Clin Exp Immunol 1994; 98:270–278

Activation of granulocytes by anti-neutrophil cytoplasmic antibodies (ANCA): a FcγRII-dependent process

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(Accepted for publication 27 July 1994)

SUMMARY

ANCA have been demonstrated to induce the respiratory burst in primed neutrophils. In this study we have extended the investigations on neutrophil activation by ANCA directed against proteinase 3 (PR3), myeloperoxidase (MPO) and lactoferrin (LF), and we have analysed the underlying mechanisms. All three ANCA antigens were expressed on the cell surface of primed neutrophils. Superoxide production assayed by both cytochrome c reduction and oxidation of dihydrorhodamine 123, was induced by heterologous polyclonal anti-MPO and anti-LF antibodies, and ANCA-positive plasma samples. Induction of superoxide production was dose-dependent. $F(ab')_2$ fragments did not induce the respiratory burst. Blockade of Fc receptors by specific MoAbs showed that anti-FcγRII antibodies were able to turn off the ANCA-induced respiratory burst, whereas anti-FcγRIII antibodies did not. Plasma samples that induced the respiratory burst did not differ from samples that did not induce superoxide production with respect to ANCA titre, but had higher levels of the IgG3 subclass of ANCA. Levels of the other subclasses of ANCA were comparable between those samples. We conclude that ANCA-induced activation of primed neutrophils is FcγRII-dependent, and appears to be facilitated by antibodies of the IgG3 subclass.

Keywords ANCA neutrophil activation Fcγ receptor

INTRODUCTION

Antibodies directed against cytoplasmic constituents of the neutrophilic granulocyte (ANCA) have extensively been described as markers for systemic vasculitis and (idiopathic) crescentic glomerulonephritis (reviewed in [1,2]). By indirect immunofluorescence (IIF) on ethanol-fixed granulocytes two types of ANCA can be distinguished: cytoplasmic or C-ANCA and perinuclear or P-ANCA. C-ANCA are directed, in most cases, against proteinase 3 (PR3), and are strongly associated with Wegener's granulomatosis (WG). A considerable number of P-ANCA-positive sera contain antibodies to myeloperoxidase (MPO), which are associated with idiopathic crescentic glomerulonephritis and different forms of necrotizing systemic vasculitis [3–5]. P-ANCA may, however, also be directed against lactoferrin (LF), cathepsin G, or other still unknown antigens, and are detected in various diseases characterized by chronic inflammation such as rheumatoid arthritis (RA), primary sclerosing cholangitis, autoimmune chronic active hepatitis, ulcerative colitis (UC) and Crohn's disease (CD) [6–11].

A role for ANCA in the pathogenesis of WG has been suggested by the studies of Cohen Tervaert *et al.* [12,13]. They demonstrated that disease activity of WG is preceded by increase in C-ANCA titre [12], and that early treatment of WG based on changes in C-ANCA levels prevents relapses of the disease [13]. The underlying mechanisms remained, however, unclear. Recently, Falk *et al.* [14] demonstrated that ANCA directed against PR3 or MPO induce, *in vitro*, the respiratory burst and degranulation of normal donor granulocytes primed with tumour necrosis factor-alpha (TNF-α). Further studies showed that ANCA enhance the adherence of neutrophils to endothelial cells [15] and neutrophil-mediated target cell destruction [16]. In addition, as PR3 and MPO also are highly cationic proteins that readily stick to endothelial cells, ANCA may induce complement-dependent cytotoxicity by binding to their localized antigens [17].

The precise mechanism of ANCA-induced neutrophil activation has, however, not been unravelled yet. In the present study we further explored the mechanisms involved in neutrophil activation by ANCA directed to PR3 and MPO as well as ANCA directed to LF which are associated with chronic inflammatory disorders. We proved that granulocyte activation by ANCA of diverse specificities is Fc-dependent and not mediated by $F(ab')_2$ fragments alone. Fc interaction occurs via the second Fc receptor (FcγRII). Accordingly, ANCA-induced

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Table 1. Characteristics of plasma samples used in this study

No.	IIF pattern*	IIF titre†	Antigenic specificity	Clinical diagnosis
1	C-ANCA	1:128	PR3	WG
2	C-ANCA	1:128	PR3	WG
3	C-ANCA	1:128	PR3	WG
4	C-ANCA	1:≥512	PR3	WG
5	C-ANCA	1:256	PR3	WG
6	C-ANCA	1:128	PR3	WG
7	P-ANCA	1:128	MPO	SV
8	P-ANCA	1:128	LF	RA
9	P-ANCA	1:≥512	LF	RA
10	P-ANCA	1:64	LF	RA
11	P-ANCA	1:64	LF	RA
12	P-ANCA	1:128	LF	RA
13	P-ANCA	1:128	LF	UC
14	P-ANCA	1:128	LF	UC
15	P-ANCA	1:128	LF	UC
16	P-ANCA	1:64	LF	UC
17	P-ANCA	1:128	LF	CD
18	P-ANCA	1:64	LF	CD
19	–			Healthy control
20	–			Healthy control
21	–			RA
22	–			RA
23	–			SLE

*Pattern of fluorescence on ethanol-fixed neutrophils; C-ANCA indicating a cytoplasmic pattern and P-ANCA indicating a perinuclear pattern; –, ANCA-negative.

†Titre of antibodies as determined by two-fold dilution started at a dilution of 1:16.

PR3, Proteinase 3; MPO, myeloperoxidase; LF, lactoferrin; WG, Wegener's granulomatosis; SV, systemic vasculitis; RA, rheumatoid arthritis; UC, ulcerative colitis; CD, Crohn's disease; SLE, systemic lupus erythematosus; IIF, indirect immunofluorescence.

granulocyte activation is particularly exerted by antibodies of the IgG3 subclass.

MATERIALS AND METHODS

Reagents

Formyl-methionyl-leucyl-phenylalanine (fMLP, F3506; Sigma Chemical Co., St Louis, MO) was dissolved in DMSO and stored in sterile, pyrogen-free containers at −80°C. All buffers and media used throughout the purification procedure and the activation experiments were pyrogen-free and contained less than 5 pg/ml endotoxin as determined by the Limulus amoebocyte assay.

