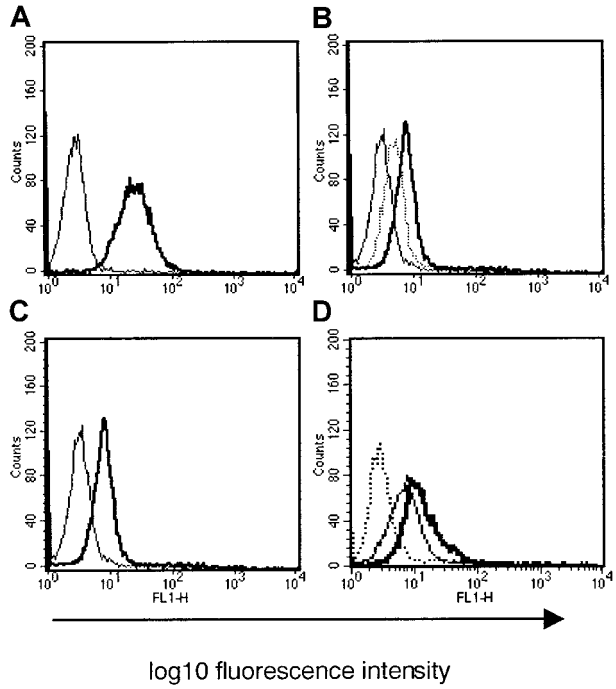

Figure 2. Cell surface expression of MPO on unstimulated PMNs incubated with supernatants containing MPO (FACScan analysis). (A) Expression of MPO after incubation with supernatant containing 600 U/mL MPO (bold curve), as compared with PMNs incubated with buffer (thin curve). (B) Dose-dependent decrease of MPO expression after immunodepletion of MPO in the supernatant from 570 U/mL (bold curve) to 135 U/mL (broken curve) and from 135 U/mL to an MPO concentration below detection limit (thin unbroken curve). (C) Expression of MPO after incubation of PMNs with purified MPO added to buffer at 450 U/mL (bold curve), as compared with PMNs incubated with buffer only (thin curve). (D) Expression of MPO after incubation of PMNs with supernatant containing 600 U/mL MPO in the presence or absence of 50% serum. The bold curve represents the expression level after incubation with supernatant diluted 1:1 with buffer; the thin curve, the expression level after incubation with supernatant diluted 1:1 with serum. The broken curve represents the expression after incubation with buffer. Similar results were obtained for 3 individual experiments.

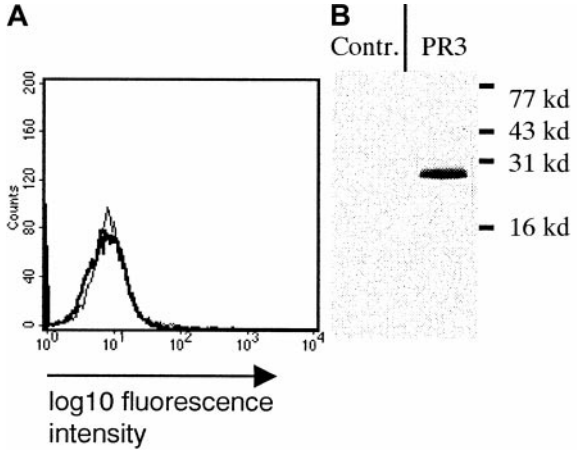

Figure 3. Detection of PR3. (A) Cell surface expression of PR3 on unstimulated PMNs incubated with supernatant from autologous degranulated PMNs (FACScan analysis). The bold curve represents expression of PR3 after incubation; the thin curve represents expression before incubation. (B) PR3 (29 kd) was easily detected in this supernatant by immunoblotting. Similar results were obtained for 3 individual experiments.

