

## Anti-neutrophil cytoplasm antibody IgG subclasses in Wegener's granulomatosis: a possible pathogenic role for the IgG4 subclass

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### SUMMARY

A characteristic feature of Wegener's granulomatosis is the presence of antineutrophil cytoplasm antibodies (ANCA) to proteinase 3 (PR3). *In vitro*, ANCA activate neutrophils by co-ligating PR3 and Fc $\gamma$ RIIa/IIIb receptors. ANCA are predominantly of the IgG isotype, and IgG1, IgG3 and IgG4 subclasses are particularly represented. To address the pathogenic role of individual ANCA-IgG subclass antibodies, patients' sera were screened using indirect immunofluorescence, enzyme-linked immunosorbent assay (ELISA) and subclass PR3-ELISA to identify patients with high titres of PR3-ANCA within the IgG1, IgG3 or IgG4 subclasses. Unfractionated ANCA-IgG and subclass fractions were isolated by affinity chromatography and compared for their capacities to stimulate superoxide production by primed human neutrophils. Donor neutrophils were analysed for constitutive and induced Fc $\gamma$ RI expression by flow cytometry. The IgG1, IgG3 and IgG4 subclass fractions, isolated from three different ANCA sera, each stimulated superoxide production from neutrophils derived from multiple donors. Subsequently, IgG4 subclass fractions isolated from a further four ANCA positive sera demonstrated varying abilities to stimulate release of superoxide; unrelated to PR3-ANCA titre, neutrophil donor, or neutrophil Fc $\gamma$ RI expression. The stimulation of superoxide release by IgG1- and IgG3-ANCA subclass fractions is consistent with the proposed mechanism of co-ligation of PR3 antigen and Fc $\gamma$ RIIa/IIIb receptors. However, the demonstration of similar activity for the IgG4-ANCA subclass fractions isolated from some sera was unexpected. This activity was independent of neutrophil donor and expression of Fc $\gamma$ RI, suggesting it was capable of activating neutrophils via constitutively expressed Fc $\gamma$ RIIa/IIIb or co-ligation of other, unidentified, cell surface molecules.

**Keywords** ANCA IgG subclasses IgG4 PR3-ANCA Wegener's granulomatosis

### INTRODUCTION

Wegener's granulomatosis (WG) is an autoimmune systemic vasculitic disease in which antineutrophil cytoplasm antibodies (ANCA) are characteristically present in the serum. The predominant specificity is for proteinase 3 (PR3), a serine protease present in cytoplasmic azurophilic granules. These antibodies can be useful diagnostic markers, because their titre correlates with disease activity, falling in remission and rising on relapse [1–3].

Indirect evidence for a pathogenic role for PR3-ANCA *in vivo* is provided by the benefit afforded by plasmapheresis for patients with severe acute disease [4]. Current understanding of possible pathogenic mechanism(s) promoted through PR3-

ANCA in WG vasculitis is based largely on *in vitro* studies. PR3-ANCA have been shown to engage PR3 antigen expressed on the surface of neutrophils, through the Fab regions and to co-ligate Fc $\gamma$  receptors [5–7] resulting in neutrophil activation with secretion of proinflammatory cytokines and the production of a respiratory burst [5,8–10]. There is evidence for ligation by ANCA of the low-affinity Fc $\gamma$ RIIa and Fc $\gamma$ RIIIb receptors, which are constitutively expressed by human neutrophils [8–10]. Involvement of the high-affinity Fc $\gamma$ RI receptor that can be induced on culture with interferon (IFN)- $\gamma$  or granulocyte macrophage colony-stimulating factor (GM-CSF) [11] has been less well studied.

The PR3 antigen is constitutively expressed, at low levels, on the surface of normal neutrophils [12], while treatment with tumour necrosis factor (TNF)- $\alpha$  or GM-CSF increases PR3 expression [13,14]. Exposure to TNF- $\alpha$  or GM-CSF also results in maturation (priming) of normal neutrophils such that on exposure to ANCA they are activated, with the generation of superoxide anions. TNF- $\alpha$  levels are increased in sera of patients with

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vasculitis, resulting in *in vivo* priming [15] and a threefold increase in the level of cell surface PR3 expression has been reported for patients with active WG [16].

ANCA are predominantly of the IgG isotype [17], but studies of their distribution within the IgG subclasses present a rather confused picture. Thus, claims have been made that pathogenicity is correlated with IgG1 and/or IgG4 levels of PR3-ANCA [18] and, similarly, for the related disorder microscopic polyangiitis which is associated with ANCA specific to myeloperoxidase (MPO-ANCA) [19]. Other studies have concluded that ANCA of the IgG3 subclass is more directly associated with pathogenicity in both WG and MPA [17,18,20,21]. By contrast, further studies concluded that IgG3-ANCA titres do not correlate with likelihood of relapse in patients with WG or potency of antibody activities [22,23]. These studies have attempted to correlate ANCA titre with disease activity and do not address deeper questions of the epitope specificity of antibody populations, their impact on immune complex formation and the activation of effector functions. An intriguing feature of autoimmune diseases is the restricted antigen specificity of the autoantibodies and the possible identification of a major antigenic determinant. In established disease the antibody response may be less focused due to the phenomenon of epitope spreading.

Four subclasses of human IgG are defined structurally, functionally and serologically. While a unique profile of effector functions has been ascribed to each IgG subclass (Table 1), such assignments have been shown to be simplistic; studies with defined immune complexes of each subclass have shown that multiple parameters may determine the effector ligand activated and the functional consequences, e.g. epitope density, antibody/antigen ratio [24–26]. In the context of this report it should be noted that IgG4 has been reported to bind the high-affinity receptor Fc $\gamma$ R1 only; however, IgG4-mediated antibody-dependent cellular cytotoxicity reactions have been observed for natural killer (NK) cells isolated from the blood of donors homozygous for the Fc $\gamma$ R1IIa-158 V allele (A. W. Morgan & J. D. Isaacs, unpublished observations). This may be added to numerous other reports of seemingly anomalous activities for IgG4 (reviewed in Aalberse & Schuurman [27]).