Sera

Plasma samples, either obtained from plasmaphoresis material or from freshly drawn blood, were obtained from patients with either WG, sytemic vasculitis, RA, UC or CD. All samples were positive for either C-ANCA or P-ANCA. Characteristics of the samples are given in Table 1. Control plasma samples consisted of fresh samples from healthy volunteers, samples positive for antinuclear antibodies (ANA) and negative for ANCA derived from patients with RA, and a pool of plasma samples from patients with systemic lupus erythematosus (SLE) that contained antibodies to double-stranded DNA and were negative for ANCA.

Purified IgG fractions were prepared from all of the above mentioned plasma samples by sequential ammonium sulphate precipitation and protein G chromatography (fast flow protein G; Pharmacia Fine Chemicals AB, Uppsala, Sweden).

Antibodies

Rabbit anti-human MPO polyclonal antibodies (A398), rabbit anti-human LF polyclonal antibodies (A186), and normal rabbit immunoglobulins (X903) were obtained from Dakopatts (Copenhagen, Denmark). MoAb IV.3 (anti-FcγRII, IgG2b) was derived from Medarex Inc. (West Lebanon, NH), and MoAbs CD16 FcR gran 1 (anti-FcγRIII, IgG2a) and 12.8 (anti-PR3, IgG1) were derived from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB, Amsterdam, The Netherlands). For some experiments $F(ab')_2$ were prepared by digestion with pepsin (P7012; Sigma) in 0·1 M NaAc pH 3·6 for 16 h at 37°C. To end the reaction the vials were centrifuged for 10 min at 13 000 *g* and the supernatant was dialysed against Hanks' balanced salt solution (HBSS; Gibco BRL, Breda, The Netherlands). SDS–PAGE was performed to ensure that the digestion was complete. FITC-conjugated $F(ab')_2$ fragments of rabbit immunoglobulins to human IgG (F315; Dakopatts) and FITC-conjugated swine immunoglobulins to rabbit immunoglobulins (F205; Dakopatts) were used as secondary antibodies.

Detection of ANCA by IIF

Detection of ANCA was done as described before [18]. Test or control sera were applied in 1:16 to 1:512 serial dilutions. Slides were read by two independent observers, and a titre ≥ 1:32 was considered positive.

Characterization of ANCA specificities by ELISA

An antigen capture ELISA, as previously described [19], was used to test sera for the presence of antibodies to either PR3, MPO, or elastase. The presence of LF antibodies was detected by ELISA on plates directly coated with LF (5 μg/ml; Serva, Heidelberg, Germany). Peroxidase-conjugated rabbit anti-human immunoglobulin, diluted 1:500, was used for detection (P212; Dakopatts). Results by ELISA were considered positive when the value obtained exceeded the mean of 30 normal control sera by more than 3 s.d.

Isolation of granulocytes

Peripheral blood from normal volunteers was drawn into vacutainer tubes containing 0·34 M EDTA. The blood was diluted 1:1 in 0·9% NaCl, and polymorphonuclear granulocytes were separated by centrifugation on a Lymphoprep density gradient (Nycomed Pharma AS, Oslo, Norway). Contaminating erythrocytes were removed by hypotonic lysis. Following two washsteps with ice-cold PBS, the granulocytes were suspended in HBSS. Before the activation experiments the granulocytes were warmed gradually to 37°C, and treated for 5 min with cytochalasin B, 5 μg/ml (18015; Serva). Priming of the granulocytes was performed by incubation with recombinant TNF-α (rTNF-α; Genzyme, Cambridge, MA), 2 ng/ml for 15 min.

Surface expression of ANCA antigens

Surface expression of ANCA antigens was investigated by IIF,

followed by flow cytometric analysis. Freshly drawn blood or freshly isolated granulocytes, either primed or not, were fixed with 1% paraformaldehyde for 10 min on ice. Incubation with the primary antibody in a dilution of 1:50 was performed for 45 min at 4°C, followed by incubation with the FITC-conjugated secondary antibody in a dilution of 1:100 for 30 min at 4°C. After each step the cells were extensively washed with and finally resuspended in PBS/bovine serum albumin (BSA). Flow cytometric analysis was performed with 10×10^6 cells/ml using a FACSSTAR (Becton Dickinson, San Jose, CA).

Detection of superoxide production using the ferri-cytochrome C reduction assay

Superoxide production by granulocytes was determined by measuring the superoxide dismutase (SOD) (S9636; Sigma) inhibitable reduction of ferri-cytochrome C, discontinuously, according to the method of Pick & Mizel [20] with minor modifications. In short, 96-well microtitre plates (F-form; Greiner BV, Alphen a/d Rijn, The Netherlands) were incubated with freshly purified granulocytes (1×10^6 cells/ml), 690 μM ferri-cytochrome C (C7752; Sigma), either 590 U/ml SOD or an equal volume HBSS, and stimulus, IgG preparations were added at a concentration of 80 μg/ml, fMLP was used at a concentration of 0·1 μM. The plates were scanned repeatedly at 550 nm using a Titertek multiscan MCC 340 apparatus. Between the readings the plates were kept at 37°C. Each test was performed in quadruplicate.

Detection of superoxide production using flow cytometry

Superoxide production by granulocytes was determined additionally by measuring the oxidation of dihydrorhodamine 123 (DHR) (D632; Molecular Probes, Eugene, OR). Freshly isolated cells, either primed or non-primed (1×10^6 cells/ml) were incubated for 15 min with DHR (1 μg/ml) at 37°C. Next, stimuli were added to the cells followed by an incubation step of 30 min. Cells were pelleted at 200 *g* and resuspended in ice-cold HBSS at a concentration of 10×10^6 cells/ml. Emission was measured at 530 nm at a FACSSTAR apparatus. The concentrations of the stimuli were identical to those used in the cytochrome C reduction assay.

