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# Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals *in vitro*

**(vasculitis/glomerulonephritis/immunopathogenesis)**

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**ABSTRACT** **Anti-neutrophil cytoplasmic autoantibodies (ANCA) are in the circulation of most patients with pauci-immune necrotizing vasculitis and pauci-immune crescentic glomerulonephritis. The current study demonstrates an effect of these autoantibodies on neutrophil function *in vitro*. ANCA cause normal human neutrophils to undergo an oxidative burst and degranulate. Both ANCA phenotypes (i.e., cytoplasmic-pattern ANCA and myeloperoxidase-specific ANCA) induce neutrophil activation. ANCA sera and purified immunoglobulins significantly increase the release of reactive oxygen species when compared with controls. ANCA, in a dose-dependent manner, induce the release of primary granule contents. These effects are markedly enhanced by priming neutrophils with tumor necrosis factor. Flow cytometry studies demonstrate the presence of myeloperoxidase on the surface of neutrophils after cytokine priming, indicating that primed neutrophils have ANCA antigens at their surfaces to interact with ANCA. These observations suggest an *in vivo* pathogenetic role for ANCA. We propose that, in patients with necrotizing vasculitis, ANCA-induced release of toxic oxygen radicals and noxious granule enzymes from cytokine-primed neutrophils could be mediating vascular inflammation.**

Systemic necrotizing vasculitis and crescentic glomerulonephritis can be categorized on the basis of three immunohistologic patterns of vascular immunoglobulin deposition: (*i*) granular deposition indicative of immune complex-mediated disease, (*ii*) linear deposition indicative of anti-basement membrane antibody-mediated disease, and (*iii*) no or scanty deposition, also referred to as pauci-immune disease (1). This third category is the most common (1–4) and includes Wegener granulomatosis and most polyarteritis nodosa. Therefore, one of the most perplexing issues with respect to systemic necrotizing vasculitis and crescentic glomerulonephritis is the pathogenesis of vascular inflammation in the absence of immunoglobulin deposition in vessel walls.

Anti-neutrophil cytoplasmic autoantibodies (ANCA) are in the serum of ≈80% of patients with pauci-immune necrotizing vasculitis (1). The vascular lesions form a continuum from renal-limited necrotizing and crescentic glomerulonephritis to systemic vasculitis (1, 5–15). ANCA react with constituents of neutrophil primary granules and monocyte lysosomes (1, 6, 16–18). Two different ANCA types have been recognized by indirect immunohistology. Within each category there are multiple specificities. One ANCA type, C-ANCA, produces cytoplasmic staining and is usually specific for the serine proteinase, proteinase 3 (19, 20). The second ANCA type (perinuclear-pattern ANCA), which usually has specificity for myeloperoxidase (MPO-ANCA), produces artifactual perinuclear staining on alcohol-fixed neutrophils but produces cytoplasmic staining on formalin-fixed cells (1, 6, 18).

We postulated that these autoantibodies are capable of activating neutrophils and may be pathogenic; this idea was tested by measuring *in vitro* the release of reactive oxygen species (ROS) and primary granule contents from ANCA-stimulated human neutrophils. The data support the hypothesis that ANCA may be involved in the pathogenesis of necrotizing vasculitis and glomerulonephritis.

## MATERIALS AND METHODS

**Serum and Immunoglobulin Samples.** ANCA sera were obtained from patients with pauci-immune necrotizing and crescentic glomerulonephritis, 75% of whom had extrarenal vasculitis. A positive ANCA serology was determined by using a standard indirect immunofluorescence microscopy assay and by ELISA (6, 21). Of the 13 ANCA sera used in this study, 7 were C-ANCA and 6 were MPO-ANCA. All of the perinuclear-pattern ANCA were found to have specificity for MPO with an MPO-specific ELISA (6). None of the C-ANCA sera reacted with MPO, but 4 of these sera reacted by ELISA with proteinase 3 (20).