By contrast, no significant increase in cell surface expression of PR3 was found on PMNs incubated under the same conditions (Figure 3A), although PR3 was easily detected in these supernatants by Western blotting (Figure 3B). Importantly, PR3 expression on freshly isolated PMNs varied considerably more than that of MPO. This observation is in agreement with the recent report of PR3 expression on the cell surface of circulating PMNs.[31,32]

The expression pattern of MPO versus PR3 was different when PMNs were primed for 10 minutes at 37°C with TNF-α (2 ng/mL). While we were unable to detect a significant increase in MPO expression on the cell surface of primed PMNs, PR3 expression clearly increased (Figure 4). In accordance with this expression pattern is the recently revealed presence of a readily plasma membrane–mobilizable pool of PR3 contained within the secretory vesicles.[31]

Superoxide anion production

We next asked whether MPO that had bound to unstimulated PMNs not only was recognized by anti-MPO antibodies, but whether PMNs would become activated by antibody binding, therefore releasing O_2^- radicals.

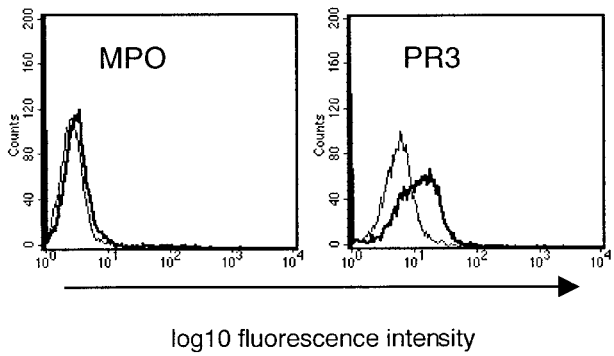

Figure 4. Cell surface expression of MPO and PR3 on PMNs primed with TNF-α (2 ng/mL) for 10 minutes (FACScan analysis). The bold line represents expression after priming; the thin line represents before priming. Increased PR3 but not MPO cell surface expression was detected as shown by 2 typical shifts observed after priming. Similar results were obtained for 4 individual experiments.

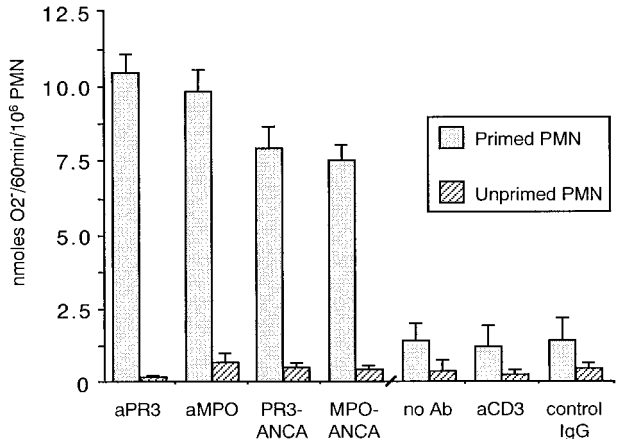

Figure 5. Release of O_2^- radicals by unstimulated and TNF-α primed PMNs. While primed PMNs were responsive to antibodies recognizing PR3 and MPO, unstimulated PMNs were not.

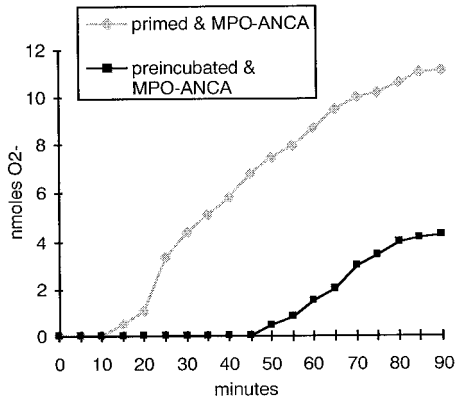

Figure 7. Example of an online measurement of O_2^- release in PMNs primed with TNF-α, as compared with unstimulated PMNs preincubated with supernatant from autologous degranulated PMNs. Release of O_2^- radicals over 90 minutes is faster and greater in primed PMNs incubated with MPO-ANCA than in preincubated unstimulated PMNs exposed to MPO-ANCA. Similar results were obtained for 4 individual experiments.