IgG subclass detection of ANCA by ELISA

IgG subclass detection of ANCA was performed as described previously [21]. In brief, a crude granule extract was isolated by nitrogen cavitation of neutrophils as described by Borregaard *et al.* [22]. Microtitre plates were coated either with the extract at a protein concentration of 20 μg/ml in 0·1 M carbonate buffer pH 9·6, or with LF (5 μg/ml) in PBS for 1·5 h at 37°C. The plates were incubated with human sera for 1 h at a dilution of 1:100 and subsequently with subclass-specific MoAbs (anti-human IgG1 clone MH161-1, anti-human IgG2 clone HP6014, anti-human IgG3 clone MH163-1-Mo5, anti-human IgG4 clone MH164-4; CLB) at a dilution of 1:250. Antibody binding was detected with alkaline phosphatase-conjugated goat anti-mouse IgG (A7157; Sigma). Values were expressed in OD units after subtraction of blanks.

Statistical analysis

Differences in levels of IgG subclasses were statistically analysed using two-tailed *t*-tests.

RESULTS

Expression of ANCA antigens on the cell surface

The ANCA antigens PR3, MPO, and LF were not present on the surface of neutrophils in freshly drawn blood immediately fixed with paraformaldehyde. A representative example of LF expression is shown in Fig. 1a. Isolation of neutrophils resulted in low surface expression of the ANCA antigens, as shown in Fig. 1b for the expression of LF. Priming of neutrophils with 2 ng/ml rTNF-α for 15 min at 37°C resulted in significantly increased expression of PR3, MPO, and LF on the cell surface (Fig. 1c–f). The presence of those ANCA antigens was demonstrated at the cell surface using heterologous polyclonal immunoglobulins to the respective antigens (shown in Fig. 1c

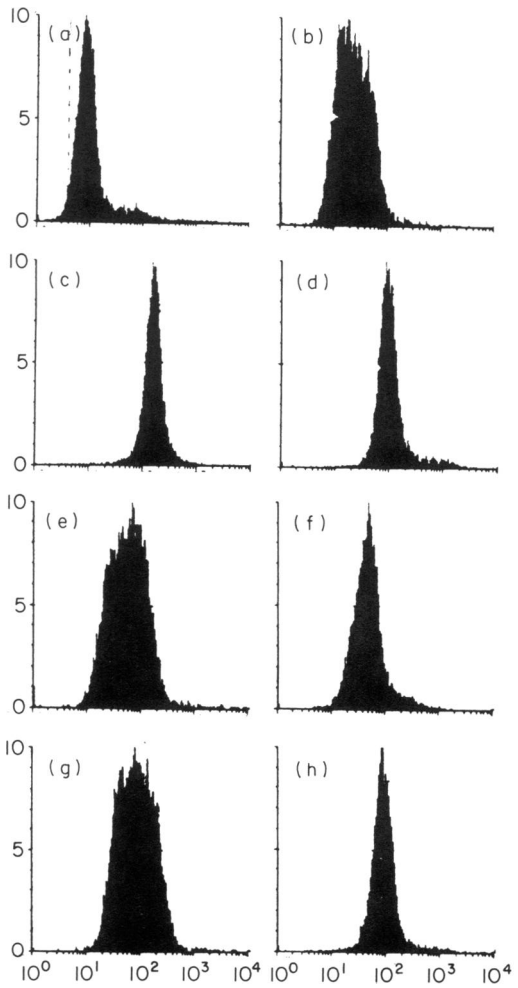

Fig. 1. (a) Expression of lactoferrin on the surface of granulocytes in freshly drawn peripheral blood. The dotted line represents the mean fluorescence intensity obtained after incubation with normal rabbit immunoglobulins. (b) Expression of lactoferrin on the surface of freshly purified granulocytes. (c–f) Expression of lactoferrin (LF), (c, heterologous; d, patient IgG), myeloperoxidase (MPO) (e), and proteinase 3 (f) on the surface of recombinant tumour necrosis factor-alpha (rTNF-α)-primed granulocytes as detected by IgG from plasma samples. (g, h) Expression of myeloperoxidase (g) and lactoferrin (h) on the surface of rTNF-α-primed granulocytes as detected with $F(ab')_2$ preparations from plasma samples positive for MPO–ANCA and LF–ANCA, respectively.

for LF) as well as using purified IgG derived from plasma samples positive for PR3-ANCA, MPO-ANCA or LF-ANCA, respectively (Fig. 1d–f). Also, $F(ab')_2$ fragments of those IgG fractions recognized the respective antigens on the cell surface. Figure 1g,h demonstrate the surface expression of MPO and LF as detected by $F(ab')_2$ fragments from plasma samples positive for MPO-ANCA and LF-ANCA, respectively.

Superoxide production as measured by cytochrome c reduction

Superoxide production was measured by the SOD-inhibitable reduction of ferri-cytochrome c. The chemotactic peptide fMLP which is known to induce the respiratory burst in granulocytes was used as positive control. As shown in Fig. 2a, fMLP induced superoxide production by purified granulocytes, whereas buffer did not. Heterologous polyclonal anti-LF (Fig. 2a) or anti-MPO antibodies induced the respiratory burst in primed granulocytes. In freshly isolated non-primed granulocytes induction of the respiratory burst did not occur during the first 30 min. After 120 min of incubation with primed neutrophils, heterologous anti-LF, heterologous anti-MPO and heterologous anti-PR3 antibodies reached ΔOD 550 values of $0{\cdot}332 \pm 0{\cdot}040$, $0{\cdot}214 \pm 0{\cdot}015$, and $0{\cdot}213 \pm 0{\cdot}042$, respectively (mean ± s.d. of three experiments). ΔOD 550 values of non-primed neutrophils after incubation with anti-LF, anti-MPO or anti-PR3 antibodies were $0{\cdot}118 \pm 0{\cdot}018$, $0{\cdot}108 \pm 0{\cdot}029$, and $0{\cdot}083 \pm 0{\cdot}024$, respectively (mean ± s.d. of three experiments). Normal rabbit immunoglobulin did not induce the respiratory burst in primed neutrophils (Fig. 2a).

Next, we tested IgG fractions from ANCA-positive plasma samples (anti-PR3, $n = 3$, anti-MPO, $n = 1$, anti-LF, $n = 11$) for their capacity to induce the respiratory burst in primed neutrophils. All three anti-PR3-positive IgG fractions, the anti-MPO-positive fraction, and two out of 11 anti-LF-positive IgG fractions induced superoxide production in primed but not in non-primed cells (Fig. 2b shows a representative experiment for one anti-MPO-positive IgG fraction and one anti-LF IgG-positive fraction). The remaining nine IgG fractions, all positive for anti-LF, did not induce the respiratory burst, not even when tested at higher concentrations (the concentration was increased stepwise from 80 μg/ml up to 1 mg/ml). The amount of superoxide production induced by the ANCA samples capable of inducing neutrophil activation proved to be dose-dependent (Fig. 2c). However, the capacity to induce the respiratory burst and ANCA titre, either measured by IIF or by antigen-specific ELISA, did not correlate (Fig. 3).