Purified immunoglobulins were prepared by sequential ammonium sulfate precipitation and DEAE-Sephacel chromatography (Pharmacia LKB) from five C-ANCA, six MPO-ANCA, and two normal human sera as well as from serum from patients with active lupus nephritis ($n = 4$), anti-glomerular basement membrane antibody-induced glomerulonephritis ($n = 1$), ANCA-negative pauci-immune necrotizing glomerulonephritis ($n = 1$), and other glomerular lesions ($n = 3$). The IgGs were negative for endotoxin by a Limulus amoebocyte lysate assay (sensitivity, 0.1 ng/ml). Prior to use, the IgGs were centrifuged to remove aggregates. For comparative purposes, a polyclonal anti-human MPO antibody (Dako, Carpinteria, CA), a monoclonal anti-human MPO antibody (Dako), and a monoclonal anti-human DNA antibody (Chemicon) were used.

$F(ab')_2$ from an MPO-ANCA IgG were prepared by using pepsin cleavage followed by a protein A separation column (ImmunoPure Fab preparation kit; Pierce).

**Chemiluminescence Studies.** Chemiluminescence studies (22, 23) were performed with normal human neutrophils (6). Assays were performed with $5 \times 10^6$ cells per ml pretreated with cytochalasin B (5 μg/ml) (24) and then placed in cold luminol (4°C). Chemiluminescence measurements were performed by an integrating photometer (model 3000; SAI Technology, San Diego). A 1:20 dilution of serum or IgG (50

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Abbreviations: ANCA, anti-neutrophil cytoplasmic autoantibody(ies); C-ANCA, cytoplasmic-pattern ANCA; MPO-ANCA, myeloperoxidase-specific ANCA; NAG, *N*-acetylglucosaminidase; ROS, reactive oxygen species; MPO, myeloperoxidase; TNF, tumor necrosis factor.

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μg/ml) was used for each time point, and each condition was assayed in quadruplicate (measurements differed by <10%). Analysis of unstimulated cells provided a baseline, whereas phorbol 12-myristate 13-acetate (1 μg/ml)-treated neutrophils were used as positive controls. Duplicate or triplicate experiments (which differed by <20%) were compared, and the mean cpm were calculated for each sample at 30 min.

To assess the specificity of these chemiluminescence assays for the products of a respiratory burst, the inhibitory effects of catalase (final concentration of 15 units/ml; no. C-100, Sigma) were analyzed. Since chemiluminescence may also measure the activation of several mixed-function oxidases, ANCA-induced ROS production was measured from the neutrophils of a patient with chronic granulomatous disease (25).

**Superoxide Generation Assay.** Superoxide was measured by the superoxide dismutase-inhibitable reduction of ferricytochrome *c* as determined by scanning between 540 and 560 nm with a double-beam spectrophotometer (model 118C; Cary, Palo Alto, CA) (26, 27). In these experiments cytochalasin B was not used. For each experiment, $1 \times 10^6$ cells were incubated with IgG at 100 μg/ml. The effects of MPO-ANCA, C-ANCA, and control IgG in four replicate experiments were compared with the effect of phorbol 12-myristate 13-acetate (1 μg/ml). The selection of these particular IgGs was made on the basis of availability, similarity of the clinical course of the patients, and a comparable degree of activation in the chemiluminescence studies. In these studies we explored the effects of priming neutrophils for 15 min at 37°C with tumor necrosis factor α (TNF-α; Genzyme) at 2 ng/ml (28–31).

**Degranulation Assays.** β-glucuronidase activity was assessed by the cleavage of phenolphthalein from phenolphthalein glucuronic acid and compared with a standard curve (32). Results are expressed as the amount of phenolphthalein released per $3.6 \times 10^6$ cells per ml. Lactate dehydrogenase assays assessed cell viability by monitoring the decrease in absorbance at 340 nm obtained by the conversion of pyruvate to lactate and concomitant conversion of NADH to $NAD^+$ (33). Lactate dehydrogenase values are reported in units/liter; the values of assay supernatants were compared to a Triton X-100 cell lysate. *N*-Acetylglucosaminidase (NAG) (another marker of primary granule degranulation) was assessed by using a modified fluorometric assay (34).