Control experiments showed that unstimulated PMNs were unreactive and thus did not release significant amounts of O_2^- radicals when incubated with anti-MPO mAb (IgG1), anti-PR3 mAb (IgG1), the control mAb anti-CD3 (IgG1), or normal control human IgG as well as MPO-ANCA$^+$ or PR3-ANCA$^+$ human IgG (Figure 5). As described in the literature, PMNs primed for 10 minutes with TNF-α (2 ng/mL) were found to release significant amounts of O_2^- radicals when incubated with anti-MPO mAb, anti-PR3 mAb, or MPO-ANCA$^+$ or PR3-ANCA$^+$ human IgG. Primed PMNs released only a fraction of O_2^- radicals when incubated with TNF-α alone, with control mAb (anti-CD3 [IgG1]), or with control IgG (Figure 5). In these experiments the release of O_2^- radicals occurred after a mean lag period of 19 minutes ± 6 minutes (± SEM of 4 independent experiments for each condition).

In a last set of control experiments, no significant amount of O_2^- was released when the microtiter plates used to incubate PMNs were constantly shaken, thereby preventing interaction of β-2 integrins with the plate[7] (data not shown).

Having confirmed the reaction pattern of TNF-α–primed and unstimulated PMNs as expected from the literature, unstimulated PMNs were preincubated with the supernatant of degranulated autologous PMNs for 10 minutes at 4°C, washed, and only then tested in the same O_2^- radical release assay. In these pre-exposed, unstimulated PMNs, anti-MPO mAb but not anti-PR3 mAb or control mAb induced a significant release of O_2^- radicals (Figure 6). Incubation of PMNs with purified MPO added to medium at the same concentration as detected in the supernatant of degranulated PMNs also made these PMNs sensitive to anti-MPO mAb. Furthermore, immunodepletion of MPO prevented the activatory effect of anti-MPO mAb. Purified IgG from a patient with MPO-ANCA and a patient with PR3-ANCA provided similar results: While unstimulated PMNs were unreactive to both types of ANCA (Figure 5), pre-exposure of these PMNs to supernatant of degranulated autologous PMNs made them reactive to MPO-ANCA$^+$ but not PR3-ANCA$^+$ IgG (Figure 6). As in the assays where mAbs were tested for their stimulatory activity on PMNs, immunodepletion of MPO in the incubating supernatant reduced PMN responsiveness back to the levels of unstimulated PMNs (Figure 6).

Two additional obvious differences were present between the release of O_2^- radicals by primed PMNs as compared with unstimulated PMNs preincubated with supernatant of degranulated, autologous PMNs. First, TNF-α–primed PMNs released approximately 2 to 3 times more O_2^- radicals than preincubated PMNs (Figure 7). Second, the kinetics of the release was clearly different. In contrast to the release of O_2^- radicals by primed PMNs, where the release of O_2^- radicals started after a mean lag period of 19 minutes, the lag period in unstimulated but preincubated PMNs was significantly longer at 49 ± 13 minutes (± SEM of 4 independent experiments for each condition).

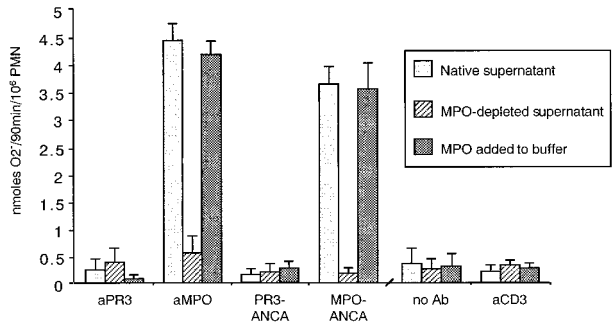

Figure 6. Release of O_2^- radicals by unstimulated PMNs preincubated with native supernatant of autologous degranulated PMNs, MPO-immunodepleted supernatant, or purified MPO added to buffer. In the presence of soluble MPO during preincubation, PMNs became responsive to anti-MPO antibody, resulting in the release of O_2^- radicals.

