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Activation of granulocytes by anti-neutrophil cytoplasmic antibodies (ANCA) in Wegener's granulomatosis: a predominant role for the IgG3 subclass of ANCA

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SUMMARY

To study ANCA-induced granulocyte activation in relation to disease activity in Wegener's granulomatosis (WG), serum samples taken from patients with WG at the time of active ($n = 17$) and inactive ($n = 17$) disease were analysed for their capacity to activate primed normal donor granulocytes. Compared with control sera ($n = 6$), the capacity of IgG fractions from patients with WG to induce the respiratory burst was significantly higher ($P < 0{\cdot}0001$). Furthermore, the capacity to induce the respiratory burst significantly correlated with ANCA titre ($r = 0{\cdot}499$, $P = 0{\cdot}003$). IgG fractions from patients with active extensive disease induced the respiratory burst significantly more stongly than IgG fractions from patients with limited disease ($n = 7$) ($P < 0{\cdot}01$) or patients during disease remission ($n = 17$) ($P < 0{\cdot}001$). As ANCA-induced neutrophil activation is Fc-dependent and different IgG subclasses are involved in the interaction with various Fc receptors from neutrophils, we assessed changes in ANCA titre, total IgG and IgG subclass distribution of ANCA during active disease and remission in relation to the neutrophil-activating capacity of ANCA. Changes in capacity to activate granulocytes were related neither to changes in titre nor to changes in levels of total IgG, IgG1, IgG3, or IgG4 subclass of ANCA. However, changes in capacity to induce the respiratory burst were significantly related to changes in the relative amount of the IgG3 subclass of ANCA ($P < 0{\cdot}001$), and not to changes in the relative amount of IgG1 or IgG4 subclass of ANCA. These data suggest that the increase in neutrophil-activating capacity of ANCA from inactive to active disease is, at least in part, based on the relative increase of the IgG3 subclass of ANCA that occurs during active disease.

Keywords ANCA neutrophil activation FcγR IgG3

INTRODUCTION

ANCA directed against the third serine proteinase of human granulocytes, proteinase 3 (PR3), have been described as sensitive and specific markers for Wegener's granulomatosis (WG) [1–3]. The autoantibodies produce a cytoplasmic staining pattern (C-ANCA) on ethanol-fixed neutrophils by indirect immunofluorescence (IIF), in contrast to the predominantly perinuclear pattern (P-ANCA) produced by ANCA directed against myeloperoxidase (MPO). The latter antibodies are associated with different forms of vasculitis and idiopathic crescentic glomerulonephritis [4]. A role for C-ANCA in the pathogenesis of WG has been suggested by various longitudinal studies demonstrating that disease activity of WG is preceded by increase in C-ANCA titre [3,5], and early treatment based on changes in C-ANCA levels prevents relapses of the disease [6]. The possible pathophysiological role of C-ANCA has, however, not been elucidated.

Falk *et al.* [7] have demonstrated that ANCA directed against PR3 and MPO induce, *in vitro*, the respiratory burst and degranulation of normal donor granulocytes primed with tumour necrosis factor-alpha (TNF-α). These data were confirmed by others [8,9]. Further studies showed that ANCA enhance the adherence of neutrophils to endothelial cells and neutrophil-mediated target cell destruction [10,11]. In addition, as PR3 and MPO are cationic proteins that readily stick to endothelial cells or may even be expressed by these cells [12], ANCA may induce complement-dependent cytotoxicity by binding to their localized antigens [13].

The precise mechanisms of ANCA-induced neutrophil activation have not been unravelled yet. We and others have demonstrated that ANCA-induced neutrophil activation is dependent on Fc receptors on the neutrophils [9,14], in

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particular the second Fc receptor for IgG (FcγRII). In addition, our data suggested a predominant role for ANCA of the IgG3 subclass in neutrophil activation, since sera capable of inducing the respiratory burst had increased levels of IgG3 subclass of ANCA, compared with sera that did not induce granulocyte activation [9]. The latter findings were, however, based on a transsectional study in only a few patients.

Following our previous study we evaluated whether active disease in WG is associated not only with an increase in C-ANCA titre but also with changes in functional characteristics of C-ANCA. In addition, we studied in a longitudinal set of serum samples the neutrophil-activating capacity in relation to the IgG subclass distribution of C-ANCA.

