Crosslinking of ANCA-Antigens Stimulates Superoxide Release by Human Neutrophils

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Abstract. Anti-neutrophil cytoplasmic antibodies (ANCA) activate primed human polymorphonuclear neutrophils (PMN) in vitro, resulting in a respiratory burst and degranulation. In this study, the hypotheses that the initiation of this process requires engagement of the F(ab')2 portion of ANCA, and that crosslinking of ANCA target antigens is necessary to trigger superoxide (O2−) release, were explored. It is speculated that Fcγ receptor engagement is a modulator of ANCA-mediated activation. Flow cytometry demonstrated that intact human ANCA immunoglobulin (Ig), their corresponding F(ab')2 and Fab fragments, as well as a murine monoclonal to human PR3 and its F(ab')2 fragment, bind to ANCA antigens on the surface of PMN primed with tumor necrosis factor (TNF) α. Intact Ig of patients with PR3-ANCA or with MPO-ANCA stimulate O2− release from TNFα-primed normal PMN (2.6 ± 3.57 to 15.3 ± 7.39 nmol O2−/2.5 × 106 PMN/30 min). Corresponding F(ab')2 fragments result in similar O2− production (10.2 ± 4.34 to 36.9 nmol) in a dose-dependent manner. ANCA Fab fragments do not stimulate O2− generation until these fragments are crosslinked with F(ab')2 of goat anti-human Ig F(ab')2, or when fragments are biotinylated and crosslinked with avidin. In contrast with these human autoantibody data, a mouse monoclonal anti-human PR3 antibody (25.7 ± 8.55 nmol O2−), but not its corresponding F(ab')2 fragment, activates TNFα-treated human PMN. When the FcγIIa receptors were blocked, superoxide production was reduced by 33% using human PR3-ANCA Ig (P < 0.05). In conclusion, PMN activation by ANCA occurs when intact ANCA or ANCA F(ab')2 fragments crosslink target antigens on the neutrophil cell surface. ANCA F(ab')2 fragments result in PMN activation when crosslinked by secondary reagents. (J Am Soc Nephrol 8: 386–394, 1997)

Anti-neutrophil cytoplasmic antibodies (ANCA) are found in microscopic polyangiitis, Wegener’s granulomatosis, Churg-Strauss syndrome, and pauci-immune necrotizing crescentic glomerulonephritis (NCGN) (1,2). Two principal types of ANCA are directed against proteinase 3 (PR3): cytoplasmic ANCA (3-5) and myeloperoxidase (MPO) perinuclear ANCA (6). Both target antigens are localized in the azurophilic granules of PMN and monocytes and play an important role in the effector arm of the immune system.

ANCA were first reported in 1982 by Davies (7). Since then, many investigators have described the association of ANCA with inflammatory vascular diseases and have proposed that ANCA may participate in the pathogenesis of these diseases (1,2).

The results of the intensive investigations of recent years have shed light on the interactions between ANCA and their target antigens on granulocytes. PR3-ANCA and MPO-ANCA are able to activate TNFα-treated PMN, causing respiratory burst and degranulation (8-13). These effector functions of PMN damage endothelial cells (14,15) and may participate in the reactions leading to vascular inflammation.

The exact nature of the interaction between ANCA antibody and neutrophils remains unclear. Priming results in the translocation of target antigens from the cytoplasm to the extracellular membrane, where the antigen becomes accessible to circulating antibodies (8,9). ANCA possibly interact with the neutrophil surface by recognizing and binding to the presented epitope through the F(ab')2 portion of the molecule. PMN also express Fcγ receptors (reviewed in References 16 and 17). An engagement of these receptors by the Fc portion of the ANCA immunoglobulin (Ig) G—presumably after binding to the target antigen—is considered to be an important condition (10) or even a condition sine qua non (11,13) for the induction of activation by ANCA.

The published reports have stimulated a controversy about which part of the ANCA molecule is responsible for the initiation of the neutrophil activation (e.g., FcγIIa receptor, or F(ab')2, or both). Additional confusion in this arena arises by the use in some experiments of monoclonal mouse antibodies to ANCA antigens and, in others, of human autoantibody preparations.