In order to assess the Fc-dependency of ANCA-induced neutrophil activation, $F(ab')_2$ fragments were applied in molar base equal to the IgG preparations. $F(ab')_2$ fragments either from heterologous polyclonal immunoglobulins or derived from patients' IgG (samples 1–11) were not able to induce superoxide production (shown in Fig. 2d for samples 7 and 9), although they were able to recognize their respective antigens on the surface of primed neutrophils.

IgG derived from patients with ANA (samples 21–23) or from normal healthy controls (samples 19 and 20) did not induce the respiratory burst in primed neutrophils (Fig. 2b). No

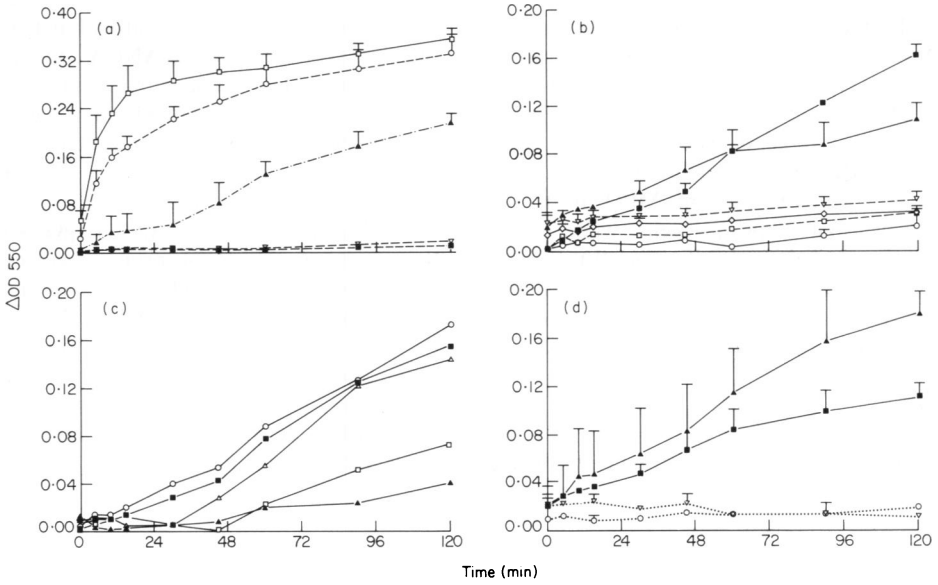

Fig. 2. Superoxide production by granulocytes as measured by the superoxide dismutase (SOD)-inhibitable cytochrome c reduction assay. The abscissa is the time axis of superoxide production (in min), the ordinate shows the SOD-inhibitable cytochrome c reduction as expressed as the difference in OD units. Data are presented as the mean + s.d. of three respective experiments. (a) Superoxide production of primed (recombinant tumour necrosis factor-alpha (rTNF-α); 2 ng/ml) granulocytes induced by formyl-methionyl-leucyl-phenylalanine (fMLP) (□), buffer (■), heterologous lactoferrin (LF) IgG (○), and normal rabbit immunoglobulin (▽). Superoxide production in non-primed cells by heterologous anti-lactoferrin (▲) IgG is also shown. (b) Superoxide production of primed (——) and non-primed (- - - -) cells induced by patients' IgG directed against myeloperoxidase (MPO) (▲, ▽) and LF (■, □), and superoxide production of primed cells by normal IgG (○) or antinuclear antibodies (ANA) containing IgG (◇). (c) Dose dependency of superoxide production of primed granulocytes induced by LF–ANCA in primed granulocytes. Doses shown are 80 (○), 40 (■), 20 (△), 10 (□) and 5 (▲) μg IgG/ml respectively. (d) Superoxide production of primed neutrophils induced by IgG (——) or $F(ab')_2$ fragments (·····) from anti-MPO (■, ○) and anti-LF (▲, ▽)-positive plasma samples.

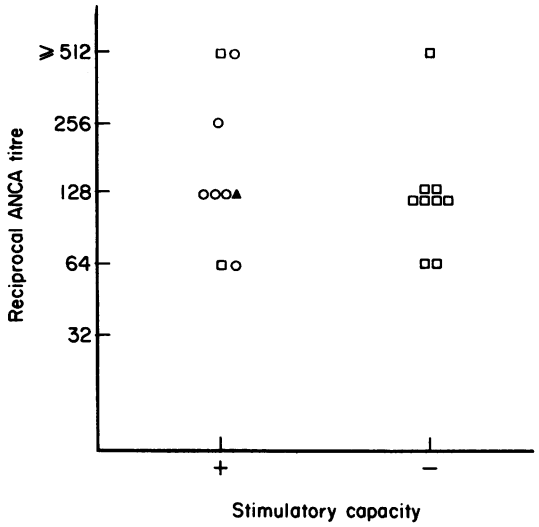

Fig. 3. Titre of ANCA as measured by indirect immunofluorescence (IIF) and capacity of IgG to induce superoxide production in plasma samples from patients with ANCA of diverse specificities. □, Anti-lactoferrin IgG; ▲, anti-myeloperoxidase IgG; ○, anti-proteinase 3 IgG.

relation was observed between the presence of IgG rheumatoid factor in the plasma samples and the capacity of plasma samples to induce the respiratory burst (data not shown).

Superoxide production as measured by the oxidation of dihydrorhodamine 123

Since the ferri-cytochrome c reduction assay measures the amount of extracellular superoxide, this method may not be sensitive enough to measure low amounts of superoxide production. Therefore, we used the superoxide-specific oxidation of dihydrorhodamine as detected by flow cytometry. As shown in Fig. 4a, fMLP induced superoxide production, whereas buffer did not. Anti-LF and anti-MPO antibodies induced the respiratory burst in primed cells, while hardly any production was seen in unprimed cells (Fig. 4b). $F(ab')_2$ fragments did not induce superoxide production in primed cells (Fig. 4c). IgG derived from patients with ANA or from normal healthy controls did not induce oxidation of dihydrorhodamine (data not shown).