For each assay, $3.6 \times 10^6$ purified neutrophils were preincubated for 5 min with cytochalasin B (final concentration of 5 μg/ml). The cells were primed with TNF (2 ng/ml) (27–30) or, for comparative purposes, Hanks' balanced salt solution with calcium (13 mM) and magnesium (4 mM) without TNF and incubated at 37°C for 15 min. Analysis of unstimulated cells provided a baseline, whereas fMet-Leu-Phe (0.1 μM)- or Triton X-100 (0.5%)-stimulated cells served as positive controls. To demonstrate a dose-dependent relationship, ANCA IgG (500–50 μg/ml), mouse anti-human MPO (15.2–0.475 μg/ml), polyclonal rabbit anit-human MPO (750–0.95 μg/ml), and mouse anti-human DNA (15.2–0.475 μg/ml) were used to stimulate polymorphonuclear leukocyte degranulation during a 1-hr incubation.

**Flow Cytometry Studies.** Flow cytometry was used to demonstrate the presence of ANCA antigen, specifically MPO, on the surface of neutrophils. Isolated neutrophils were suspended in 1.0% bovine serum albumin (Sigma)/phosphate-buffered saline at $0.5 \times 10^6$ cells per ml. Cells were preincubated with either 0.1 μM fMet-Leu-Phe or TNF (2 ng/ml) from 30 sec to 30 min. The reactions were terminated with 1.0% paraformaldehyde, which immobilized proteins at the cell surface, and centrifuged at 4°C. The pellet was resuspended and incubated for 15 min in a 1:100 dilution of polyclonal anti-human MPO, MPO-ANCA IgG, or the $F(ab')_2$ MPO-ANCA preparation. The cells were washed with 1.0% bovine serum albumin/phosphate-buffered saline (4°C) and incubated with goat $F(ab')_2$ anti-rabbit immunoglobulin conjugated to fluorescein isothiocyanate (Tago) or with a goat $F(ab')_2$ anti-human immunoglobulin conjugated to fluorescein isothiocyanate (Tago). After another 15-min incubation period at 4°C, the cells were washed twice. Analysis of surface antigen expression was performed on a EPICS Coulter model 753 cell sorter (Coulter) at 488 nm. Control studies were performed without a primary antibody, with normal rabbit serum, and with purified normal and disease control human IgGs.

**Statistical Analysis.** All statistical differences between groups were determined by Student's *t* test. Differences between multiple groups were found by one-way analysis of variance. When the one-way analysis of variance showed significant differences between groups, Fisher's least significant difference method was performed to compare individual pairs of group means. In all cases, raw data were used for analysis without the use of transformed data.

## RESULTS

**Release of ROS.** The release of ROS from neutrophils was measured by using luminol-dependent chemiluminescence (Fig. 1). Thirteen ANCA-positive (7 C-ANCA and 6 MPO-ANCA) sera were compared with 5 normal sera (Fig. 1*A*). ROS release was markedly enhanced when neutrophils were incubated with ANCA sera ($35.7 \times 10^3 \pm 28.6$ cpm) as compared with normal sera ($2.2 \times 10^3 \pm 2.0$ cpm; $P < 0.02$). MPO-ANCA results ($44.1 \times 10^3 \pm 32.1$ cpm) were not statistically different from C-ANCA results ($28.4 \times 10^3 \pm 25.4$ cpm), but both were different from the normal sera ($P < 0.05$).

ANCA IgGs caused increased ROS release from neutrophils ($28.8 \times 10^3 \pm 27.2$ cpm) as compared with disease controls ($4.5 \times 10^3 \pm 4.3$ cpm; $P < 0.02$) (Fig. 1*A*). MPO-ANCA results ($39.6 \times 10^3 \pm 31.3$ cpm) were not different from C-ANCA results ($15.9 \times 10^3 \pm 15.9$ cpm), but both were greater than control IgGs ($P < 0.01$). The time course of activation was similar for both MPO-ANCA and C-ANCA (Fig. 1*B*).