In the present report IgG was isolated from the sera of WG patients and assayed to determine the relative titres of IgG subclass ANCA present. Serum samples from the patients with high IgG1, IgG3 or IgG4 ANCA titres were fractionated by affinity chromatography to yield polyclonal IgG1, IgG3 and IgG4

subclass fractions with ANCA activity. We report that polyclonal IgG1, IgG3 and IgG4 fractions containing PR3-ANCA exhibit differential capacity to stimulate a superoxide response in fresh, primed human neutrophils, independent of the neutrophil donor. This may be indicative of differing epitope specificities between the IgG subclass fraction and the sera from which they were isolated. The stimulatory activity of IgG4 ANCA was unexpected since IgG4 has not been reported to ligate/activate Fc $\gamma$ R1IIa or Fc $\gamma$ R1IIb. The possible involvement of Fc $\gamma$ R1 was excluded by its absence from primed neutrophils; further, induction of Fc $\gamma$ R1 expression by overnight stimulation with IFN- $\gamma$  did not result in a significant increase in superoxide production. Deglycosylation of PR3-ANCA IgG resulted in a loss of its activating ability, consistent with Fc $\gamma$ R1IIa or Fc $\gamma$ R1IIb involvement. These findings suggest that IgG4 ANCA can activate neutrophils via constitutively expressed Fc $\gamma$ R1IIa and Fc $\gamma$ R1IIb or co-ligation of other, unidentified, cell surface molecules. This report adds to an increasing literature in which IgG4 antibodies have been shown to exhibit unexpected activities [27].

## PATIENTS AND METHODS

### Sample selection

Fifty-five plasma-derived serum samples from patients with ANCA-associated vasculitis were screened to select samples with PR3-ANCA but not MPO-ANCA or antiglomerular basement membrane (GBM) antibody activity. The final panel was comprised of sera from 14 patients with PR3-ANCA who were diagnosed as suffering from active WG (six female, eight male, mean female age 66.8 years, mean male age 57.9 years). These patients had clinical evidence of WG and were new presentations. The Birmingham Vasculitis Activity Score was >10 in all patients indicating active disease, mean score was 18. All had renal biopsy confirmation of active vasculitis and accorded with the Chapel Hill Consensus Conference definition of WG [28]. All were on prednisolone and cyclophosphamide therapy for a maximum of 24–48 h prior to plasma exchange. The first litre of plasma exchange fluid was retained, aliquoted and stored at –20°C. Thawed plasma was converted to serum by the addition of 1 ml of sterile, 1 M calcium chloride per 50 ml of plasma. The plasma was incubated at 37°C for 1 h and left to clot at 4°C, overnight. The fibrin clots were pelleted by centrifugation at 3000 rpm for 10 min. Clarified serum was removed, passed through 0.2  $\mu$ m filters, dispensed into 5 ml aliquots and frozen at –20°C. Studies were conducted under permission from the Local Research Ethics Committee.

### Indirect immunofluorescence (IIF)

IIF was performed as described [29]. Indirect immunofluorescence results were reported as cANCA, pANCA or negative.

### Anti-PR3, anti-MPO and anti-GBM antibody enzyme-linked immunosorbent assay (ELISA)

Samples were tested for PR3-ANCA, MPO-ANCA and anti-GBM using Bind-azyme kits (Binding Site, Birmingham, UK). The ELISA was performed according to the manufacturer's instructions. Briefly, standards, controls and samples, diluted 1 : 50, 1 : 100 with phosphate buffered saline (PBS)-0.05% Tween were added to wells of PR3-coated plates. Following incubation at room temperature (30 min) the plates were washed three times

**Table 1.** Specificity of human IgG subclasses for Fc $\gamma$ R: + refers to affinity of binding of a subclass for the stated Fc $\gamma$ R, – denotes that a subclass is reported not to bind to the stated Fc $\gamma$ R [44]

	IgG1	IgG2	IgG3	IgG4
Fc $\gamma$ R1 (CD64)	+++	–	+++	+
Fc $\gamma$ R2 (CD32)				
Fc $\gamma$ R2a-H131	++	++	+++	–
Fc $\gamma$ R2a-R131	++	–	+++	–
Fc $\gamma$ R2b	++	–	+++	+
Fc $\gamma$ R3 (CD16)				
Fc $\gamma$ R3a	++	+	++	+
Fc $\gamma$ R3b	++	+	++	–

with 300  $\mu$ l of 1 $\times$  kit wash buffer and 100  $\mu$ l of antihuman IgG (Fc-specific) conjugate was added and incubated for 30 min at room temperature. The plates were washed again three times with 300  $\mu$ l of 1 $\times$  kit wash buffer and 100  $\mu$ l of 3,3',5,5'-tetramethylbenzidine (TMB) substrate added per well. The plates were incubated at room temperature for 30 min and 100  $\mu$ l of 3 M phosphoric acid stopping solution was added to each well. The optical density of the plate was read within 30 min at 450 nm.

#### PR3-ANCA IgG subclass ELISA

ELISA plates precoated with PR3 (Binding Site Birmingham, UK) were incubated with PR3-ANCA positive serum at dilutions of 1 : 50–1 : 10 000 in PBS-0.05% Tween 20 (PBS-T). All samples were run at a minimum of four dilutions. After a 30-min incubation at room temperature, the plates were washed three times with 300  $\mu$ l of the kit wash buffer and 100  $\mu$ l of antihuman IgG1, 2, 3 or 4 horseradish peroxidase-conjugated subclass specific antibody diluted 1 : 1000 with PBS-T, was added (Binding Site, Birmingham, UK). The optical density of the plate was read within 30 min at 450 nm. Anti-PR3 activity was estimated using the calibration samples supplied with the kit. Titres determined at all dilutions were normalized to a 1 : 50 dilution, and the mean was calculated.