In this study, we explored the hypothesis that F(ab')2 fragments activate PMN by crosslinking target antigens using human MPO-ANCA and human PR3-ANCA, as well as a murine monoclonal antibody against human PR3. We applied intact ANCA immunoglobulins, F(ab')2 fragments, and Fab fragments to PMN, and learned that crosslinking of their target autoantigens on the cell surface results in PMN activation when human reagents are used. When the FcγIIa receptors...
were blocked with the appropriate monoclonal antibody, a modulation of the response was seen. When the same studies were performed using a mouse monoclonal antibody to proteinase 3, F(ab')2-triggered activation did not occur.

Materials and Methods

Isolation of Human Neutrophils

Polymorphonuclear neutrophils (PMN) of healthy human volunteers were isolated from heparinized whole blood by red blood cell sedimentation with plasma gel (Cellular Products Inc., Buffalo, NY), followed by Ficoll-Hypaque (Sigma, St. Louis, MO) density gradient centrifugation. Erythrocytes were lysed by incubation with hypotonic saline for 15 s. PMN were spun down (200 g, 7 min) and reconstituted in HBSS with calcium and magnesium (HBSS++; University of North Carolina, Cancer Center Tissue Culture Facility, Chapel Hill, NC). The cell viability was detected in every cell preparation by trypan blue dye exclusion and found to be greater than 99%. The percentage of PMN in the suspension was >95% by a Wright-Giemsa staining and by light microscopy.

Preparation of Immunoglobulins and Immunoglobulin Fragments

Human PR3-ANCA sera from Patients KM and RR (with biopsy-proven Wegener's granulomatosis), MPO-ANCA sera from Patient AA with microscopic polyangiitis, and Patient TR with Wegener's granulomatosis (both diagnoses biopsy-proven), and sera from three healthy individuals were used for the isolation of human immunoglobulins. After treating the sera with equal volumes of saturated ammonium sulfate, the samples were dialyzed overnight at 4°C (Spectra/Por membrane, molecular weight cutoff of 50,000; Spectrum Medical Industries, Inc., Laguna Hills, CA) against 20 mM sodium phosphate buffer, pH 7.0 (binding buffer). The solutions were filtered through a 0.2-μm syringe filter (Gelman Sciences, Ann Arbor, MI) and applied to a protein G affinity column. The column was prepared from protein G Sepharose 4 Fast Flow (Pharmacia, Uppsala, Sweden) connected to FPLC (Pharmacia). Bound immunoglobulins were eluted with 0.1 M glycine-HCl buffer, pH 2.75 (elution buffer). After the antibodies emerged from the column, the pH was immediately adjusted to pH 7.0 by using 1 M Tris-HCl, pH 9.0.

A mouse monoclonal anti-human PR3 antibody (IgGlκ) previously developed in our laboratory was concentrated from tissue culture supernatant by using dia-flo ultrafilter YM 100 (Amicon, Beverly, MA) and purified by protein G column chromatography. A mouse monoclonal IgGlκ (15 μg/mL) produced in a hybridoma cell line (MOPC21) (Sigma) and served as isotype-matched control for the experiments with the murine anti-PR3 antibodies.

F(ab')2 of human ANCA, mouse monoclonal anti-PR3 and normal human immunoglobulins were prepared as described by Lamoyi (18). In brief, the purified immunoglobulins were dialyzed overnight against 0.1 M sodium acetate (pH 7.0). The pH of the antibody-containing suspension (protein concentration, 2.0 mg/mL) was adjusted at 4.0 for the mouse monoclonal or at 4.25 for the human immunoglobulins. Pepsin (Worthington Biochemical Corp., Freehold, NJ) was added to maintain an enzyme/antibody ratio of 1:33. The mixture was incubated on a rotator at 37°C for 7.5 h. To stop the reaction, 1 N NaOH was added to raise the pH to 8.0. The F(ab')2-containing digest of the mouse IgG, one human ANCA, and one normal human control were dialyzed against binding buffer for the protein A column (Pierce, Rockford, IL) and chromatographed on a protein A column (Pierce). Bound protein was removed using protein A elution buffer (Pierce). Three other ANCA-F(ab')2 and two normal F(ab')2 were dialyzed against 20 mM sodium phosphate, pH 7.0, and were purified by protein G affinity column as described above. The samples were repetitively run over the absorption column (2–4 x) until no remaining elution peak was detectable.

Fab of human ANCA were generated by an Immunopure Fab Preparation Kit (Pierce). Papain digestion was performed at 37°C for 9 h, and the solution was dialyzed against protein A–binding buffer (Pierce). Contaminating intact immunoglobulins and intact Fab parts were removed by the protein A affinity column. To achieve a highly purified Fab preparation, samples were run repetitively (two to three times) over the column until no elution peak was detectable. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) chromatography demonstrated the purity of intact immunoglobulin, F(ab')2, and Fab preparations.