The specificity of the chemiluminescence assay for ROS release was explored. In luminol-dependent chemiluminescence experiments, catalase diminished ANCA-induced ROS release by 70% ($20 \times 10^3$ cpm without versus $4.3 \times 10^3$ cpm with catalase at 30 min). These results are not surprising since luminol-dependent chemiluminescence measures an MPO-dependent pathway of oxygen metabolism (20). In another series of experiments, the most potent MPO-ANCA IgG could not induce ROS release from neutrophils from a patient with chronic granulomatous disease (CGD) (Fig. 1*B*), whereas this MPO-ANCA IgG was capable of degranulating CGD neutrophils. The results of the catalase and CGD experiments render unlikely the possibility that the chemiluminescence experiments measured nonspecific emission of light.

An $F(ab')_2$ was prepared from an MPO-ANCA IgG. The $F(ab')_2$ fraction had no effect on neutrophils when used by itself. In the presence of TNF, however, the $F(ab')_2$ fraction was able to release ROS with a time course similar to that of the parent IgG (Fig. 1*B*). The release of ROS by TNF alone was not different than that observed for resting cells.

When superoxide release was assessed by superoxide dismutase-inhibitable reduction of ferricytochrome *c*, TNF priming was an important facilitator of activation, as has been observed by others (28–31). Four ANCA IgGs were compared with four control IgGs. When ANCA IgGs were incubated without TNF, no superoxide release was observed. In a continuous assay, ANCA IgGs in the presence of TNF resulted in superoxide release, whereas a control IgG had no effect (Fig. 2). Similar activation kinetics were ob-