#### IgG subclass purifications

All subclass IgG purifications were performed using an automated system composed of a peristaltic pump, fraction collector, UV monitor and chart recorder (Amersham Biosciences, Bucks, UK) connected to the affinity column. Affinity columns were prepared using subclass specific monoclonal antibodies for IgG1, IgG3 or IgG4 (8C/6, ZG4 and RJ4, respectively [30]) and immobilized to CNBr activated Sepharose-6B (Amersham Biosciences, Bucks, UK). Serum was loaded onto columns equilibrated in PBS and the breakthrough fraction was collected following washing with PBS. The bound IgG was eluted with 0.1 M glycine, pH 2.6 and fractions collected in tubes containing 1 M Tris base, pH 9.0 (100  $\mu$ l Tris per 1 ml of eluent) to neutralize the solution. The eluted IgG subclass was dialysed extensively against changes of PBS at 4°C. The flowthrough ANCA-IgG subclass-depleted serum fraction was then loaded onto a HiTrap protein G column (Amersham Biosciences, Bucks, UK) pre-equilibrated with PBS. The column was washed with PBS and, as before, the bound IgG eluted with 0.1 M glycine, pH 2.6. The eluted IgG was extensively dialysed against changes of PBS at 4°C. The isolated ANCA-IgG subclass fractions were concentrated using Vivaspinn concentrators with a 10 000 Da cut-off (Vivascience, Hanover, Germany). The fractions were 0.2  $\mu$ m filtered into a sterile container and the protein concentration of both fractions determined by UV absorption at 280 nm and  $E_{280\text{nm}}^{1\%} = 14.5$ . The samples were aliquoted and stored at 4°C until use.

#### Total IgG and IgG subclass concentrations

The total IgG and IgG subclass concentrations of each PR3-ANCA positive serum was determined in the routine clinical immunology laboratory employing nephelometry, as described by Grange *et al.* [31]. The proportion of each subclass within each sample was calculated.

#### Analysis of the purity of IgG subclass fractions

Sheep red blood cells (SRBC) were sensitized with purified monoclonal antibodies specific for each IgG subclass, by the

chromium chloride method [32]. The appropriate IgG subclass protein agglutinates such cells. Each IgG subclass fraction was titrated in a twofold dilution series in 2% HEPES buffer in the wells of a round-bottomed 96-well microtitre plate; control wells contained 2% HEPES buffer only. Positive control plates were prepared as above using dilutions of known concentrations of purified IgG subclass protein. Sensitized SRBC (25  $\mu$ l) were added and plates incubated at room temperature for 1 h. Relative concentrations of the IgG subclass proteins were determined by comparison of the haemagglutination titre end-points obtained for the IgG subclass fractions and the corresponding purified monoclonal subclass protein.

#### Human neutrophils

Neutrophils were isolated as described previously employing a discontinuous Percoll density gradient [33] from peripheral blood taken from healthy volunteers following informed consent and with permission from the Local Research Ethics Committee. Isolated neutrophils were diluted with Trypan blue solution and counted using a haemocytometer to determine cell viability. All preparations were >95% viable.

Neutrophils were primed for superoxide assays by exposure to cytochalasin B 1 U/ml (Sigma-Aldrich Company Ltd, Dorset, UK) for 5 min followed by 2 U/ml TNF- $\alpha$  (NISBC, Potters Bar, UK) for 15 min.

#### Culture of neutrophils with IFN- $\gamma$

Freshly isolated neutrophils were resuspended in Iscove's DMEM (Dulbecco's modified Eagle's medium) (Sigma-Aldrich Company Ltd, Dorset, UK), with added 10% autologous human serum to maintain the cells viability, in the presence or absence of 400 U/ml IFN- $\gamma$  (R&D Systems Europe Ltd, Abingdon, Oxon, UK) for 18 h at 37°C. Following incubation cells were analysed by single or dual stain flow cytometry (FacScan, BD Biosciences, Oxford, UK). IFN- $\gamma$  treated neutrophils were also primed as described above and used in superoxide assays; neutrophil counts were based on viable cells only.

#### Superoxide generation

Superoxide assays were performed as described previously using a kinetic microplate assay that measures the superoxide dismutase inhibitable reduction of ferricytochrome C [7]. The plates were scanned at 550 nm at 0, 5, 30, 60 and 100 min using a Multiscan® Bichromatic plate reader (Thermo Labsystems Oy, Helsinki, Finland), between readings the plates were incubated at 37°C. Each test was performed in triplicate.

The activity of the purified IgG subclass preparations was determined at concentrations equivalent to those that were present in the whole serum IgG. Thus, if the input total IgG was at a concentration of 200  $\mu$ g/ml and the IgG4 content represented 10% of the total IgG, the purified IgG4 fractions was assayed at an initial concentration of 20  $\mu$ g/ml.

#### Single stain flow cytometric analysis for Fc $\gamma$ RI or PR3 expression

Aliquots of neutrophils ( $5 \times 10^5$ ) were exposed to saturating concentrations of FITC-conjugated anti-Fc $\gamma$ RI (Medarex Inc, Princeton, NJ, USA) or unconjugated 4A5-anti-PR3 antibody (Wieslab, Lund, Sweden), the latter being followed by FITC-conjugated goat antimouse F(ab')<sub>2</sub> (Dako Ltd, Ely, Cambridgeshire, UK) for 30 min at 4°C. The cells were fixed in 1%

paraformaldehyde solution and flow cytometric analysis performed (FacScan, BD Biosciences, Oxford, UK).

#### Dual stain flow cytometric analysis

Aliquots of freshly isolated neutrophils ( $5 \times 10^5$ ), cultured overnight in the presence or absence of IFN- $\gamma$  (R&D Systems Europe Ltd), were stained for 45 min at 4°C with saturating concentrations of FITC conjugated anti-Fc $\gamma$ RI (Medarex Inc.). Cells were washed with PBS, pelleted at 400 g for 5 min and resuspended in 100  $\mu$ l of annexin V kit binding buffer. Cells were incubated with saturating concentration of PE conjugated annexin V (BD Biosciences, Oxford, UK) for 15 min in the dark. Following incubation, 400  $\mu$ l of annexin V kit binding buffer was added and flow cytometric analysis was performed (FacScan, BD Biosciences).

#### Preparation of deglycosylated IgG

Purified IgG was dialysed into 40 mM potassium phosphate, 10 mM EDTA, pH 7.4. PNGase F (1 U/0.5 mg IgG) (Roche Diagnostics Ltd, East Sussex, UK) was added and the sample incubated at 37°C for 72 h. Control IgG was incubated for the same time period without the addition of enzyme. The deglycosylated and control antibody samples were purified on a 1 ml HiTrap protein G column (Amersham Biosciences, Bucks, UK) and frozen at -20°C until purification. The extent of deglycosylation was assessed by SDS-PAGE analysis of the heavy chain mass [33].