Blockade of Fcγ receptors

Since it appeared that the presence of Fc fragments on the immunoglobulins was required to induce the ANCA-induced respiratory burst, blockade of Fcγ receptors was supposed to prevent the induction of the respiratory burst. As shown in Fig. 5, $F(ab')_2$ fragments of the MoAb IV.3 directed against FcRII (10 μg/ml) prevented the respiratory burst in its initial phase as measured by the cytochrome c reduction assay of primed neutrophils stimulated with polyclonal anti-MPO or anti-LF antibodies. fMLP-induced respiratory burst was not inhibited when the anti-FcγRII MoAb was added. The $F(ab')_2$ fragments of the MoAb CLB gran1 directed against FcRIII (20 μg/ml) did not influence anti-LF-induced respiratory burst. Figure 5 shows that blockade of the FcγRII inhibits heterologous polyclonal antibodies to induce the respiratory burst, while Fig. 6 shows the same for IgG derived from ANCA containing plasma samples. Using the dihydrorhodamine assay, similar results were obtained. Blockade of FcγRII prevented the ANCA-induced respiratory burst, whereas blockade of FcγRIII had no effect (Table 2). Simultaneous blockade of both receptors had no additional effect (data not shown).

If the Fc fragment of ANCA IgG is necessary to induce the respiratory burst of primed neutrophils, than $F(ab')_2$ fragments of ANCA would be able to block the activation of primed neutrophils by ANCA IgG. Table 3 shows that indeed the addition of $F(ab')_2$ fragments of IgG from ANCA-positive plasma samples results in delay of the respiratory burst induced by the corresponding IgG fraction when applied in a 1:1 molar ratio, and results in the inhibition of the respiratory burst when applied in a 3:1 molar ratio.

Table 2. Fc-dependency of ANCA-induced superoxide production as measured by the dihydrorhodamine assay

	Mean fluorescence intensity		
Stimulus	+ buffer	+ Fc RII*	+ Fc RIII*
fMLP	150	152	142
α-LF	235	122	230
Normal rabbit immunoglobulins	91	94	92

Given is a representative example of three experiments.

α-LF, Heterologous polyclonal anti-lactoferrin antibodies; fMLP, formyl-methionyl-leucyl-phenylalanine.

*Addition of $F(ab')_2$ fragments of anti-FcRII (MoAb IV.3) and anti-FcRIII (CLB gran1), respectively.

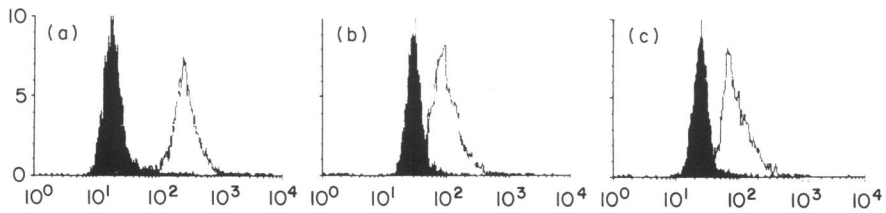

Fig. 4. Superoxide production by granulocytes as measured by the oxidation of dihydrorhodamine. (a) Formyl-methionyl-leucyl-phenylalanine (fMLP)-induced superoxide production in comparison with buffer. (b) Heterologous polyclonal anti-lactoferrin-induced superoxide production in primed granulocytes in comparison with non-primed cells. (c) Heterologous polyclonal anti-lactoferrin-induced superoxide production in primed cells by IgG in comparison with $F(ab')_2$ fragments.

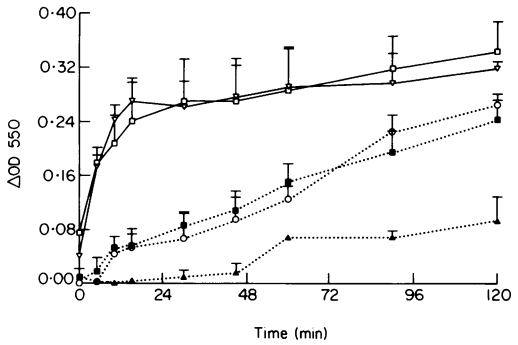

Fig. 5. Superoxide production as measured by the superoxide dismutase (SOD) inhibitable cytochrome c reduction assay. Induction of the respiratory burst by formyl-methionyl-leucyl-phenylalanine (fMLP) in the presence of $F(ab')_2$ fragments of anti-FcRII (▽) and FcRIII (□) MoAbs, and induction of the respiratory burst by heterologous anti-lactoferrin (○) in the presence of either $F(ab')_2$ fragments of anti-FcRII (▲) or anti-FcRIII (■) MoAbs.

IgG subclass distribution

Since the various IgG subclasses have different affinities for the Fc receptors, we studied the IgG subclass distribution in IgG preparations that induced the respiratory burst and in preparations that did not. ANCA titres between both groups were comparable (Fig. 3). As shown in Fig. 7, ANCA IgG preparations that induced the respiratory burst in primed neutrophils ($n = 8$) had higher levels of IgG3 than ANCA IgG preparations that did not induce the respiratory burst ($n = 7$) ($P < 0{\cdot}005$). Levels of the other IgG subclasses were comparable between both groups.

DISCUSSION

This study confirms and extends data that the intracellularly localized antigens recognized by ANCA become accessible at the cell surface after priming of granulocytes, and that interaction of the antigens with the respective autoantibodies results in induction of the respiratory burst. In addition, we demonstrate that this process is FcRII-dependent and may be preferentially achieved by IgG3 subclass antibodies.