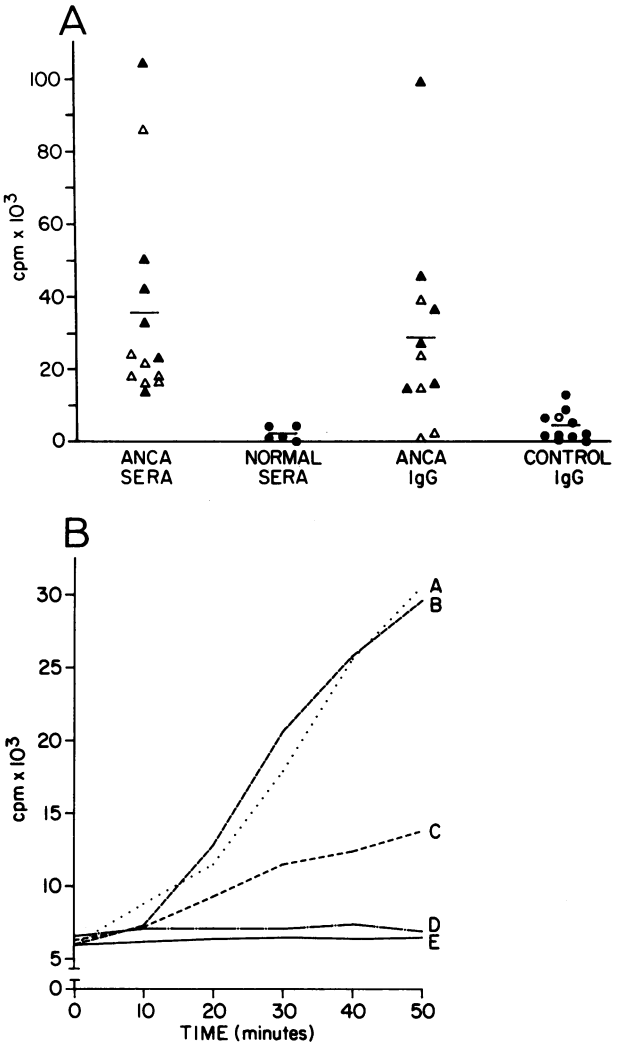


FIG. 1. ANCA-induced release of ROS measured by chemiluminescence. (*A*) Sera or purified immunoglobulins were incubated with neutrophils. Replicate experiments were compared, and the mean cpm at 30 min were calculated. Thirteen ANCA sera were compared with 5 normal sera. Eleven ANCA IgG were compared with 2 normal IgG as well as with IgG from 9 disease control patients. ▲, MPO-ANCA; △, C-ANCA; ○, normal IgGs; ●, normal serum samples in the normal sera column and disease controls in the control IgG column. The mean cpm for each of the four groups is indicated by a horizontal line. (*B*) The time courses of ROS release from neutrophils incubated with C-ANCA IgG (line A), MPO-ANCA IgG (line B), and Goodpasture syndrome IgG (line D) are compared. The most potent MPO-ANCA did not stimulate neutrophils obtained from a patient with chronic granulomatous disease (line E). MPO-ANCA $F(ab')_2$ in the presence of TNF stimulated ROS release (line C), whereas there was no stimulation without TNF. Values for unstimulated cells were comparable to the observations described by lines D and E. TNF was not used in experiments A, B, D, or E.

served when an MPO-ANCA IgG-induced superoxide release was compared with ROS release by using luminol-dependent chemiluminescence (Fig. 3).

In view of the observations that ANCA react with primary granule constituents and that MPO-ANCA react in particular with purified MPO (1, 6, 17, 18), we explored the effects of a commercial polyclonal anti-human MPO antibody in several activation assays. This anti-MPO antibody at 100 μg/ml caused the release of ROS in the chemiluminescence assay ($44.8 \times 10^3 \pm 17.0$ cpm; $n = 5$) and the superoxide assay (7.3 nmol). Although TNF priming facilitated these effects, at the concentration of antibody used in these experiments, TNF was not essential for activation.

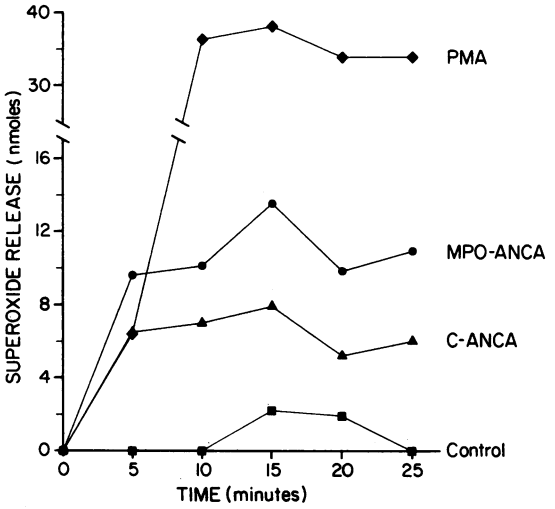


FIG. 2. ANCA-induced superoxide release. Superoxide was measured in a continuous assay by the superoxide dismutase-inhibitable reduction of ferricytochrome *c*. The effects of an MPO-ANCA, C-ANCA, and control IgG in four replicate experiments are shown in comparison with the effects of phorbol 12-myristate 13-acetate (PMA) at 1 μg/ml.

**Degranulation of Primary Granules.** In addition to ANCA-induced oxygen metabolism, we wondered whether release of noxious enzymes from primary granules could also be triggered by ANCA. Primary granule degranulation was assessed by β-glucuronidase (Fig. 4) and NAG release. The effects of 10 ANCA IgGs (500 μg/ml) were compared to that of 6 control IgGs (500 μg/ml) in the presence and absence of TNF (Fig. 4*A*). β-Glucuronidase release was increased with TNF-primed ANCA-stimulated cells (17.5 ± 16.6 μg of phenolphthalein) when compared with neutrophils incubated with ANCA alone (5.0 ± 4.6 μg of phenolphthalein; $P < 0.02$). Control IgGs with TNF (4.9 ± 4.8 μg of phenolphthalein) or without TNF (2.9 ± 1.2 μg of phenolphthalein) did not significantly release β-glucuronidase. In the absence of TNF there was no difference between ANCA and control IgG stimulation. The effects of ANCA IgGs on β-glucuronidase release was dose dependent (Fig. 4*A*). Similarly, polyclonal anti-MPO induced a dose-dependent increase in β-glucuronidase release (Fig. 4*B*). A monoclonal anti-MPO antibody produced a dose-dependent response, with degranula-