MATERIALS AND METHODS

Sera

Serum samples were obtained from patients with biopsy-proven WG at the moment of diagnosis ($n = 17$), and from patients during clinical remission within a period of 3–12 months after the time of diagnosis ($n = 17$). Paired serum samples from the moment of diagnosis and from clinical remission were available from 13 patients. All samples were positive for C-ANCA at the time of diagnosis. The diagnosis of WG was established according to clinical and histological criteria [15], and all patients fulfilled the criteria for the classification of WG as described by the American College of Rheumatologists [16]. Disease activity of WG was scored using a disease activity index, and a distinction between extensive and limited disease activity was made as described before [6,17]. Briefly, limited disease activity was defined as active lesions of WG in the upper or lower airways without evident vasculitic activity in other organs. Extensive disease activity was defined by renal involvement with deteriorating renal function, with erythrocyte casts or biopsy-proven necrotizing glomerulonephritis, pulmonary involvement with impending respiratory failure, cerebral vasculitis, or acute abdomen or massive gastrointestinal haemorrhage due to vasculitis. Remission was defined as the complete absence of signs or symptoms of disease activity in combination with a normal C-reactive protein (CRP) level. Characteristics of the patients are given in Table 1.

Table 1. Characteristics of the patient ($n = 17$) at the moment of diagnosis of Wegener's granulomatosis (WG) grouped according to extensive or limited disease activity

	Extensive WG ($n = 10$)	Limited WG ($n = 7$)
Male/female	7/3	5/2
Age, years*	61 (20–72)	44 (35–70)
Disease activity*†	28·5 (17–35)	10 (6–16)
Organ involvement (%)		
Ear, nose, throat	10 (100)	7 (100)
Lungs	8 (80)	2 (29)
Kidney	10 (100)	—
Eyes	6 (60)	4 (57)
Skin	8 (80)	1 (14)
Joints	10 (100)	4 (57)
Nervous system	4 (40)	—
Gastrointestinal tract	2 (20)	—
Constitutional symptoms	10 (100)	4 (57)

* Median (range).
† Scored as reported in [16].

Control plasma samples consisted of freshly drawn samples from healthy volunteers ($n = 4$), or samples positive for antinuclear antibodies (ANA) ($n = 2$).

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).

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 < 5 pg/ml endotoxin as determined by the Limulus amoebocyte assay.

Detection of ANCA by IIF

Detection of ANCA was performed as described previously [1]. Test or control sera were applied in 1:16–1:512 serial dilutions, and C-ANCA was found to be present when IIF showed a cytoplasmic pattern with accentuation of the fluorescence intensity in the area between the nuclear lobes. 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 [18], was used to test sera for the presence of antibodies to either PR3, MPO, or elastase. Results by ELISA were considered positive when the value obtained exceeded the mean of 30 normal control sera by > 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 wash steps with ice-cold PBS, the granulocytes were suspended in Hanks' balanced salt solution (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, Heidelberg, Germany). Priming of the granulocytes was performed by incubation with recombinant TNF-α (rTNF-α; Genzyme, Cambridge, MA), 2 ng/ml for 15 min.

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 [19], 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 readings the plates were kept at 37°C. Each test was performed in quadruplicate. Superoxide production of the neutrophils was expressed as the difference in OD 550 nm between the ferri-cytochrome C reduction in the absence and in the presence of superoxide dismutase; this ΔOD is directly proportional to the amount of superoxide produced.

IgG subclass detection of ANCA by ELISA

IgG subclass detection of ANCA was performed as described previously [20]. Since in previous studies we were unable to detect significant amounts of IgG2 subclass of ANCA [9,20], only levels of IgG1, IgG3, and IgG4 subclass of ANCA were analysed. In brief, a crude granule extract was isolated by nitrogen cavitation of neutrophils as described by Borregaard *et al.* [21]. Microtitre plates were coated with the extract at a protein concentration of 20 μg/ml in 0·1 M carbonate buffer pH 9·6 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 IgG3 clone MH163–1-Mo5, anti-human IgG4 clone MH164–4; CLB, Amsterdam, The Netherlands) at a dilution of 1 : 250. Antibody binding was detected with alkaline phosphatase-conjugated goat anti-mouse IgG (A7157; Sigma). Values are expressed in OD units after subtraction of blanks.