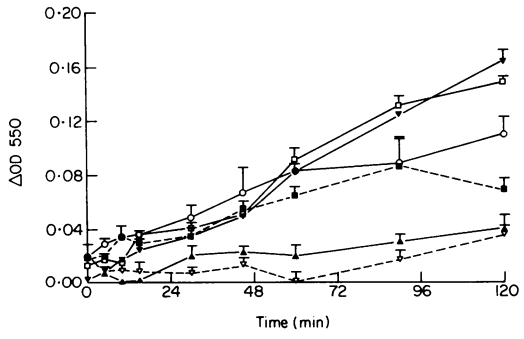

Fig. 6. Superoxide production as measured by the superoxide dismutase (SOD)-inhibitable cytochrome c reduction assay. Induction of the respiratory burst by IgG fraction from an anti-lactoferrin-positive patient (▼) in the presence of $F(ab')_2$ fragments of anti-FcRII (▽) and anti-FcRIII (□), and induction of the respiratory burst by IgG fraction anti-myeloperoxidase patients' IgG (○) in the presence of either anti-FcRII (▲) or anti-FcRIII (■) MoAbs.

Table 3. Inhibition of ANCA IgG-induced neutrophil activation by addition of their respective $F(ab')_2$ fragments

Sample no.*	$F(ab')_2$: IgG ratio	Per cent inhibition at time point† 30 min	75 min
1	1:1	34·4	7·1
2	1:1	59·4	22·2
3	3:1	77·9	60·9
4	1:1	39·8	12·9
5	1:1	32·2	25·1

* Numbers according to Table 1.

† Given is the percentage of inhibition obtained in comparison with IgG stimulation on primed neutrophils.

Neutrophil activation is thought to contribute significantly to tissue destruction in several diseases, including vasculitis, RA, and inflammatory bowel disease. Falk *et al.* [14] were the first to report that PR3- and MPO-ANCA may play an active role in neutrophil activation. They demonstrated, *in vitro*, that after a 'priming' event the ANCA antigens are expressed on the surface of the neutrophil, which makes them available for interaction with ANCA. Subsequently, this interaction leads to activation of the neutrophil, resulting in the production of oxygen radicals and release of lytic enzymes which may contribute to tissue destruction as seen in ANCA-associated diseases. As such, the presence of ANCA might be directly related to neutrophil activation. The mechanisms by which neutrophils are activated by ANCA have not been extensively studied yet. Fujimoto & Lockwood [23] demonstrated that after incubation with ANCA neutrophils show translocation of protein kinase C (PKC) to the cell membrane. This translocation is the initial step in the activation of PKC, which participates in the assembly and the activation of the NADH oxidase system.

We tested ANCA of various specificities for their capacity to induce the respiratory burst in primed neutrophils. We observed, in accordance with previous studies [14,24], that the ANCA antigens PR3, MPO and LF were expressed on the surface of rTNF-α-primed neutrophils, whereas these antigens were hardly expressed on freshly purified neutrophils. Incubation of primed granulocytes with ANCA of defined specificities, either heterologous or derived from plasma samples from ANCA-positive patients, resulted in induction of the respiratory burst. The largest amount of superoxide production was induced by heterologous anti-LF antibodies, probably related to the higher expression of LF on the cell surface in comparison with MPO and PR3. ANCA-induced superoxide production was dose-dependent, and others [24] have shown that ANCA-induced superoxide production is antigenic-specific. It is presently not known whether other antibodies that recognize surface antigens are able to stimulate primed neutrophils as well.

It has been suggested that neutrophil activation by ANCA results from $F(ab')_2$-dependent binding of surface antigens

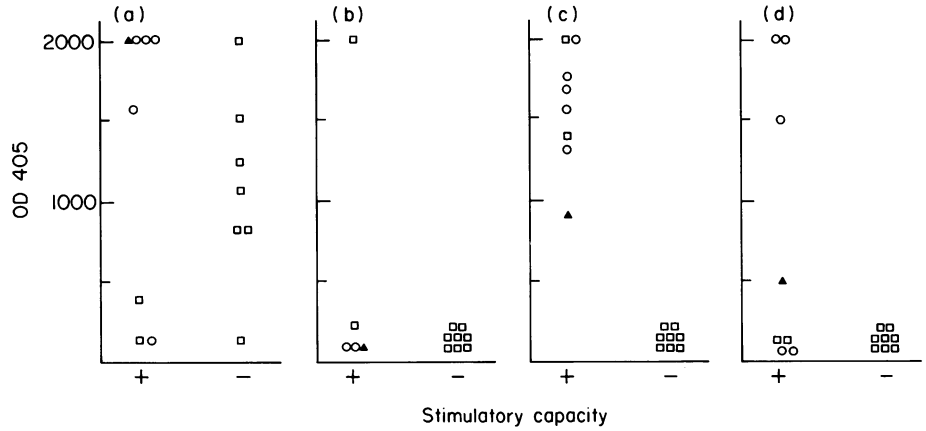

Fig. 7. IgG subclass distribution of ANCA IgG as determined by ELISA in relation to their capacity to induce the respiratory burst. On the abscissa the IgG fractions are grouped in samples that can induce granulocyte activation (+) and samples that cannot induce granulocyte activation (−). On the ordinate OD units (after subtraction of blanks) for antigen-specific IgG subclass ANCA are given. (a) IgG1. (b) IgG2. (c) IgG3. (d) IgG4 ANCA. □, Anti-lactoferrin IgG; ▲, anti-myeloperoxidase IgG; ○, anti-proteinase 3 IgG.

only [14]. Using the cytochrome c reduction assay, we could, however, not detect induction of the respiratory burst by $F(ab')_2$ fragments from ANCA of diverse specificities, although those $F(ab')_2$ preparations still recognized the ANCA antigens at the cell surface of primed granulocytes. To control for a possibly low sensitivity of the cytochrome C reduction assay, superoxide production was also detected by the oxidation of dihydrorhodamine as measured by flow cytometry. This system might be more sensitive, as it measures the intracellular superoxide production. Using this system the $F(ab')_2$ preparations from ANCA-positive IgG fractions again failed to induce the respiratory burst in primed granulocytes. In their initial report Falk *et al.* [14] mentioned only one $F(ab')_2$ preparation from an anti-MPO-positive serum that induced the respiratory burst as tested by the chemiluminescence assay. We tested IgG as well as $F(ab')_2$ preparations from ANCA-positive plasma samples for their capacity to induce the respiratory burst using the chemiluminescence assay in which either a fluorimeter or a scintillation counter was used for detection. Again, all $F(ab')_2$ fragments failed to induce the respiratory burst in primed neutrophils, whereas IgG fractions did (data not shown). Recently, Keogan *et al.* [24] found five out of six $F(ab')_2$ ANCA preparations produced neutrophil activation as demonstrated by a chemiluminescence assay. They could, however, not inhibit this response with SOD, but did inhibit their reaction with azide and salicylhydroxamic acid. This demonstrates that their assay does not measure superoxide production but the active MPO system.