Human ANCA, as well as the murine monoclonal anti-PR3 antibody and all corresponding F(ab')2 fragments, were tested on alcohol-fixed neutrophils and showed the appropriate C-ANCA or P-ANCA pattern on indirect immunofluorescence, indicating the ability to recognize and bind the target antigens. Intact C-ANCA and P-ANCA were further tested for reactivity to purified proteinase 3 or myeloperoxidase by standard clinical ELISA.

The protein concentrations of the antibodies and their fragments were measured using the Coomassie protein assay (Pierce). All immunoglobulin preparations were found to be negative for endotoxin when a Limulus amoebocyte lysate assay with a sensitivity of 0.1 ng/mL was used (Biowhittaker, Walkersville, MA).

ELISA for the Assessment of the Purity of ANCA

All commercial antibodies used in this ELISA were purchased from Jackson ImmunoResearch Lab, West Grove, PA. Microtiter plates (Costar, Cambridge, MA) were coated with 0.75-μg/well affinity-purified goat anti-human IgG (Fc fragment-specific). The antibody fragments were diluted in 100 μL coating buffer (50 mM sodium acetate, 100 mM sodium chloride, pH 6.0). Remaining binding sites were blocked with 200 μL/well blocking buffer (phosphate-buffered saline [PBS]/0.05% Tween/0.5% goat serum). In a dose-dependent fashion, 1 ng to 5 μg of Chromo Pure human IgG or Fab fragments, diluted in blocking buffer, were added to duplicate wells. Simultaneously, duplicate samples of ANCA F(ab')2 and ANCA Fab preparations at a concentration of 1 μg/well were evaluated. After incubating for 1 h at room temperature, 100 μL/well of alkaline phosphatase–conjugated affinity-purified mouse anti-human IgG (Fcγ fragment specific, 1:20,000 dilution) was added. Each step was followed by three washings with PBS/0.05% Tween. One hundred micromgrams/well of p-nitrophenyl phosphate disodium (Sigma) was added. The OD was measured at 405 nm after 1 h. The OD of F(ab')2 and Fab preparations were compared with the standard curve, revealing that 1-μg ANCA F(ab')2 fragments contain 1 to 2 ng Fab fragments and 1-μg Fab fragments contain less than 1 ng Fab fragments.

Flow Cytometry Studies

Flow cytometry was used to evaluate the PMN binding properties of intact human ANCA immunoglobulins and the corresponding F(ab')2 and Fab preparations, and of intact immunoglobulins and F(ab')2 from normal human subjects. Binding of an intact murine monoclonal antibody to human PR3 and its F(ab')2 fragment was also studied by flow cytometry.

Antibodies of interest were biotinylated with the N-hydroxysuccinimide ester form of biotin coupled to an extended spacer arm (NHS-LC-Biotin) (Pierce). Biotin incorporation was detected with a 2-(4-
hdroxyazobenzene) benzoic acid (HABA) test. The ratio of biotin to immunoglobulin or immunoglobulin fragment was as follows: PR3-ANCA Ig, 6; PR3-ANCA F(ab')2, 4.8; PR3-ANCA Fab, 4.3; normal human Ig, 3.8; and normal human F(ab')2, 6.

2.5 × 10^6 PMN were treated with 1.33 ng/mL TNFα for 15 min at 37°C and then incubated with 50 μg biotinylated antibodies in a total volume of 500 μL HBSS++ for 20 min at 4°C. Unbound antibodies were removed by washing with the cells with 1.5 mL cold PBS (University of North Carolina, Cancer Center Tissue Culture Facility, Chapel Hill, NC). PMN were pelleted at 200 g for 5 min at 4°C, resuspended in 200 μL (2 μg) fluorescein-isothiocyanate (FITC)-conjugated avidin (Pierce) and incubated for 20 min on ice. PMN were washed in 1.5 mL ice-cold PBS and reconstituted in 1 mL 1% paraformaldehyde (Fluka, Ronkonkoma, NY).