Statistical analysis

Data are given as mean ± s.d. unless stated otherwise. Statistical calculations were carried out with an MS-DOS computer using the InStat V 2.04 software package (GraphPad Software, San Diego, CA). Statistical analysis was performed using the Mann–Whitney rank sum test or Student's *t*-test when appropriate. More than two groups were compared with ANOVA with the Tukey–Kramer multiple comparison test to correct for multiple comparisons. Correlation was studied by Spearman's rank correlation.

RESULTS

In vitro *neutrophil activation and ANCA titre*

IgG fractions isolated from serum samples from 17 patients during active WG, 10 with extensive and seven with limited disease activity, and from 17 patients during remission of WG at 3–12 months after diagnosis, were assayed for their capacity to induce the respiratory burst using the SOD-inhibitable reduction of ferri-cytochrome C. As shown in Fig. 1, all but one of the samples induced the neutrophil respiratory burst in primed normal donor granulocytes compared with normal control sera. Mean ΔOD 550 nm, a measure for the amount of superoxide production induced by all IgG fractions from patients with WG, was 0·078 ± 0·049 ($n = 34$) compared with 0·007 ± 0·003 ($n = 6$) as induced by control IgG fractions ($P < 0·0001$). The capacity to induce the neutrophil respiratory burst was significantly related to C-ANCA titre as

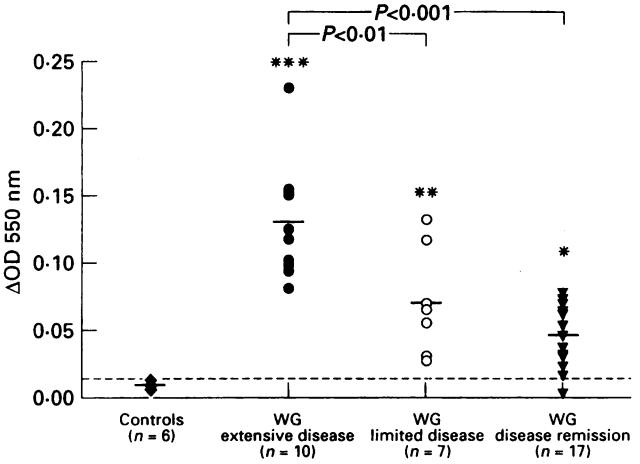

Fig. 1. ΔOD 550 nm as a measure for the amount of superoxide production of normal donor polymorphonuclear neutrophils (PMN) induced by IgG fractions derived from sera from either controls ($n = 6$), patients with active Wegener's granulomatosis (WG) and extensive ($n = 10$) or limited ($n = 7$) disease activity, or patients with WG during disease remission ($n = 17$). The dotted line indicates the upper limit of the ΔOD 550 nm induced by the six control sera (mean + 3 s.d.). $*P < 0·05$; $**P < 0·01$; $***P < 0·001$ compared with controls.

determined in the corresponding serum sample ($r = 0·499$, $P = 0·0027$, $n = 34$) (Fig. 2). This correlation between C-ANCA titre and capacity to induce the neutrophil respiratory burst was caused by the correlation derived from the 17 samples taken during active disease ($r = 0·688$, $P = 0·0023$), while no significant correlation was found when evaluating the results from the 17 samples taken during disease remission ($r = 0·286$). No significant correlation was observed between levels of total IgG, IgG1, IgG3, or IgG4 subclass of ANCA and the capacity to induce neutrophil respiratory burst, either when the results from all samples were evaluated, or when the evaluation was restricted to the samples taken at the moment of active disease only.