## RESULTS

#### PR3-ANCA IgG subclass ELISA

Serum samples from 14 PR3-ANCA WG patients selected for study and six controls were analysed to determine IgG subclass PR3-ANCA titres, using a modified PR3-ELISA as described. A summary of the mean IgG subclass anti-PR3 titres for each patient is given in Table 2. The titres of subclass restricted anti-PR3 antibodies can be compared between samples for each individual IgG subclass. Comparisons between titres for each subclass within a given sample are more problematic due to possible differences between the IgG subclass specific antibodies, e.g. affinity, number of epitopes recognized, etc.

**Table 2.** Summary of IgG PR3-ANCA subclass ELISA results

P	IgG1 PR3 titre (U/ml)	IgG2 PR3 titre (U/ml)	IgG3 PR3 titre (U/ml)	IgG4 PR3 titre (U/ml)
1	10	2	200	90
2	400	3	190	10
3	50	40	10	1800
4	10	4	40	10
5	10	2	20	90
6	20	3	180	10
7	5	2	5	20
8	50	6	2000	10
9	300	15	70	20
10	600	9	150	50
11	30	5	290	10
12	70	3	160	10
13	10	3	70	5
14	50	4	1500	200

The profile of the IgG subclass anti-PR3 titres differed for each serum sample with mainly one subclass predominating. Thus, IgG1 anti-PR3 predominated in samples P2, P9 and P10; IgG3 in P1, P4, P6, P8, P11-14; IgG4 in P3, P5 and P7. IgG2 did not predominate in any sample and significant titres were observed for P3 and P9 only.

#### Affinity purification and characterization of ANCA positive IgG subclass fractions

IgG1, IgG3 and IgG4 subclass fractions were isolated from sera P2, P8 and P3, respectively. Each IgG subclass preparation was shown to be free of contaminating serum proteins, by SDS-PAGE, and comprised essentially of a single subclass (>95% pure as determined by the haemagglutination assay). Samples of total unfractionated IgG, IgG subclasses fractions, and IgG from healthy controls were assayed by PR3-ELISA (antihuman IgG Fc specific conjugate) at 100  $\mu$ g/ml; the IgG subclass fractions were also assayed at concentrations proportional to their concentrations in the original serum sample (Table 3), i.e. for sample P2 this comprised 49% of the total IgG, therefore anti-PR3 titre was determined at 49  $\mu$ g/ml.

Importantly, anti-PR3 binding was detected in isolated subclass fractions for P2, P8 and P10 but not in normal IgG preparations.

#### Activation of primed human neutrophils with IgG1-, IgG3- and IgG4-ANCA

The ability of each ANCA-IgG positive fraction to stimulate generation of superoxide from freshly isolated primed neutrophils was determined; each sample was tested against neutrophils from a minimum of three different donors. The baseline concentration used for testing the purified subclass isolates was that which was proportional to the concentration in the original serum samples, as described above.

**Table 3.** Anti-PR3 ELISA results for (A) P2 IgG1 (B) P8 IgG3 and (C) P3 IgG4 fractionations. Results are shown for samples of unfractionated IgG, isolated subclasses and normal IgG for each ELISA. Each was tested at 100  $\mu$ g/ml and the purified subclass antibodies were also tested at concentrations equivalent to their percentage within the original serum sample

Sample	Anti-PR3 titre (U/ml)
(a)	
P2 IgG 100 $\mu$ g/ml	77
P2 IgG1100 $\mu$ g/ml	52
P2 IgG1 49 $\mu$ g/ml	22
Normal IgG 100 $\mu$ g/ml	5.4
(b)	
P8 IgG 100 $\mu$ g/ml	156
P8 IgG3100 $\mu$ g/ml	152
P8 IgG3 13 $\mu$ g/ml	115
Normal IgG 100 $\mu$ g/ml	0.8
(c)	
P3 IgG 100 $\mu$ g/ml	133
P3 IgG4100 $\mu$ g/ml	120
P3 IgG4 22 $\mu$ g/ml	86
Normal IgG 100 $\mu$ g/ml	0.8



The IgG1-ANCA fraction isolated from serum P2 elicited a superoxide response in a dose-dependent manner at 100 min (Fig. 1a) and at the earlier time-points of 5, 30 and 60 min (not shown). However, it was not as vigorous as that seen within the original unfractionated ANCA-IgG. Even increasing the concentration of IgG1-ANCA to 100 and 200  $\mu\text{g}/\text{ml}$  did not increase the response ( $4.4 \pm 0.9$  and  $4.9 \pm 1.28$  nmol superoxide, respectively, at 100 min) above that seen with unfractionated P2 ANCA-IgG.

The IgG3-ANCA fraction isolated from serum P8 elicited rapid and vigorous responses ( $7.3 \pm 1.3$  nmol superoxide at 100 min using 13  $\mu\text{g}/\text{ml}$  as present in the unfractionated P3 IgG) (Fig. 1b). Increasing the concentration of IgG3 above that present in the original sample led to a further increase in superoxide production, so that at 25, 50 and 100  $\mu\text{g}/\text{ml}$  the response was  $13.4 \pm 2.6$ ,  $16.8 \pm 2.3$  and  $22.2 \pm 2.2$  nmol, respectively, at 100 min, exceeding that obtained for unfractionated P8 IgG at 100  $\mu\text{g}/\text{ml}$  ( $10.4 \pm 3.6$  nmol at 100 min).