Binding of intact monoclonal antibody to human PR3 (4 μg/mL) and its F(ab')2 fragment (4 μg/mL) was studied by immunofluorescence staining using 20 μg/mL of FITC-conjugated goat F(ab')2 fragment to mouse IgG (Cappel) as secondary antibody. MOPC-21 (4 μg/mL), a mouse IgG1 fragment to mouse IgG (Cappel), served as an isotype control. Flow cytometry was performed on the same day by using a FACScan (Becton Dickinson, Mountain View, CA), and 20,000 events per sample were collected.

**Superoxide Generation Assay**

Superoxide was measured using the standard assay of superoxide dismutase (Sigma)-inhibitable reduction of ferricytochrome C (Sigma) (19,20). PMN were pretreated with 5 μg/mL cytochalasin B (Sigma) for 15 min at 4°C. 2.5 × 10^6 cells were treated with 1 ng/mL TNFα (Genzyme Corp., Boston, MA) (experiments with monoclonal antibodies) or 1.3 ng/mL (experiments with ANCA) for 15 min at 37°C before Ig, F(ab')2, Fab fragments, or HBSS++ were added. The final assay volume was 500 μL with a ferricytochrome C concentration at 50 μM and a cell concentration at 2.5 × 10^6/mL. The mixture was incubated for 30 min at 37°C. The reaction was stopped when cells were placed on ice for 3 min and pelleted down in a refrigerated centrifuge at 4°C. The absorption of the supernatant of samples with and without 125 μg/mL superoxide dismutase (SOD) was detected at 550 nm, using a Microplate Autoreader (BioTek Instruments). Sodium dithionite (Fisher, Fair Lawn, NJ) was added to the experimental tubes containing no SOD, to fully reduce all residual ferricytochrome C, and the results were expressed in mmol O2-7/2.5 × 10^6 cells/30 min (21). All experiments were performed in duplicate. The baseline activity of TNFα-treated PMN was determined in every experiment and was factored for each condition.

**Crosslinking Studies**

After 2.5 × 10^6 PMN were incubated with TNFα for 15 min at 37°C, ANCA Fab fragments (125 μg/mL) or F(ab')2 fragments of the murine monoclonal anti-PR3 antibody (4 μg/mL) were added for 10 min, washed with 1.5 mL cold HBSS++, and then incubated for 30 min at 37°C with F(ab')2 fragments of a goat anti-human (15 μg/mL) or anti-mouse (4 μg/mL) IgG F(ab')2 (Cappel, Organon Teknika Corp., Durham, NC). In two crosslinking experiments, biotinylated ANCA Fab fragments (125 μg/mL) (Patient R.R.) were used and exposed to avidin (15 μg/mL) (Pierce). The biotinylation procedure is described below. Superoxide production was measured as previously described. The baseline activation for all of these experiments was washing of the TNFα-treated PMN exposed to the secondary antibody alone.

**FcγIIa Receptor Blockade Studies**

TNFα-treated PMN were simultaneously incubated with saturating doses of intact IgG2b monoclonal anti-FcγIIa receptor antibody or, in other studies, with the Fab fragment of anti-FcγIIa receptor antibody (10 μg/mL). After 15 min, human PR3-ANCA (125 μg/mL) were added, and superoxide production detection was continued as described above. A time course of the effect of FcγIIa blockade on superoxide release to PR3-ANCA (Patient K.M.) was established in two experiments, each performed in duplicate. TNFα-treated neutrophils (1.33 ng/mL) were incubated either with Fab fragments of anti-FcγIIa receptor antibody (10 μg/mL) or with an equal volume of HBSS++ and consecutively exposed to 125 μg/mL PR3-ANCA. Samples were incubated at 37°C; the reaction was stopped after 5, 15, 30, and 60 min, and the amount of superoxide was determined as described above. In another series of experiments, different concentrations of PR3-ANCA (50, 75, 125, and 175 μg/mL) were applied simultaneously using a constant concentration of FcγIIa receptor blocker (10 μg/mL).

**Statistical Analyses**

Wilcoxon’s rank-sum test was used to test differences in distributions between groups. For paired groups, the centered signed rank statistic was used to test if the differences were unequal to 0.