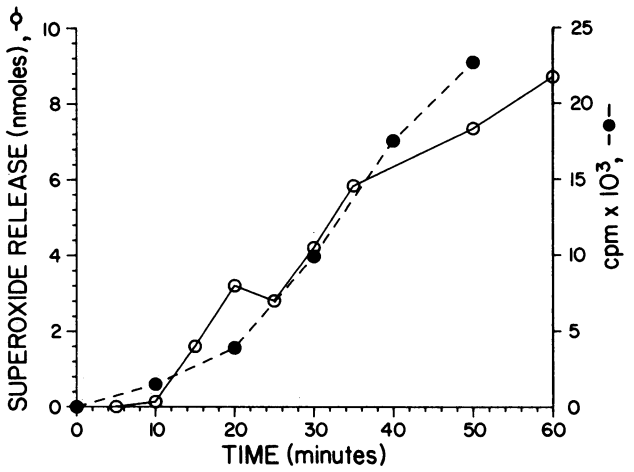


FIG. 3. Comparative kinetics of ANCA-induced chemiluminescence and superoxide release. The kinetics of a continuous chemiluminescence assay (●) and a continuous superoxide dismutase-inhibitable superoxide release assay (○) were compared after neutrophil activation with an MPO-ANCA IgG at 50 μg/ml.

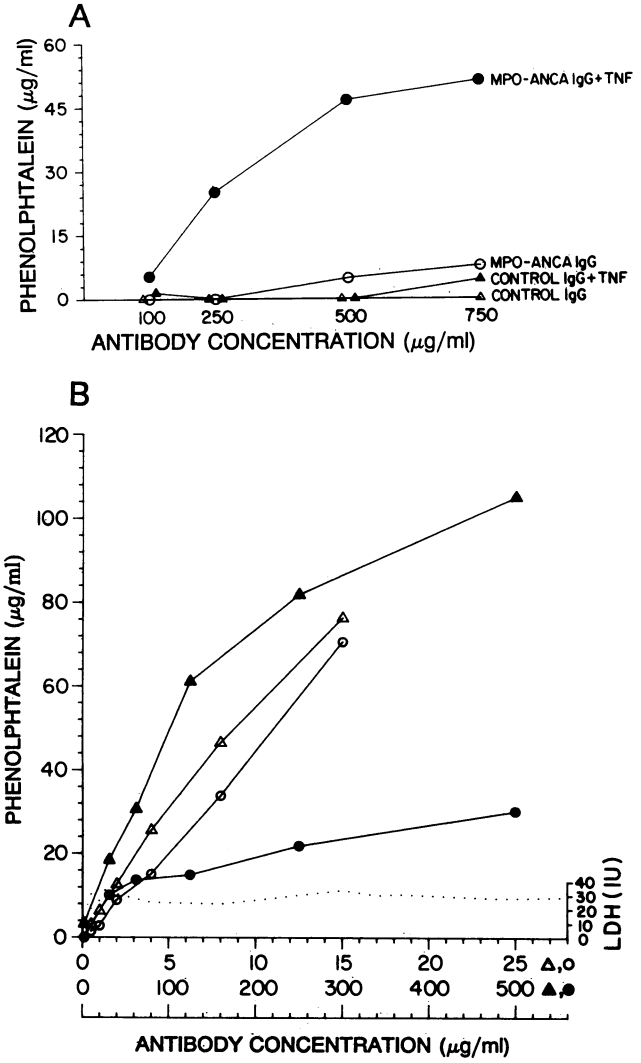


FIG. 4. ANCA induced β-glucuronidase release. (*A*) Primary granule release was measured by the release of β-glucuronidase from neutrophils pretreated with cytochalasin B (5 μg/ml) as measured by the phenolphthalein cleavage from phenolphthalein glucuronic acid relative to a standard curve. An MPO-ANCA IgG and a control IgG were incubated for 60 min at 37°C with neutrophils that were unprimed or primed with TNF (2 ng/ml). (*B*) Polyclonal anti-human MPO (100–500 μg/ml) with TNF (▲) or without TNF (●) produced dose-dependent β-glucuronidase release. Monoclonal anti-MPO (0.5–15 μg/ml) with TNF (△) or without TNF (○) also released β-glucuronidase. Only a small and constant amount of lactate dehydrogenase (LDH) was released.