The IgG4-ANCA fraction isolated from serum P3 also elicited a vigorous superoxide response ( $5.5 \pm 2.2$  nmol at 100 min using 22  $\mu\text{g}/\text{ml}$  as present in the unfractionated P3 ANCA-IgG) (Fig. 1c). Increasing the concentration of IgG4-ANCA to 100  $\mu\text{g}/\text{ml}$  and 200  $\mu\text{g}/\text{ml}$  increased superoxide production to  $12.4 \pm 5.3$  and  $13.6 \pm 2.9$  nmol, respectively, at 100 min, more than that obtained with the original unfractionated ANCA-IgG

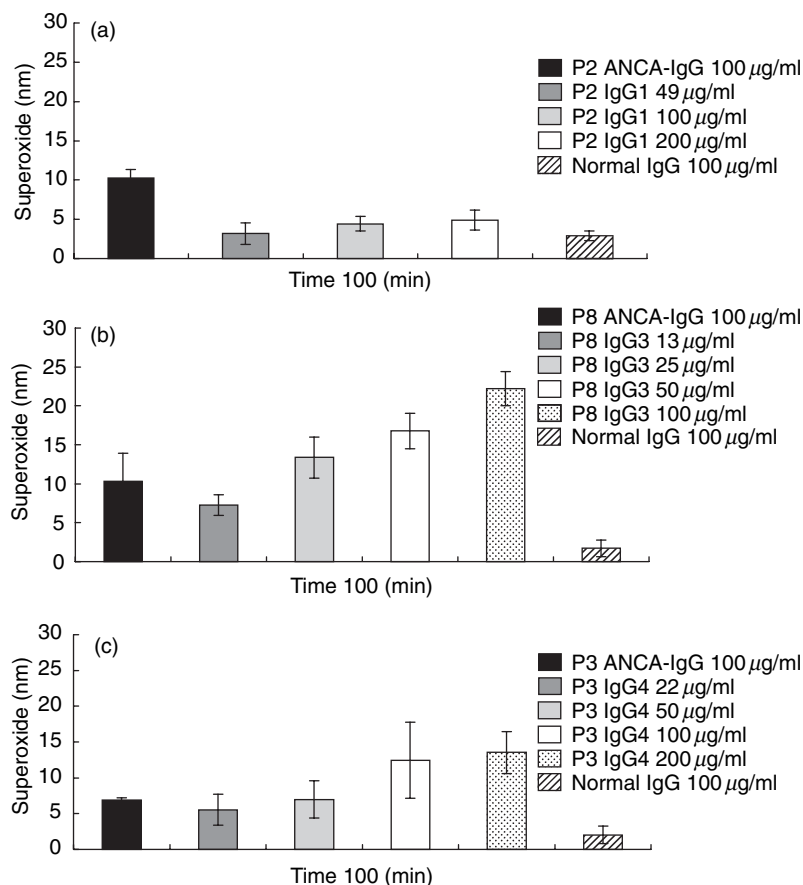
( $6.9 \pm 0.28$  nmol at 100 min). It was apparent from these studies that the binding activity in the PR3-ANCA ELISA did not correlate with the ability of any sample or sample fraction to activate neutrophils in the superoxide assay. It was also apparent that IgG4-ANCA was able to activate freshly isolated primed neutrophils.

#### Activation of primed human neutrophils with IgG4-ANCA isolated from four individual WG patients

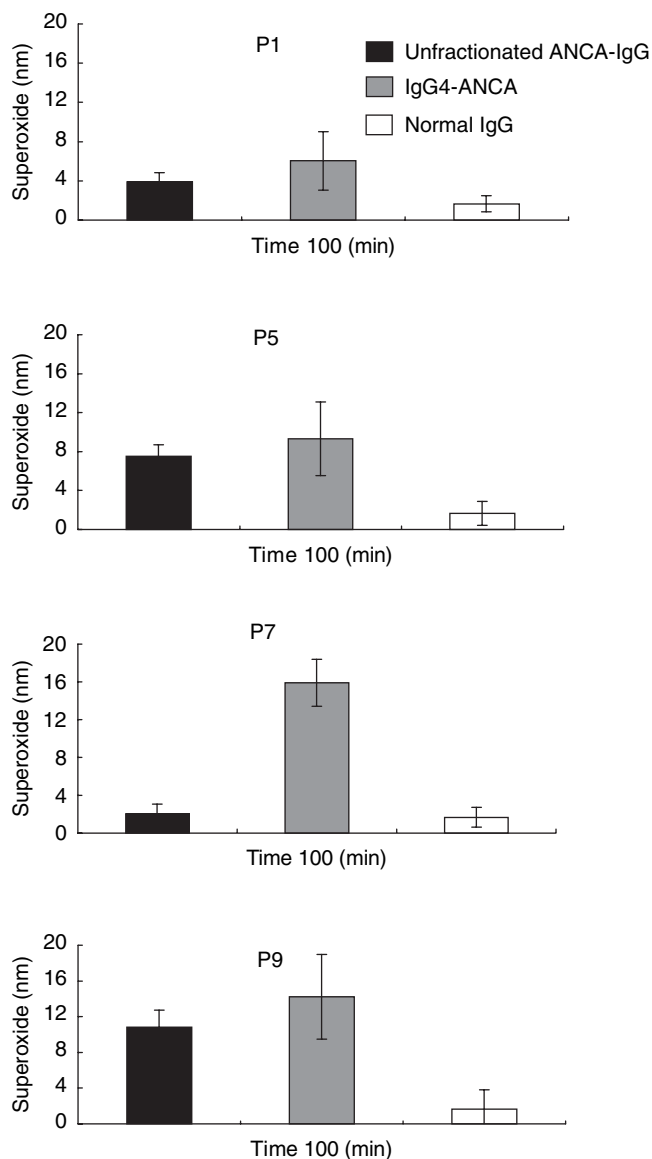
The generality of IgG4-ANCA activation of neutrophils was investigated following the isolation of the IgG4 subclass fraction from sera P1, P5, P7 and P9. Unfractionated ANCA-IgG and the IgG4-ANCA subclass fractions were assayed for superoxide production using four different neutrophil donors (Fig. 2). All four IgG4-ANCA fractions, tested at 200  $\mu\text{g}/\text{ml}$ , produced a superoxide response. The purified P7 IgG4-ANCA was particularly activating, compared with the unfractionated ANCA-IgG from which it was purified.