**Results**

**Binding of ANCA, Monoclonal Antibody to Human PR3 and Their Fragments to Human Neutrophils Detected by Flow Cytometry**

Activation of PMN by ANCA Ig, F(ab')2, or Fab is dependent on binding to target antigens expressed on the cell surface. We evaluated the binding properties of intact human ANCA and their digestion products by indirect immunofluorescence microscopy on normal human neutrophils. Both MPO-ANCA and PR3-ANCA and their digestion products provided the expected P-ANCA or C-ANCA staining pattern. Normal immunoglobulin did not stain alcohol-fixed human neutrophils. By flow cytometry, biotinylated intact PR3-ANCA, ANCA F(ab')2, and ANCA Fab, as well as normal Ig and their F(ab')2 fragments, were examined. Representative results from one of three experiments on neutrophils of different donors are depicted in Figure 1A, showing that intact biotinylated PR3-ANCA, as well as the corresponding F(ab')2 and Fab fragments, bound to the surface of TNFα-treated PMN. Removal of the Fc-portion resulted in diminished binding. Similar binding of ANCA F(ab')2 and ANCA Fab fragments was observed. Although intact normal human immunoglobulins bound to the surface of TNFα-treated PMN by FACS, normal F(ab')2 did not bind to these cells. Normal immunoglobulin probably bind to TNFα-treated PMN via the Fc fragment.

Binding of intact murine monoclonal antibody to PR3 and its F(ab')2 fragment to the surface of TNFα-treated human neutrophils was demonstrated by flow cytometry. In three experiments carried out on neutrophils of three different donors, comparable binding of intact monoclonal antibody to PR3 and
Figure 1. Flow cytometry studies demonstrate binding properties of biotinylated human PR3-ANCA, PR3 Fab', and PR3 Fab fragments compared with Ig and F(ab')$_2$ fragments from a healthy person (A). Binding of intact monoclonal antibody to human PR3 and its corresponding F(ab')$_2$ fragment are shown in Panel B. The region of interest was set to include less than 5% of the control cells not treated with a primary antibody. The percentage of cells with increased fluorescence is indicated for each condition.
its corresponding F(ab')$_2$ fragment was detected. Results of a typical experiment are shown in Figure 1B.

**Initiation of PMN Activation Using Intact PR3-Anca, Mpo-ANCA, F(ab')$_2$, or Fab Fragments**

Once we were confident that our preparations bound to neutrophils, we assessed the ability of these reagents to activate neutrophils. Purified immunoglobulins from Patients K.M. and R.R. with PR3-ANCA and Patients A.A. and T.R. with MPO-ANCA, and their corresponding F(ab')$_2$- and Fab preparations, were used to stimulate superoxide production (Table 1). O$_2^-$ generation by human PMN in the presence of 1.33 ng/mL TNFα (3.4 ± 1.83 nmol O$_2^-$/2.5 × 10$^6$ PMN/45 min; N = 34) or in the absence of TNFα (2.3 ± 1.10 nmol O$_2^-$/2.5 × 10$^6$ PMN/45 min; N = 34) was determined in each experiment. TNFα treatment of PMN was used as a baseline. Intact ANCA immunoglobulin preparations from different patients resulted in different amounts of O$_2^-$ production (Table 1), triggering less O$_2^-$ release than 50 ng/mL PMA (44.8 ± 2.53 nmol O$_2^-$/2.5 × 10$^6$ PMN/30 min).

F(ab')$_2$ preparations from normal controls did not activate PMN O$_2^-$, whereas ANCA F(ab')$_2$ initiated a significant O$_2^-$ production (Table 1). O$_2^-$ release by F(ab')$_2$ fragments of ANCA was dependent on treating PMN with TNFα. Without TNFα, neither intact PR3- or MPO-ANCA containing preparations nor F(ab')$_2$ caused "un-primed" PMN to produce O$_2^-$ (data not shown). For comparative purposes, intact PR3-ANCA and F(ab')$_2$ from Patient R.R. were titrated in a dose-response curve in two simultaneous experiments (Figure 2). These results demonstrate that ANCA F(ab')$_2$ initiate a dose-dependent production of O$_2^-$.

ANCA Fab fragments from all four patients did not initiate neutrophil activation. The differences in O$_2^-$ production between ANCA F(ab')$_2$ and Fab fragments were statistically significant, with P < 0.05 (Table 1). In contrast, when Fab fragments of PR3- or MPO-ANCA were crosslinked by anti-F(ab')$_2$ of a goat anti-human F(ab')$_2$, or when biotinylated Fab fragments were crosslinked by avidin, O$_2^-$ was produced in 18 of 19 experiments (Figure 3) and reached statistical significance in three of the four patients (P < 0.05).