tion detected with as little as 0.5 μg of antibody per ml (Fig. 4*B*). A monoclonal anti-human DNA antibody did not induce degranulation. Lactate dehydrogenase measurements indicated that the antibodies were not releasing enzymes as a result of cytotoxicity (Fig. 4*B*).

To confirm these β-glucuronidase results, NAG release was also measured. Four representative ANCA IgGs released 0.0020 ± 0.0008 μmol of NAG per ml per min as compared with 0.0007 ± 0.0003 μmol of NAG per ml per min for four control IgGs ($P < 0.05$). Furthermore, a dose–response curve similar to that shown in Fig. 4 for β-glucuronidase release was found for NAG release at 100 μg/ml. MPO-ANCA IgG released 0.0008 μmol of NAG per ml per min, and at 750 μg/ml, it released 0.0031 μmol of NAG per ml per min. In the presence of TNF, a polyclonal anti-MPO antibody at 100 μg/ml also induced the release of 0.0053 μmoles of NAG per ml per min. Thus, by using two

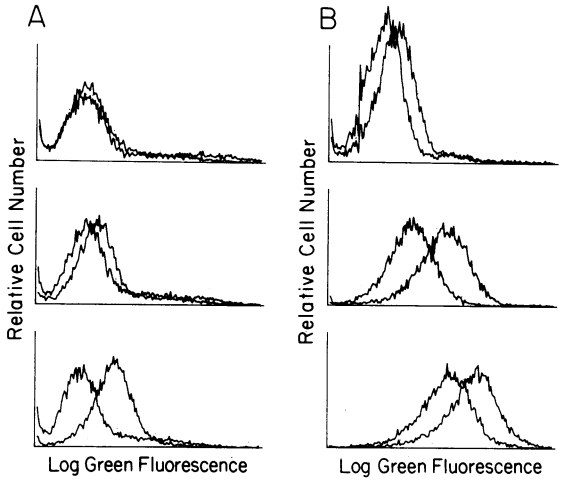


FIG. 5. Flow cytometric analysis of MPO expression on activated neutrophil cell surfaces. (*A*) By using a polyclonal anti-human MPO antibody as the primary reagent and a goat anti-rabbit IgG–fluorescein isothiocyanate conjugate as the secondary reagent, the presence of MPO on the surface of neutrophils was measured with an EPICS Coulter model 753 cell sorter. The presence of MPO on the surface of the cells with and without TNF stimulation was measured at 2 min (*Top*), 5 min (*Middle*), and 30 min (*Bottom*). In each panel, the control measurement of cells without TNF is to the left and that with TNF stimulation is to the right. (*B*) By using polyclonal anti-human MPO antibody (*Top*), MPO-ANCA IgG (*Middle*), and MPO-ANCA $F(ab')_2$ (*Bottom*), MPO was identified on the surface of the cells within 1 min of fMet-Leu-Phe stimulation. In each panel, the control measurement of cells without TNF is to the left and that with TNF stimulation is to the right.

different assays for primary granule release, ANCA IgGs in the presence of TNF induced dose-dependent degranulation.

**Flow Cytometry Studies.** Flow cytometry studies were performed to determine whether ANCA antigens, and in particular MPO, were expressed at the surface of neutrophils during stimulation. When TNF was used as the primer at 2 ng/ml, MPO was expressed on the surface of neutrophils by 5 min, with a marked increase in antigen expression by 30 min (Fig. 5*A*). When fMet-Leu-Phe was used as the activator, MPO was observed on the cell surface within 30 sec of stimulation, with a peak effect within 1–2 min (Fig. 5*B Top*). An MPO-ANCA IgG and its $F(ab')_2$ derivative also recognized increased MPO expression on the surface of fMet-Leu-Phe-activated cells (Fig. 5*B Middle* and *Bottom*). These results are similar to those reported by Pryzwansky *et al.* (35).