#### Fc $\gamma$ RI expression and superoxide production by human neutrophils cultured with IFN- $\gamma$

ANCA-IgG is believed to activate neutrophils via co-ligation of Fc $\gamma$ RIIa and/or Fc $\gamma$ RIIIb [10]; however, IgG4 would not be expected to activate freshly isolated neutrophils through these



**Fig. 1.** Mean superoxide production stimulated by ANCA-IgG and fractionated ANCA-IgG subclasses from patients P2, P8 and P3 in primed neutrophils. Superoxide production was measured for 100 min in response to 100  $\mu\text{g}/\text{ml}$  of ANCA-IgG or normal IgG. For ANCA-IgG subclasses, IgG1-ANCA from patient P2, shown in (a), was used at 49, 100 and 200  $\mu\text{g}/\text{ml}$ ; IgG3-ANCA from P8, shown in (b), was used at 13, 25, 50 and 100  $\mu\text{g}/\text{ml}$  and IgG4-ANCA from P3, shown in (c), was used at 22, 50, 100 and 200  $\mu\text{g}/\text{ml}$ . Results are expressed as nmol/ $1 \times 10^5$  cells. Data shown are mean  $\pm$  s.e.m. for experiments performed in triplicate on four neutrophil donors.



**Fig. 2.** Mean superoxide production stimulated by unfractionated ANCA-IgG and IgG4-ANCA samples from patients P1, P5, P7 and P9 in primed neutrophils. Superoxide production was measured for 100 min in response to 100  $\mu\text{g/ml}$  of unfractionated ANCA-IgG (black bars) or normal-IgG (white bars) and 200  $\mu\text{g/ml}$  IgG4-ANCA (dark grey bars). Results are expressed as  $\text{nmol/l} \times 10^5$  cells. Data shown are mean  $\pm$  s.e.m. for experiments performed in triplicate on four neutrophil donors.s.e.m.

receptors. Because IgG4 can ligate  $\text{Fc}\gamma\text{RI}$ , a possible role for  $\text{Fc}\gamma\text{RI}$  in IgG4-ANCA-induced neutrophil activation was investigated. Unfortunately, effective blocking antibodies to  $\text{Fc}\gamma\text{RI}$  are not available, so more indirect approaches had to be taken to investigate this.

The expression of  $\text{Fc}\gamma\text{RI}$  was evaluated on freshly isolated,  $\text{TNF-}\alpha$ -primed neutrophils and on neutrophils that had been cultured for 18 h in the presence of  $\text{IFN-}\gamma$  prior to priming. As established previously, freshly isolated neutrophils primed with  $\text{TNF-}\alpha$  showed increased bimodal surface PR3 expression compared with unprimed neutrophils [34,35], indicating that PR3 expression increases but the overall proportion of low and high PR3-

**Table 4.** Mean superoxide production stimulated by unfractionated ANCA IgG, deglycosylated ANCA IgG and sham deglycosylated ANCA IgG samples in fresh, primed human neutrophils. Superoxide production was measured in response to 100  $\mu\text{g/ml}$  of all IgG samples. Data are shown at 100 min  $\pm$  standard error. Results are expressed as  $\text{nmol/l} \times 10^5$  cells. Experiments performed in triplicate on three neutrophil donors

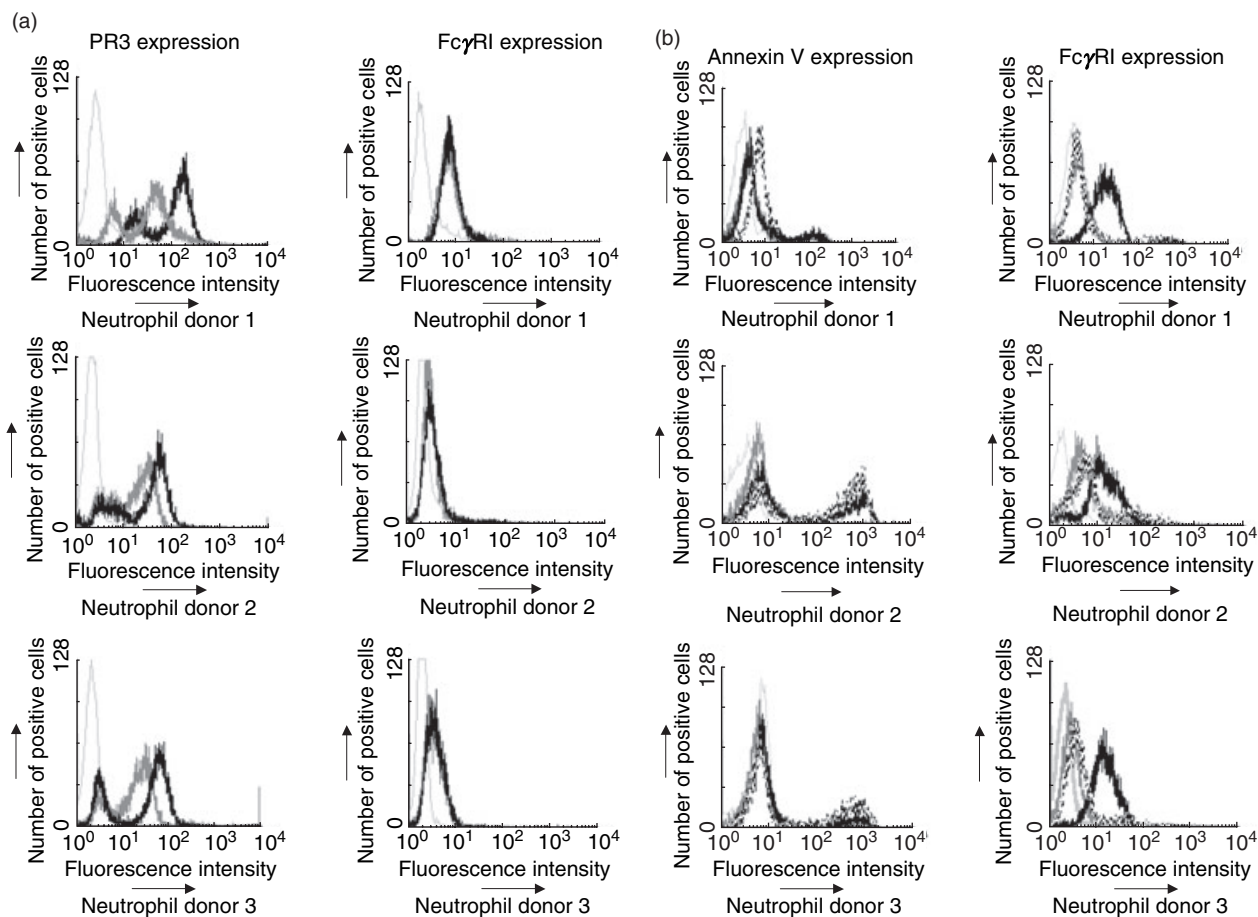
Sample	Superoxide production at 100 min (nm)
P8 IgG	6.3 ( $\pm$ 1.3)
P8 IgG sham deglycosylation	6.5 ( $\pm$ 1.4)
P8 IgG deglycosylated	0.7 ( $\pm$ 0.9)
P3 IgG	2.3 ( $\pm$ 1.0)
P3 IgG sham deglycosylation	2.6 ( $\pm$ 0.9)
P3 IgG deglycosylated	0.7 ( $\pm$ 0.7)
Normal IgG	0.7 ( $\pm$ 0.4)
Normal IgG sham deglycosylation	1.2 ( $\pm$ 0.2)
Normal IgG deglycosylated	0.1 ( $\pm$ 0.2)