The degree of activation seen with ANCA and their corresponding fragments were highly dependent on the source of the donor PMN. This result is similar to that previously observed (13). For comparative purposes, we simultaneously tested each ANCA, F(ab')$_2$, or Fab fragment with the same donor PMN. As seen in Figure 4, the amounts of O$_2^-$ released by ANCA

![Figure 2](image-url). A comparison of the dose-response curve of superoxide release from TNFα-primed normal human PMN treated with 5, 50, 125, and 250 µg/mL intact PR3-ANCA and 0.5, 1, 5, 10, 15, 25, 50, and 125 µg/mL Fab'$_2$ fragments (Patient R.R.).

**Table 1.** Oxygen superoxide production from TNFα-treated human PMN with human ANCA, mouse anti-human PR3 antibodies, and corresponding antibody fragments

<table>
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<tr>
<th>Stimulus (patient)</th>
<th>Intact Ig (N) (P Value)</th>
<th>Fab'$_2$ Fragment (N) (P Value)</th>
<th>Fab Fragment (N) (P Value)</th>
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<tr>
<td>PR3-ANCA (K.M.)</td>
<td>15.3 ± 7.39 (N = 21)</td>
<td>10.2 ± 4.34 (N = 9)</td>
<td>-1.4 ± 2.47 (N = 4)</td>
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<td>PR3-ANCA (R.R.)</td>
<td>8.4 ± 4.87 (N = 6)</td>
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<td>MPO-ANCA (A.A.)</td>
<td>10.2 ± 6.70 (N = 9)</td>
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<td>(NS)</td>
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<td>MPO-ANCA (T.R.)</td>
<td>2.6 ± 3.57 (N = 9)</td>
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<tr>
<td>Human controls</td>
<td>1.0 ± 1.62 (N = 9)</td>
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<td>Mouse PR3-ANCA</td>
<td>25.7 ± 8.55 (N = 11)</td>
<td>-0.01 ± 1.31 (N = 6)</td>
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<tr>
<td>Control IgG$_{1K}$</td>
<td>0.97 ± 0.59 (N = 4)</td>
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* 125 µg/mL human antibodies, 4 µg/mL murine monoclonal anti-PR3, and the same amount of corresponding antibody fragment were used. O$_2^-$ release (nmol O$_2^-$/2.5 × 10$^6$ PMN/30 min) was compared between:

$^b$ intact ANCA and control antibodies;

$^c$ ANCA-Fab$_2$ and control Fab$_2$;

$^d$ ANCA-Fab$_2$ and ANCA-Fab. NS, not statistically significant; ND, not done.
F(ab')2 were at least equivalent to intact immunoglobulin, and Fab fragments did not cause O2− production from PMN.

**Initiation of Neutrophil Activation Using Murine Monoclonal PR3-ANCA or Its F(ab')2 Fragment**

Previous studies of ANCA activation used murine monoclonals as surrogates for human ANCA. We used intact mouse monoclonal IgG1κ anti-PR3 antibodies and F(ab')2 fragments to induce a respiratory burst in TNFa-treated PMN. To select the appropriate doses, we applied different amounts of murine monoclonal antibody. Based on these results, 2, 4, and 15 μg/mL were chosen for comparative experiments with intact IgG and F(ab')2 fragments. In contrast to intact murine anti-PR3 antibodies, the F(ab')2 fragments failed to initiate a respiratory burst at any concentration (Figure 5). TNFa-treated PMN produced 0.9 ± 0.59 nmol O2− when incubated with 15 μg/mL MOPC IgG1κ.

Attempts at crosslinking the F(ab')2 of the murine monoclonal anti-PR3 antibody using a F(ab')2 of a goat anti-human IgG (F(ab')2) antibody (N = 5) did not lead to a measurable O2− production: −0.1 ± 1.27 nmol with 15 μg/mL F(ab')2 alone and 0.2 ± 0.96 after exposure to the secondary crosslinking antibody.

**Figure 3.** (A through D) Superoxide release from primed human PMN incubated with 125 μg/mL of human ANCA Fab without and with exposure to the crosslinking F(ab')2 of a goat anti-human IgG (F(ab')2). In two experiments (Panel D), biotinylated ANCA Fab were crosslinked with avidin-FITC (+) instead of a secondary antibody.