## DISCUSSION

ANCA were first reported in 1982 in eight patients with pauci-immune segmental necrotizing glomerulonephritis and evidence for systemic vasculitis (5). Since then, ANCA have been identified in patients with a spectrum of necrotizing vasculitis ranging from renal-limited necrotizing glomerulonephritis to widespread systemic vasculitis, including the syndromes of Wegener granulomatosis and polyarteritis nodosa (1, 5–15).

In the current study we learned that ANCA, in addition to being important serological markers of vasculitis, are capable of activating neutrophils *in vitro*. The interaction of MPO-ANCA and C-ANCA with neutrophils resulted in the production of ROS. ROS were produced when either sera or purified IgGs were employed. Interestingly, ROS were not released by purified IgGs from patients with lupus nephritis, a typical immune complex-mediated nephritis, or by an IgG from a patient with Goodpasture syndrome, the prototype of anti-basement membrane antibody-mediated nephritis. The

release of ROS in the chemiluminescence assay was corroborated by release of superoxide measured on an entirely different system.

Both ANCA phenotypes also induce, in a dose-dependent manner, neutrophil degranulation. Primary granule release was demonstrated by using two independent assay systems that measure different granule constituents (i.e., β-glucuronidase and NAG). Commercial polyclonal and monoclonal anti-human MPO antibodies produced comparable neutrophil activation as compared with MPO-ANCA.

The mechanism by which ANCA interact with their target antigens is not readily apparent since their antigens are found within cytoplasmic primary granules. ANCA-induced degranulation was markedly enhanced by TNF priming. That ANCA-induced neutrophil activation is facilitated by TNF priming is consistent with previous studies in which short periods of TNF pretreatment enhance the effects of other soluble stimuli to induce an oxidative burst and degranulation (28–31). The flow cytometry studies demonstrate MPO on the surface of neutrophils after TNF or fMet-Leu-Phe stimulation. Thus, in the process of priming, small quantities of ANCA antigens, such as MPO, are expressed at the cell surface and are available to interact with ANCA and initiate neutrophil activation. Since neutrophils are presumably activated to some degree during their procurement and isolation, small amounts of ANCA-related antigens may always be expressed on the surface of isolated cells. Cytokine priming invariably enhanced, and in some assays was required for, ROS production, and degranulation.

The flow cytometry results also shed light on the kinetics of ANCA activation. When compared to stimuli that induce activation within a matter of seconds to 1–2 min (35), ANCA activation is delayed. It is interesting to note that the delayed surface expression of MPO with TNF pretreatment correlates well with the delayed release of ROS caused by ANCA stimulation.

MPO is capable of inhibiting neutrophil secretory products and activation (36, 37). It is conceivable that ANCA, especially MPO-ANCA, interact with extracellular MPO and abrogate the inhibitory effects of MPO-catalyzed oxidation of neutrophil secretory products.

Since TNF as well as other cytokines (30) are released into the circulation during inflammatory diseases, such as infections, it is possible that they provide an *in vivo* priming of neutrophils in ANCA-positive patients that then allows for ANCA-induced neutrophil activation. This might explain the observation that the majority of patients with ANCA-associated disease describe a "flu-like" prodrome before the onset of vasculitic and nephritic symptoms. Additionally, we have observed a seasonal variation in the onset of clinical symptoms of necrotizing vasculitis, with a preponderance of cases during winter months (38).

The pathogenesis of pauci-immune necrotizing vasculitis and glomerulonephritis is not attributable to immune complex-mediated or anti-basement membrane antibody-mediated injury. We propose that ANCA, probably in concert with leukocyte priming factors, induce neutrophils, and possibly mononuclear phagocytes, to release toxic oxygen radicals and noxious granule constituents that cause vascular injury in patients with pauci-immune necrotizing vasculitis and pauci-immune crescentic glomerulonephritis.

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