To demonstrate further the inability of $F(ab')_2$ fragments of IgG–ANCA to induce the respiratory burst we evaluated whether those fragments in fact could inhibit the IgG–ANCA-induced respiratory burst. Indeed, $F(ab')_2$ fragments were able to inhibit this process, suggesting that these fragments do bind to the ANCA antigens at the surface of the neutrophil and subsequently inhibit the interaction of the complete IgG molecules with those antigens. Due to shortage of the antibodies we could apply most $F(ab')_2$ fragments only in a 1:1 molar ratio with their corresponding IgG fraction, which accounts for the only partial inhibition of the respiratory burst observed.

From the above mentioned data we conclude that ANCA-induced activation of primed neutrophils is dependent on the Fc fragments of the IgG molecule. Along these lines we hypothesize that interaction of the ANCA antigens at the neutrophil surface with the autoantibodies leads to an *in situ* immune complex formation which activates neutrophils by way of their Fcγ receptors. Since rTNF-α-primed neutrophils mainly express FcγRIII and FcγRII, we next investigated the effect of blockade of these receptors on ANCA-induced neutrophil activation. We used $F(ab')_2$ fragments of MoAbs against these receptors, which have been proved to block the receptor without inducing activation [25]. Indeed, we found that the induction of the respiratory burst by ANCA could be prevented by blockade of FcγRII, while blockade of FcγRIII had no effect. Blockade of both receptors had no additional effect, and the fMLP-induced respiratory burst was not affected by the addition of MoAbs to FcγRII and -RIII. Thus, ANCA-induced neutrophil activation is dependent on the FcγRII receptor. This conclusion is in accordance with the studies of Reibman *et al.* [26] and Huizinga *et al.* [25], who showed that FcγRII is the most important receptor in eliciting the respiratory burst, while FcγRIII is involved more preferentially in binding of immune complexes and is unable by itself to transduce transmembrane signals. Other studies, however, have shown that both receptors are equally important for superoxide production [27,28], or suggest that FcγRIII might even be the main pathway in immune complex activation of granulocytes [29,30].

During our study we observed that not all of the ANCA-positive sera were able to induce the respiratory burst. Although activation of granulocytes was dependent on the concentration of ANCA in a particular sample, ANCA titres in samples capable of stimulation were comparable to those that were incapable of stimulation. Out of the 11 sera positive for anti-LF, only two IgG fractions were found to be able to induce the respiratory burst, despite the fact that in general anti-LF antibodies gave rise to the strongest stimulation. Preliminary data show, however, that the distribution of IgG subclasses of ANCA in plasma samples that stimulate primed granulocytes appears to be different from that in plasma

samples that do not stimulate. Stimulating samples contained high concentrations of IgG3–ANCA, whereas non-stimulating samples did not. Since IgG3 binds strongly to FcγRII, this IgG subclass may preferentially be involved in ANCA activation. In this regard it is interesting that Huizinga *et al.* [31] observed that human neutrophils bind IgG3 complexes approximately three times faster than IgG1 complexes, whereas IgG2 and IgG4 complexes are not bound. IgG3 complexes were bound both by FcγRII and FcγRIII. The IgG subclass distribution of ANCA in WG has been studied by Brouwer *et al.* [21]. They found that ANCA in WG are predominantly of the IgG1 and IgG4 class, but the additional presence of IgG3 was associated with the development of renal involvement, one of the most serious sequelae of the disease. Since only a limited number of plasma samples have been studied, further longitudinal studies have to be performed to investigate the relationship between IgG subclasses of ANCA, neutrophil activation and disease activity of the ANCA-associated diseases.

In conclusion, IgG from ANCA-positive samples with specificity for PR3, MPO, and LF are capable of inducing the respiratory burst in primed neutrophils, although not all ANCA-positive samples do stimulate neutrophils. The activation process is dependent of the expression of the ANCA antigens on the cell surface and the presence of FcγRII receptors. Preliminary data suggest that the IgG3 subclass, in particular, is involved in the induction of the respiratory burst.

REFERENCES

1 Jennette JC, Charles LA, Falk RJ. Anti-neutrophil cytoplasmic autoantibodies: disease associations, molecular biology and pathophysiology. Int Rev Exp Pathol 1991; **32**:193–221.

2 Kallenberg CGM, Mulder AHL, Cohen Tervaert JW. Anti-neutrophil cytoplasmic antibodies: a still growing class of autoantibodies in inflammatory disorders. Am J Med 1992; **93**:675–82.

3 Falk RJ, Jennette JC. Anti-neutrophil cytoplasmic autoantibodies with specificity for myeloperoxidase in patients with systemic vasculitis and idiopathic necrotizing and crescentic glomerulonephritis. N Eng J Med 1988; **318**:1651–7.

4 Cohen Tervaert JW, Goldschmeding R, Elema JD *et al.* Autoantibodies against myeloid lysosomal enzymes in crescentic glomerulonephritis. Kidney Int 1990; **37**:795–806.

5 Cohen Tervaert JW, Goldschmeding R, Elema JD *et al.* Association of autoantibodies to myeloperoxidase with different forms of vasculitis. Arthritis Rheum 1990; **33**:1264–72.

6 Coremans IEM, Hagen EC, Daha MR, van der Woude FJ, van der Voort EAM, Kleijburg-van der Keur C, Breedveld FC. Anti-lactoferrin antibodies in patients with rheumatoid arthritis are associated with vasculitis. Arthritis Rheum 1992; **35**:1466–75.

7 Mulder AHL, Horst G, van Leeuwen MA, Limburg PC, Kallenberg CGM. Anti-neutrophil cytoplasmic antibodies in rheumatoid arthritis: characterization and clinical correlates. Arthritis Rheum 1993; **36**:1054–60.