**Engagement of the Fcγ II Receptor in ANCA-Induced PMN Activation**

Several investigators have suggested that the FcγIIa receptor plays a critical role in ANCA activation. We were interested in whether blockade of this receptor modulates or prevents TNFa-treated neutrophil activation by intact ANCA. Whole IgG of monoclonal anti FcγII receptor antibody did not induce superoxide production using un-primed PMN (1.1 ± 2.07 nmol O2−/2.5 × 10^6 PMN/30 min; N = 3), whereas TNFa-treated PMN incubated with intact anti-FcγII receptor antibody resulted in 3.5 ± 0.75 nmol O2− and with Fab fragments of intact anti-FcγII receptor antibody produced 2.6 ± 0.87 nmol O2−. The differences in superoxide release between intact antibody and its Fab fragment were not statistically significant.

We established a time course of superoxide release of primed neutrophils exposed to 125 μg/mL PR3-ANCA with and without blockade of the FcγIIa receptor (N = 2) (Figure 5). The results demonstrate an inhibitory effect of FcγIIa blockade on superoxide release occurring as early as 5 min after stimulation and apparently at all time points.

To determine the extent of inhibition, a total of seven experiments was performed, with 30 min of incubation with intact human PR3-ANCA in the absence or presence of FcγIIa.
Discussion

This study improves our understanding about the nature of the interaction between ANCA and their target antigens resulting in PMN activation. It has been shown previously that exposure of primed human PMN to ANCA results in vitro in superoxide release and degranulation (8–13). Our results are in agreement with these observations and show that human ANCA trigger the release of superoxide in a dose-dependent manner. Controversy exists as to what portion of the ANCA molecule initiates the process of activation. Fcγ receptor-mediated leukocyte activation processes are well described. Whether F(ab’)2 or even Fab fragments of Ig can activate leukocytes is uncertain. Our goal was to identify which part of the human ANCA molecule is responsible for the initiation of neutrophil activation. We approached this goal by studying binding and activating properties of intact ANCA Ig, as well as ANCA F(ab’)2 and Fab fragments. We explored the influence of the FcγIIa receptor on the release of reactive oxygen species. It has been suggested that this receptor class participates in the induction of ANCA-mediated respiratory burst (10) or even provides a condition sine qua non (11,13). Finally, we compared the results of PMN activation by human ANCA to murine monoclonal anti-human PR3-antibody.

The priming process, here simulated by incubation of PMN with TNFα, results in translocation of PR3 and MPO from the primary granules to the surface membrane (9). This expression is a necessary condition for activation of PMN by ANCA (8,10,11). In fact, PMN from patients with Wegener’s granulomatosis have ANCA antigen on their surface (10). Using flow cytometry, we analyzed the binding properties of human ANCA and their corresponding F(ab’)2 and Fab fragments. Our results indicate the binding of intact human ANCA and intact murine monoclonal against human PR3 to primed PMN. This finding is comparable with other reports (8,10,11). ANCA F(ab’)2 and ANCA Fab, as well as F(ab’)2, of a murine monoclonal against PR3 bound to the cell surface. Intact ANCA Ig bound to PMN to a greater degree than either F(ab’)2 or Fab fragments, suggesting some effect of Fc fragment engagement. PMN express all three classes of Fcγ receptors: FcγRII and III are expressed constitutively and FcγRI after incubation with interferon γ. Crosslinking Fcγ receptors mediates effector functions, including a respiratory burst, degranulation, and
phagocytosis (reviewed in References 16 and 17). That Fc engagement of human immunoglobulins occurs, yet does not cause PMN activation, is supported by the observation that intact normal human antibodies bind to PMN by FACS but do not result in $O_2^-$ production. Although significant binding of the intact normal human immunoglobulins was detected by FACS, a normal human F(ab')$_2$ preparation that did not contain Fc fragment contamination did not bind to PMN. Thus, intact ANCA Ig, as well as normal human Ig, engage Fc receptors. Despite binding to primed PMN, normal human immunoglobulin do not activate PMN.

Our data demonstrate that human ANCA F(ab')$_2$ activate TNFα-primed PMN in a dose-dependent manner. Stimulation of superoxide release of primed PMN by one human MPO-ANCA F(ab')$_2$ was shown in our laboratory as early as 1990 (8) and has been confirmed by Keogan et al. (12) using MPO-ANCA and PR3-ANCA. In contrast, Mulder et al. (11) and Remaux et al. (13) detected no superoxide production by human PMN in response to F(ab')$_2$ fragments of human ANCA (11,13), heterologous anti-lactoferrin antibodies (11), and monoclonal antibodies to MPO and PR3 (13). These disparities may be a consequence of differences in types of antibodies used: human versus mouse, and MPO-ANCA and PR3-ANCA versus anti-lactoferrin antibodies. That F(ab')$_2$ fragments, and not only intact immunoglobulins, can activate leukocytes was demonstrated by Ravid as early as 1978 (22). A biological effect—mitogenic activity—was induced using F(ab')$_2$ antibody fragments directed against antigens grafted onto lymphocytes. In Ravid's studies and in those by Shen (23), F(ab')$_2$ fragments were superior activators when compared with the intact antibody.