Polymorphonuclear neutrophil (PMN)-activating capacity

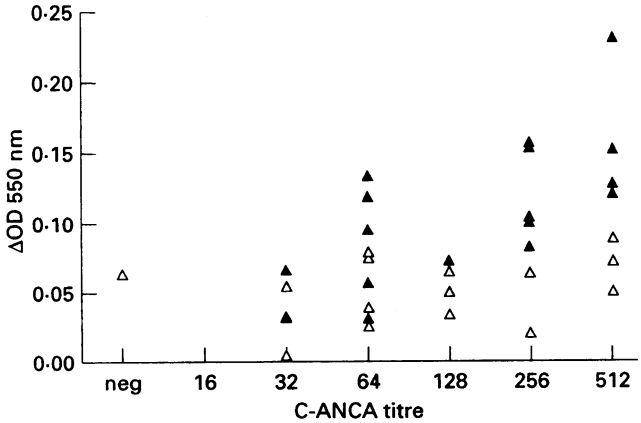

Fig. 2. ΔOD 550 nm as a measure for the amount of superoxide production of normal donor polymorphonuclear neutrophils (PMN) induced by IgG fractions derived from sera from patients during active disease (▲) or disease remission (△) in relation to the reciprocal titre of ANCA.

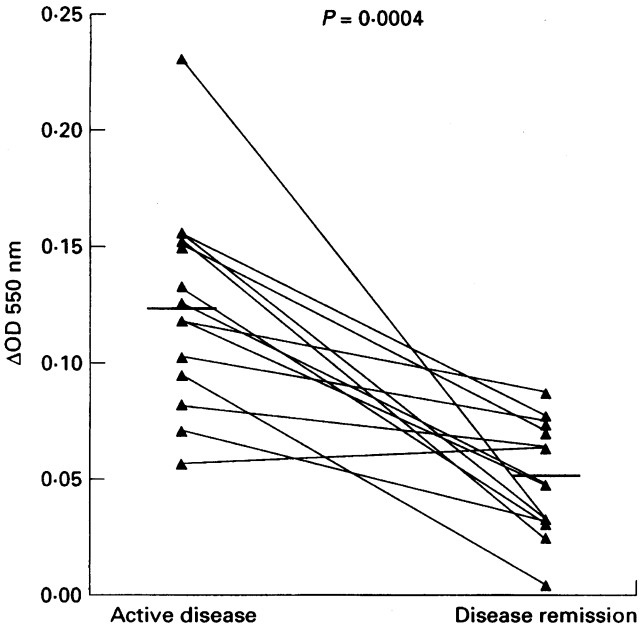

Fig. 3. ΔOD 550 nm as a measure for the amount of superoxide production of normal donor polymorphonuclear neutrophils (PMN) induced by IgG fractions derived from paired sera taken during active disease and disease remission, respectively, in 13 patients.

of C-ANCA-positive sera compared with control sera was observed both in patients with extensive disease activity of WG and in those with limited activity (Fig. 1). The capacity of the IgG fractions to induce the respiratory burst was found to be related to the extent of disease activity. Samples from patients with extensive WG disease activity showed statistically significantly higher levels of the respiratory burst than those from patients with limited disease activity or from patients in remission ($P < 0{\cdot}01$ and $P < 0{\cdot}001$, respectively) (Fig. 1). The median C-ANCA titre at the moment of active disease (256, range 32–512) was higher than that at the moment of remission (64, range 0–512), although the difference was not statistically significant. However, as shown in Fig. 2, IgG fractions isolated from sera taken at the moment of active disease tended to induce a higher level of neutrophil respiratory burst than the IgG fractions from sera with an identical C-ANCA titre drawn during disease remission.

In vitro *neutrophil activation and IgG subclasses of C-ANCA*

ANCA-induced neutrophil activation is dependent on Fcγ receptors on the neutrophil, in particular FcγRII [9]. Since the various IgG subclasses have different affinities for the individual Fcγ receptors, we studied the relation between C-ANCA IgG subclass distribution in the IgG fractions and their capacity to induce neutrophil respiratory burst from the 13 patients from whom paired sera from both active WG and disease remission were available. In this group of 13 paired sera, a statistically highly significant difference was found between sera taken at the moment of active disease compared with sera taken during disease remission (ΔOD $0{\cdot}123 \pm 0{\cdot}045$ *versus* $0{\cdot}051 \pm 0{\cdot}025$; $P = 0{\cdot}0004$) (Fig. 3). In these paired sera C-ANCA titre, and levels of total IgG and IgG1, IgG3, and IgG4 subclass of ANCA were lower during disease remission compared with the preceding active phase of WG in most patients, but differences failed to reach statistical significance.