8 Mulder AHL, Horst G, Haagsma EB, Limburg PC, Kleibeuker JH, Kallenberg CGM. Prevalence and characterization of anti-neutrophil cytoplasmic antibodies in autoimmune liver diseases. Hepatology 1993; **17**:411–7.

9 Saxon A, Shanahan F, Landers C, Ganz R, Targan S. A distinct subset of anti-neutrophil cytoplasmic antibodies is associated with inflammatory bowel disease. J Allergy Clin Immunol 1990; **86**:202–10.

10 Mulder AHL, Broekroelofs J, Horst G, Limburg PC, Nelis GF, Kallenberg CGM. Anti-neutrophil cytoplasmic antibodies in inflammatory bowel disease: characterization and clinical correlates. Clin Exp Immunol 1994; **95**:490–7.

11 Peen E, Almer S, Bodemar G, Ryden BO, Sjolin C, Tjelle K, Skogh T. Anti-lactoferrin antibodies and other types of ANCA in ulcerative colitis, primary sclerosing cholangitis, and Crohn's disease. Gut 1993; **34**:56–62.

12 Cohen Tervaert JW, van der Woude FJ, Fauci AS *et al.* Association between active Wegener's granulomatosis and anticytoplasmic antibodies. Arch Int Med 1989; **149**:2461–5.

13 Cohen Tervaert JW, Huitema MG, Hene RJ, Sluiter WJ, The TH, van der Hem GK, Kallenberg CGM. Prevention of relapses in Wegener's granulomatosis by treatment based on anti-neutrophil antibody titre. Lancet 1990; **336**:709–11.

14 Falk RJ, Terrell RS, Charles LA, Jennette JC. Anti-neutrophil cytoplasmic antibodies induce neutrophils to degranulate and produce oxygen radicals *in vitro*. Proc Natl Acad Sci USA 1990; **87**:4115–9.

15 Keogan MT, Rifkin I, Ronda N, Lockwood CM, Brown DL. ANCA increase neutrophil adhesion to cultured human endothelium (abstract). In: Groes WL, ed. ANCA-associated vasculitis: immunodiagnostic and pathogenetic value of anti-neutrophil cytoplasmic antibodies. London: Plenum Press, 1993.

16 Savage COS, Gaskin G, Pusey CD, Pearson JD. Anti-neutrophil cytoplasm antibodies can recognize vascular endothelial cell-bound anti-neutrophil cytoplasm antibody-associated autoantigens. Exp Nephrol 1993; **1**:190.

17 Ewert BH, Jennette JC, Falk RJ. Anti-myeloperoxidase antibodies stimulate neutrophils to damage human endothelial cells. Kidney Int 1992; **41**:375–83.

18 van der Woude FJ, Rasmussen N, Lobatto S *et al.* Autoantibodies to neutrophils and monocytes: a new tool for diagnosis and marker of disease activity in Wegener's granulomatosis. Lancet 1985; **i**:425–9.

19 Goldschmeding R, Cohen Tervaert JW, van der Schoot CE, Kallenberg CGM, von dem Borne AEGKr. ANCA, anti-myeloperoxidase and anti-elastase: three members of a novel class of autoantibodies against myeloid lysosomal enzymes. APMIS 1989; **97**:(Suppl. 6)48.

20 Pick E, Mizel D. Rapid microassays for the measurement of superoxide and hydrogenperoxide production by macrophages in culture using automatic enzyme immunoassay reader. J Immunol Methods 1982; **38**:211–8.

21 Brouwer E, Cohen Tervaert JW, Horst G, Huitema MG, van der Giessen M, Limburg PC, Kallenberg CGM. Predominance of IgG1 and IgG4 subclasses of anti-neutrophil cytoplasmic autoantibodies (ANCA) in patients with Wegener's granulomatosis and clinically related disorders. Clin Exp Immunol 1991; **83**:379–86.

22 Borregaard N, Heiple JM, Simons ER, Clark ER. Subcellular localization of the b-cytochrome component of the human neutrophil microbicidal oxidase: translocation during activation. J Cell Biol 1983; **97**:52–61.

23 Fujimoto T, Lockwood CM. Anti-neutrophil antibodies (ANCA) activate protein kinase C in human neutrophils and HL60 cells. Am J Kidney Dis 1991; **18**:204.

24 Keogan MT, Esnault VLM, Green AJ, Lockwood CM, Brown DL. Activation of normal neutrophils by anti-neutrophil cytoplasm antibodies. Clin Exp Immunol 1992; **90**:228–34.

25 Huizinga TWJ, van Kemenade F, Koenderman L, Dolman KM, von dem Borne AEGKr, Tetteroo PAT, Roos D. The 40-kDa Fcγ receptor (FcRII) on human neutrophils is essential for the IgG-induced respiratory burst and IgG-induced phagocytosis. J Immunol 1989; **142**:2365–9.

26 Reibman J, Haines KA, Gude D, Weissmann G. Differences in signal transduction between Fcγ receptors (FcγRII, FcγRIII) and fMLP receptors in neutrophils. J Immunol 1991; **146**:988–96.

27 Anderson CL, Shen L, Eicher DM, Wewer MD, Gill JK. Phagocytosis mediated by three distinct Fc gamma receptor classes on human leukocytes. J Exp Med 1990; **171**:1333–45.

28 Brunkhorst BA, Strohmeier G, Lazzari K, Weil G, Melnick D, Fleit HB, Simons ER. Differential roles of FcγRII and FcγRIII in immune complex stimulation of human neutrophils. J Biol Chem 1992; **267**:20659–66.

29 Hundt M, Schmidt RE. The glycosylphosphatidylinositol-linked Fcγ receptor III represents the dominant receptor structure for immune complex activation of neutrophils. Eur J Immunol 1992; **22**:811–6.

30 Crockett-Torabi E, Fantone JC. Soluble and insoluble immune complexes activate human neutrophil NADPH oxidase by distinct Fc gamma receptor-specific mechanisms. J Immunol 1990; **145**:3026–32.

31 Huizinga TWJ, Kerst M, Nuyens JH, Vlug A, von dem Borne AEGKr, Roos, D, Tetteroo PAT. Binding characteristics of dimeric IgG subclass complexes to human neutrophils. J Immunol 1989; **142**:2359–64.