expressing cells remains stable, yet there was no significant effect on  $\text{Fc}\gamma\text{RI}$  expression (Fig. 3a). In contrast,  $\text{Fc}\gamma\text{RI}$  expression was up-regulated following overnight exposure to  $\text{IFN-}\gamma$ . Prolonged culture of neutrophils enhances apoptosis, with characteristic up-regulation of annexin V expression. Therefore, following overnight culture in the presence or absence of  $\text{IFN-}\gamma$ , neutrophils were analysed by flow cytometry for the expression of annexin V and  $\text{Fc}\gamma\text{RI}$ . Freshly isolated, primed neutrophils showed low-levels of expression of annexin V and  $\text{Fc}\gamma\text{RI}$  (Fig. 3b), there is increased annexin V in donor 2 compared to other donors, which may be due to more cells being committed to apoptosis. Overall, the level of expression of annexin V was unaffected by overnight culture in the presence or absence of  $\text{IFN-}\gamma$ , however, expression of  $\text{Fc}\gamma\text{RI}$  was substantially increased following  $\text{IFN-}\gamma$  treatment.

Neutrophils cultured in the presence or absence of  $\text{IFN-}\gamma$  were assessed for their relative abilities to produce superoxide in response to activation with unfractionated ANCA-IgG and IgG4-ANCA fractions, isolated from serum P3 and a pool of sera P1, P5, P7 and P9 (Fig. 4). For these assays the viability of the cultured neutrophils was determined by Trypan blue exclusion and the same number of viable cells were used in assays with freshly isolated and cultured neutrophils. In each case, the response obtained with fresh neutrophils was greater than for cells cultured in the presence or absence of  $\text{IFN-}\gamma$ . Therefore, the induction/up-regulation of  $\text{Fc}\gamma\text{RI}$  expression following culture in the presence of  $\text{IFN-}\gamma$  did not enhance neutrophil activation by IgG4-ANCA.

#### Activation of primed human neutrophils with deglycosylated IgG-ANCA

Samples of IgG isolated from serum P3 and P8 and normal (control) IgG were subjected to sham and active deglycosylation. Glycosylation of IgG is necessary for activation of  $\text{Fc}\gamma\text{RII}$  and  $\text{Fc}\gamma\text{RIII}$ , but not  $\text{Fc}\gamma\text{RI}$ . Analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) confirmed deglycosylation of the sample exposed to PNGase F. While the P3 and P8 IgG-ANCA samples and their sham deglycosylated equivalents provoked superoxide production, the deglycosylated IgG did not (Table 4), suggesting that  $\text{Fc}\gamma\text{RII}$  and/or  $\text{Fc}\gamma\text{RIII}$ , but not  $\text{Fc}\gamma\text{RI}$ , was ligated.



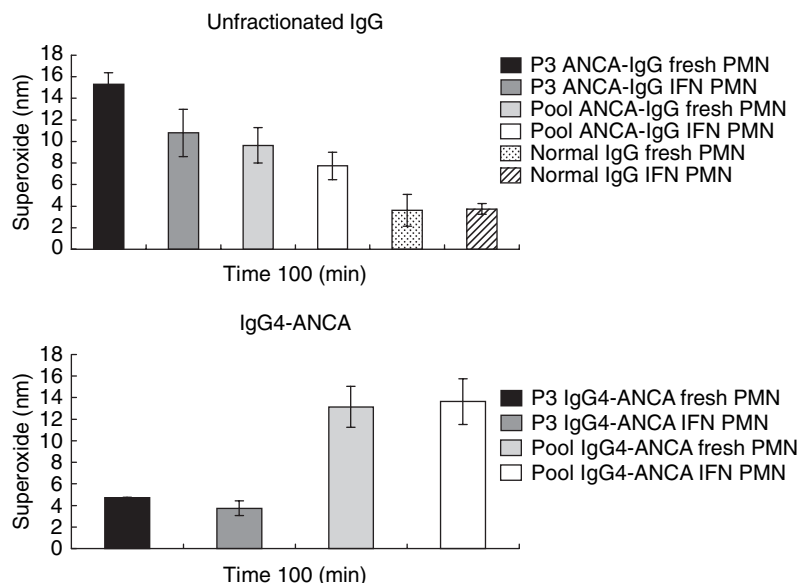
**Fig. 3** (a) FACS analysis histograms of isolated neutrophils to detect PR3 and FcγRI expression. Dark grey histograms represent expression by unprimed, freshly isolated cells and black histograms represent expression after priming of cells for 15 min with 2 U/ml of TNF- $\alpha$ . Light grey bars histograms represent isotype control IgG1 binding. (b) FACS analysis histograms of isolated neutrophils, dual stained to detect annexin V and FcγRI expression. Dark grey histograms represent expression after priming of cells for 15 min with 2 U/ml of TNF- $\alpha$ . Black histograms represent expression after treatment with 400 U/ml of IFN- $\gamma$  for 18 h while dashed line histograms represent cells cultured without IFN- $\gamma$  for 18 h. Light grey histograms represent isotype control IgG1 binding.