The changes in capacity to induce neutrophil respiratory burst between the 13 paired sera taken at the moment of active WG and during remission, respectively, showed no statistically significant correlations with changes in either C-ANCA titre, or total IgG, IgG1, IgG3, and IgG4 subclass of ANCA between the paired sera. However, a statistically significant correlation existed between the change in the 13 paired samples in relative IgG3 subclass value of ANCA, i.e. the quantity of IgG3 subclass of ANCA divided by the amount of total IgG ANCA, and the change in capacity to induce neutrophil respiratory burst ($r = 0{\cdot}687$, $P = 0{\cdot}0095$), while this was not found for IgG1 and IgG4 subclasses of ANCA (Fig. 4).

DISCUSSION

In the present study we have demonstrated that IgG fractions from C-ANCA-positive serum samples at the moment of active WG induced higher levels of the respiratory burst than IgG fractions from serum samples at remission. The amount of superoxide production appeared to be dependent on the amount of IgG class of C-ANCA present as it correlated with C-ANCA titre. Furthermore, the relative increase in activating capacity during the time of active disease correlated strongly with the increase in the relative amount of the IgG3 subclass of ANCA, but not with changes in the relative amounts of IgG1 and IgG4 subclasses of ANCA.

A pathogenic role for ANCA has been proposed by studies

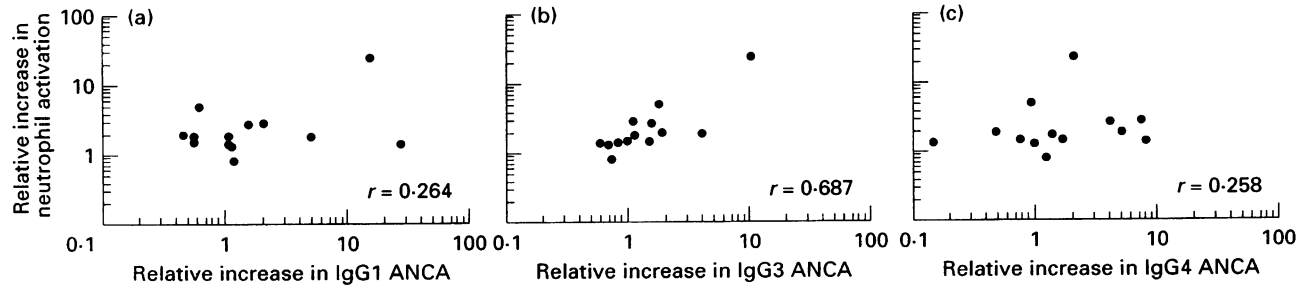

Fig. 4. Relative increase in neutrophil-activating capacity (i.e. change in the capacity to induce the neutrophil respiratory burst in paired serum samples taken at the moment of active disease and disease remission) measured as ΔOD 550 nm of 13 paired sera of Wegener's granulomatosis (WG) patients in relation to the relative increase in levels of IgG1 (a), IgG3 (b), and IgG4 subclass (c) of ANCA (i.e. change in level of IgG subclass relative to total specific IgG as measured in paired serum samples).

of Falk *et al.* [7], in which ANCA were demonstrated to activate neutrophils that were pretreated ('primed') with low dosage TNF-α to the production of reactive oxygen species and the release of lysosomal enzymes. The activation of neutrophils by ANCA is dependent on the expression of ANCA antigens at the cell surface of primed granulocytes. Falk's original report suggests that activation can be induced by $F(ab')_2$ fragments of ANCA. We and others recently demonstrated that the Fc region of ANCA is, however, involved as well [9,14]. Blockade of Fcγ receptors of the neutrophil, in particular FcγRII, inhibited neutrophil activation induced by ANCA. Sera with relatively high levels of IgG3 subclass of ANCA preferentially activated neutrophils.