In our experiments, Fab fragments of human MPO- and PR3 ANCA did not activate PMN. When bound ANCA-Fab were exposed to a crosslinking agent such as an F(ab')$_2$ of a goat anti-human IgG (F(ab')$_2$), a significant increase in $O_2^-$ release was found. Similarly, biotinylated ANCA-Fab crosslinked with avidin produced $O_2^-$ (24). These studies demonstrate that ANCA-induced neutrophil activation is dependent on crosslinking of surface molecules. This principle has other examples in the field of leukocyte activation. Crosslinking of target antigens, not only ligand binding, is an essential step in leukocyte activation with such membrane proteins as CD43 (24) and CD 59 (25) on PMN, CD53 on B-lymphocytes (26), and CD45 on natural killer cells (23). The Fcγ receptor requires crosslinking to induce neutrophil activation (27,28).

Several investigators have contended that ANCA activate leukocytes through a FcγIa receptor-dependent mechanism and that F(ab')$_2$ do not play a role (10,11,13). However, our data indicate that the Fcγ receptor is not necessary for the induction of the response. To evaluate any modulating influence of the FcγIa receptor on ANCA-induced PMN activation, we blocked this receptor class by a monoclonal antibody. This pretreatment resulted in a decrease of $O_2^-$ production by 33% when human PR3-ANCA were used. In other studies of human ANCA-induced PMN activation, it has been shown that FcγIII receptor blockade does not inhibit superoxide release (11,13). The studies by Porges et al. did not reveal any competition in binding between antibodies blocking the ligand binding site of the FcγIIIb receptor and monoclonal antibodies to PR3 or MPO. Porges et al. reported that FcγIIa receptor blockade resulted in a 27% decrease in respiratory burst, using murine monoclonal PR3- and MPO-ANCA. The influence was dependent on the high or low responder allelic phenotype of the FcγIIa receptor. Human ANCA autoantibodies were not studied (10).

In contrast to these findings and to our own data, Mulder et al. (11) and Remaux et al. (13) showed that respiratory burst was abrogated by FcγIa receptor blockade. Mulder used heterologous anti-lactoferrin antibody, one human lactoferrin-ANCA, and one MPO-ANCA, whereas Reumaux employed a monoclonal antibody to PR3 and to MPO. The antibodies applied in the latter two studies resulted in a rather weak stimulation, compared with our data. Although no definitive explanation can be given for the discrepancy to our own results and to the studies by Porges et al. (10), it is conceivable that FcγIa receptor blockade is more effective when only a weak activation is triggered.

Murine monoclonal anti-protease 3 antibodies do not behave in a manner analogous to human ANCA. Mouse monoclonal antibodies have been used to activate leukocytes as surrogates for human autoantibodies. A strong dose-dependent activation was seen when TNFα-treated PMN were incubated with intact monoclonal anti-PR3 IgG. In contrast, F(ab')$_2$ fragments bound but did not induce superoxide release in PMN. Attempts at crosslinking the F(ab')$_2$ fragments of this monoclonal anti-human PR3 by a F(ab')$_2$ of a goat anti-mouse IgG (F(ab')$_2$) failed to induce respiratory burst. We are not able to explain the difference between human ANCA and at least one mouse monoclonal antibody against human PR3. Perhaps the differences are related to differences in epitope specificity that may alter crosslinking of the target antigen.

In conclusion, our findings demonstrate that neutrophil activation by human ANCA is dependent on the expression of the target antigens on the cell membrane. Although ANCA binding is necessary for activation, it is not sufficient for activation. Only antibody binding in concert with crosslinking of the expressed target antigens triggers the release of reactive oxygen species. This process does not require the Fcγ receptors, although neutrophil activation by intact ANCA is modulated by the FcγIa receptor engagement. The observations offer further support to the hypothesis that ANCA are directly involved in the pathogenesis of necrotizing small-vessel vasculitis.

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