Discussion

PMN activation causes cell surface expression and degranulation of MPO and PR3. In the present work we analyzed whether exposure of unstimulated PMNs to supernatant of degranulated autologous PMNs affected cell surface expression of MPO and PR3 in a functionally relevant manner. The major findings were that (*a*) MPO but not PR3 expression was significantly enhanced and (*b*) exogenously acquired MPO made unstimulated PMNs responsive to both monoclonal antibody and polyclonal autoantibody against MPO. This change in cell surface expression of MPO versus PR3 is of particular interest because autoantibodies against these 2 proteins are highly specific and sensitive markers of MPA and WG, respectively.[1-5]

From these data it can be suggested that, in MPA, MPO might become accessible to MPO-ANCA independently from both apoptosis and priming. Following release by activated PMNs, soluble

MPO bound to the cell surface of unstimulated PMNs, thereby dispersing reactivity of autoantibody binding. On the same PMNs, no enhanced expression of PR3 was found. Recently the interaction of PR3 with the cell surface has been characterized.[31] Neither high salt concentrations, acidic or basic pH, nor treatment of PMNs with neuraminidase could release PR3 from the cell surface. These data suggested covalent binding of PR3, possibly involving lipid interactions. In contrast, MPO binding is probably largely charge-dependent.[33]

The enhanced expression of MPO on PMNs after exposure to supernatant from degranulated autologous PMNs was found to be functionally relevant because, when bound, MPO made these PMNs responsive to specific antibodies, as shown by the release of oxygen radicals.

Circulating MPO levels are known to be increased during inflammatory reactions,[34-37] and a fraction of the soluble MPO might bind to the cell surface of circulating PMNs. If this occurs in an individual in whom, at the same time, a critical MPO-ANCA level is reached, binding to cell surface–associated MPO might induce the release of O_2^- radicals by these PMNs. Isolated high levels of MPO-ANCA or increased concentrations of MPO alone are known to occur without any evidence of vasculitis,[35-41] while high levels of circulating MPO in the presence of MPO-ANCA correlate with severity of the disease.[42]

It is worthwhile to mention that under such circumstances circulating MPO/anti–MPO-ANCA immune complexes may be formed as well. Nevertheless, from the lack of immune complexes deposited in ANCA-associated vasculitis lesions, it seems unlikely that immune complexes contribute significantly to the pathophysiology of MPA.

The histologic differences in WG and MPA are mainly based on the presence of granulomatous lesions in the former. Marino et al have shown that TNF-α is essential for granulomatous lesions to form,[43] and TNF-α is likely to play an important role as a priming agent in vivo as well. A pool of PR3 mobilizable with priming-range concentrations of inflammatory mediators[31] may suggest that in vivo priming with TNF-α induces the expression of PR3 on the cell surface of PMNs, which thereby become the target of the specific anti-PR3 antibodies. In WG, perpetuation of the inflammation thus might depend on continuous priming of quiescent PMNs by agents such as TNF-α. Under such conditions granulomatous lesions may form. By contrast, circulating soluble MPO might disperse MPO/anti-MPO activation to resting "innocent bystander PMNs," thereby perpetuating inflammation and tissue destruction. The absence of granulomatous lesions may further indicate that MPA is a TNF-α–independent process.

The suggested differences in the pathomechanisms of WG and MPA are not exclusive. For instance, anti-MPO antibodies have been reported in patients with WG, and priming with TNF-α renders PMNs reactive to these antibodies.[7,9,21-23,26] The mechanism of this activation is unclear, and data on the expression of MPO on these anti-MPO–reactive PMNs are inconsistent. Some authors describe an enhanced expression of MPO on primed PMNs,[9,26] while others do not.[7] In our hands priming with TNF-α did not (or at best only slightly so) increase the cell surface expression of MPO. One therefore may speculate that priming induces a barely detectable increase in MPO expression, which, together with additional changes known to be induced by TNF-α,[7] might be sufficient to allow anti-MPO antibodies to activate these cells.

However, knowing that soluble MPO binds to unstimulated PMNs, thereby dispersing the target of pathogenic autoantibodies in a functionally relevant manner, may help to develop disease-specific treatments. For example, heparin, a polyanionic molecule, has been shown to interact strongly with MPO[44] and might interfere with MPO binding to quiescent PMNs.

Acknowledgments

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