To study the relation between C-ANCA-induced neutrophil activation and IgG subclass distribution of C-ANCA, plasma samples from patients with WG drawn during active and inactive disease were included. A significant correlation was observed between the amount of superoxide produced and the titre of the antibodies. This confirms data in our previous study that ANCA-induced neutrophil activation is dose-dependent for individual sera. This correlation between C-ANCA titre and capacity to induce the neutrophil respiratory burst was caused by the correlation in the 17 samples taken during active disease, while no significant correlation was found in the 17 samples during disease remission, even though disease remission sera had, in some cases, comparable ANCA titres. As a consequence, the capacity to induce neutrophil respiratory burst was not, at least not completely, related to the absolute amount of C-ANCA present as measured by IIF or antigen-specific ELISA. This is in agreement with our previous study on P-ANCA-positive sera of patients with inflammatory disorders such as rheumatoid arthritis and ulcerative colitis, in which no correlation was observed between the amount of autoantibodies present and neutrophil-activating capacity [9].

We hypothesized that the higher capacity of C-ANCA to induce the respiratory burst during active disease irrespective of titre, could be a result of changes in the affinity of C-ANCA, in the epitopes recognized, or in the subclass distribution of C-ANCA. Since all IgG subclasses have different affinities for the Fc receptors, and Fc receptors are involved in ANCA-induced neutrophil activation, we favoured the latter hypothesis. To correct for overall changes in antibody quantities, the percentage increase in neutrophil-activating capacity from inactive to active disease was related to the relative change of IgG subclasses of C-ANCA from inactive to active disease. Comparing paired plasma samples for 13 patients individually, a strong correlation was observed between the percentage increase in neutrophil-activating capacity and the relative change of IgG3 subclass of C-ANCA, but not with the relative changes in IgG1 or IgG4 subclass of C-ANCA. These data strongly suggest that IgG3 C-ANCA facilitate granulocyte activation. Two observations support the particular role of IgG3 C-ANCA in granulocyte activation. First, IgG3 antibody complexes bind to FcγII receptors approximately three times faster than IgG1 antibodies, and far faster than IgG2 and IgG4 antibodies [22]. Second, the IgG3 molecule is the most flexible molecule of the IgG family, making it conceivable that immune complex formation at the surface of the granulocyte in which IgG3 molecules are involved, facilitates neutrophil activation. Interestingly, it has been shown that renal exacerbations of WG are associated with increases of the IgG3 subclass of ANCA [20]. In addition, Jayne *et al.* [23] demonstrated that ANCA of IgG3 subclass in patients with systemic vasculitis were relatively abundant during active disease.

Measurement of differential subclass binding by ELISA in which the antigen is directly coated and detection occurs with subclass-specific MoAbs is highly dependent on both inter-subclass competition for the coated antigen and the various affinities of the subclass-specific MoAbs. However, we feel that these potential methodological errors are largely reduced by comparing the changes in relative amount of each subclass (i.e. relative to the total amount of specific IgG) from disease remission to the time of active disease.

Whether or not changes in IgG3 subclass of C-ANCA and changes in C-ANCA titre are solely responsible for the fact that C-ANCA during active disease better induced the respiratory burst in primed neutrophils than C-ANCA in inactive disease cannot be deduced from this study. Different quality of antibodies may be of importance for the pathogenesis of WG. Recently, Brouwer *et al.* [24] demonstrated that activated neutrophils, as assessed by their *in situ* H_2O_2 production, are present in renal biopsies from patients with WG. They found a strong correlation between the extent of renal impairment and the numbers of activated neutrophils producing H_2O_2, which suggests that activated PMN contribute to the pathogenesis of renal lesions in WG. However, they found no relation between the numbers of H_2O_2-producing cells present within the renal biopsy and the *in vitro* capacity of ANCA to activate primed granulocytes. This also suggests that other factors besides ANCA are probably important for the *in vivo* outcome. Dolman *et al.* [25] recently demonstrated in a small study that C-ANCA have an inhibitory effect on the complexation of PR3 and its major physiologic inhibitor, α_1-antitrypsin (α_1-AT), and, more importantly, that this inhibitory effect of C-ANCA correlates with clinical activity of WG but not with total C-ANCA levels.

The PMN-activating capacity of C-ANCA was not related to renal impairment, and occurred in patients with generalized WG as well as in patients with limited WG. In this study we demonstrate not only that C-ANCA titres change in the course from active to inactive disease, but that the IgG subclass distribution of C-ANCA changes as well. The latter changes may be of pathophysiological importance, since the relative increase in IgG3 subclass of ANCA correlated strongly with the percentage increase in activating capacity of C-ANCA IgG.

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