## DISCUSSION

We are engaged in a detailed study of parameters contributing to ANCA activation of human neutrophils. A number of sera from patients with active WG were screened and a panel of 14 were selected that contained PR3-ANCA but not MPO-ANCA. In the present report we demonstrate that IgG4 PR3-ANCA has the ability to activate cytokine-primed neutrophils. This finding is difficult to explain in terms of the consensus view that PR3-ANCA activates neutrophils following binding to surface PR3 antigen and co-ligation of FcγRIIa and/or FcγRIIIb as IgG4 is reported to bind FcγRI and not FcγRIIa/FcγRIIIb. However, close scrutiny of the literature provides evidence of numerous well-defined systems in which, unexpectedly, it has been concluded that IgG4 may act through FcγRII and/or FcγRIII receptors; these interactions may be independent of, or aided by, the presence of complement [24,27].

Initially we identified three sera (P2, P8 and P3) with high titre PR3-ANCA of IgG1, IgG3 or IgG4 subclass, respectively. Polyclonal IgG subclass fractions were isolated from patient sera by affinity purification. All IgG fractions were shown to

retain PR3-ANCA activity, as determined by ELISA, and the IgG subclass fraction shown to be >95% pure, with respect to subclass composition. The IgG1-ANCA, IgG3-ANCA and IgG4-ANCA were each shown to activate primed neutrophils with the production of superoxide anion. The activity of the IgG1 and IgG3 ANCA fractions is consistent with proposed mechanisms of neutrophil activation by PR3-ANCA, namely PR3 engagement and co-ligation of FcγRIIa and/or FcγRIIIb. However, the activity of the IgG4 fraction was unexpected. To evaluate the generality of these findings, four sera that gave positive IgG4-ANCA titres in ELISA were subject to IgG4 subclass affinity purification. The individual IgG4-subclass fractions activated primed neutrophils. Interestingly the activating potential of isolated IgG4 sample P7 was greater than that of the original unfractionated ANCA-IgG, showing that the PR3-ANCA IgG subclass titre is not a guide to functionality, indeed this was also so of the unfractionated PR3-ANCA IgG samples. This may relate to affinity of PR3-ANCA or competition for epitopes by polyclonal PR3-ANCA and purifying the IgG subclasses may have reduced this, helping to establish which subclasses are contributing to ANCA activity. *In vivo*, antibody/



**Fig. 4.** Mean superoxide production stimulated by P3 and pooled ANCA-IgG in fresh and IFN- $\gamma$ -treated neutrophils. Unfractionated ANCA-IgG and IgG4-ANCA were isolated from the serum of patient P3 and from a pool made up of serum from patients P1, P5, P7 and P9. Superoxide production was measured for 100 min in response to unfractionated 100  $\mu$ g/ml ANCA or normal IgG and 200  $\mu$ g/ml IgG4-ANCA. Neutrophils were cultured for 18 h in medium supplemented with 400 U/ml IFN- $\gamma$  or freshly isolated from the same donors. Results are expressed as nmol/l  $\times 10^5$  cells superoxide measured at 100 min. Data shown are mean  $\pm$  s.e.m. for experiments performed in triplicate on three neutrophil donors.

antigen ratios and complement activating ability may also affect the functional outcome [24].

IgG4 is known to bind and activate Fc $\gamma$ RI and there are reports that neutrophils may express Fc $\gamma$ RI constitutively, at very low levels, and that Fc $\gamma$ RI is induced/ $\mu$ p-regulated following culture of neutrophils with G-CSF or IFN- $\gamma$  [36]. To explore the possibility that IgG4-ANCA were activating primed neutrophils by ligation of Fc $\gamma$ RI, the expression of Fc $\gamma$ RI on freshly isolated primed neutrophils and on neutrophils following overnight culture in the presence of IFN- $\gamma$  was evaluated by flow cytometry. Overnight culture of neutrophils is known to promote apoptosis, which could affect neutrophil viability and their ability to be activated, so expression of annexin V, a marker of apoptotic cells, was also tested. In addition, equal numbers of live neutrophils were employed for the activation studies. PR3 expression was up-regulated following priming with TNF $\alpha$  for 15 min as expected, but Fc $\gamma$ RI expression was at background levels. Fc $\gamma$ RI expression was unaffected by priming or overnight culture in the absence of IFN- $\gamma$  but was induced significantly on culture in the presence of IFN- $\gamma$ . Despite these increases in Fc $\gamma$ RI expression, the levels of superoxide production from viable neutrophils cultured overnight with IFN- $\gamma$  and primed with TNF- $\alpha$  were less (with P3) or unchanged (with pooled IgG4 samples) than for fresh primed neutrophils. These data do not support a role for Fc $\gamma$ RI in IgG4 PR3-ANCA activation of primed neutrophils. Further, deglycosylation of PR3-ANCA IgG abolished the ability to induce a respiratory burst suggesting that IgG4-ANCA can ligate Fc $\gamma$ RIIIa/Fc $\gamma$ RIIIb but not Fc $\gamma$ RI. These results demonstrate clearly that polyclonal IgG4-ANCA isolated from the sera of some WG patients can activate neutrophils to produce a superoxide response, reproducibly and independent of neutrophil donor.

If IgG4-ANCA in common with IgG1-ANCA and IgG3-ANCA can ligate Fc $\gamma$ RIIIb on neutrophils, then yet other properties may determine the biological outcome. For example, the extent of fucosylation [37] and/or galactosylation and sialylation [38] of the IgG antibody, the glycosylation status of the Fc $\gamma$ RIII [39] or Fc $\gamma$ RIII polymorphisms that may influence Fc $\gamma$ RIII engagement and activation [38,40]. Numerous other parameters could be added including the allotype of the antibody, the epitope target and the antigen on which it is expressed. How such variables interact with IgG4 has yet to be determined. ANCA may also interact with monocytes [41] and these cells express Fc $\gamma$ RIIIa. There is some evidence that IgG4 is bound by only the Fc $\gamma$ RIIIa-158 V isoform [42,43]. This difference in binding affinity also appears to translate to alterations in IgG4-mediated effector functions, with IgG4-mediated antibody-dependent cellular cytotoxicity reactions occurring only in those individuals that carry the Fc $\gamma$ RIIIa-158 V allele (A. W. Morgan & J. D. Isaacs, unpublished observations).

In conclusion, we predict that the mere presence of PR3-ANCA in the serum of a patient with Wegener's granulomatosis will not predict detailed pathogenic potential of the autoantibody, as multiple factors, including IgG subclass and epitope specificity, will determine this.

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