Clin. exp. Immunol. (1991) **83**, 379–386 ADONIS 000991049100070H

Predominance of IgG1 and IgG4 subclasses of anti-neutrophil cytoplasmic autoantibodies (ANCA) in patients with Wegener's granulomatosis and clinically related disorders

E. BROUWER, J. W. COHEN TERVAERT*, G. HORST, M. G. HUITEMA, M. VAN DER GIESSEN, P.C. LIMBURG† & C. G. M. KALLENBERG *Departments of Clinical Immunology, *Nephrology and †Rheumatology, University Hospital, Groningen, The Netherlands*

(Accepted for publication 28 September 1990)

SUMMARY

In view of the supposed hypersensitivity, the elevated levels of IgE, and the occurrence of eosinophilia reported in Wegener's granulomatosis and related conditions, we studied the IgG subclass distribution of ANCA directed against a 29-kD serine protease and myeloperoxidase (MPO) in 41 untreated ANCA-positive patients with several forms of active vasculitis and/or glomerulonephritis. We found that both 29-kD ANCA and MPO ANCA were predominantly of the IgG1 and IgG4 subclass in all groups of patients. The additional presence of IgG3 subclass was associated with renal involvement. We compared the subclass distribution of ANCA with that of total IgG subclass levels, and with the IgG subclass distribution of antibodies to cytomegalovirus (CMV) as a persistent endogenous antigen and antibodies to tetanus toxoid (TT) as an exogenous recall antigen. Total levels of IgG4 were elevated in the majority of the patients together with elevated IgG1 levels. Antibodies to CMV and TT, however, had the same subclass distribution as found in normals and did not show enhanced IgG4 expression. ANCA belong predominantly to the IgG1 and IgG4 subclass, which may suggest that the production of ANCA is related to recurrent exposition to the antigen(s) involved, possibly as part of a hypersensitivity reaction.

Keywords ANCA anti-MPO antibodies IgG subclasses Wegener's granulomatosis

INTRODUCTION

Human IgG subclasses differ substantially in biological functions. IgG1 and IgG3 exhibit a strong complement-activating capacity and bind firmly to Fc receptors on mononuclear cells (Schur, 1987). IgG4 is involved in the manifestation of allergic states and is thought to mediate histamine release from mast cells similar to IgE (Fagan *et al.*, 1982). After immunization, quantitative differences are found in IgG subclass responses dependent on the antigen(s) involved and the balance between regulatory factors produced by T cells. In humans, antibodies against viral capsid proteins belong predominantly to the IgG1 and IgG3 subclasses (Skavril, 1986), whereas IgG2 is the predominant subclass in immune responses against polysaccharides (Schur, 1987). IgG4, like IgE, is highly responsive to repeated challenge with environmental antigens (Heiner, 1984; van der Zee, van Swieten & Aalberse, 1986).

In autoimmune diseases such as systemic lupus erythematosus (SLE), autoantibodies are in general of the IgG1 and IgG3 subclass (Rubin *et al.*, 1986a), despite continuous exposure to the autoantigens involved. In contrast, autoantibodies to glomerular basement membrane (GBM) are restricted to the IgG1 and IgG4 subclass (Bowman, Ambrus & Lockwood, 1987).

Wegener's granulomatosis (WG) is a distinct clinico-pathological entity characterized by necrotizing granulomatous inflammation of the upper and lower respiratory tract in association with necrotizing crescentic glomerulonephritis and vasculitis (Fauci *et al.*, 1983). Localized forms of the disease without kidney involvement have been described as limited WG (Carrington & Liebow, 1966; Fienberg, 1981; Fauci *et al.*, 1983). In both forms of the disease elevated IgE levels and eosinophilia have been described (Conn *et al.*, 1976), suggesting that hypersensitivity is involved in the disease process.

Recently, ANCA have been described in patients with WG and related disorders (van der Woude *et al.*, 1985; Savage *et al.*, 1987; Nölle *et al.*, 1989; Cohen Tervaert *et al.*, 1989). These antibodies comprise, among others, anti-29-kD serine protease (29-kD ANCA) antibodies (Goldschmeding *et al.*, 1989) which are highly sensitive and specific for active WG, and anti-myeloperoxidase (MPO ANCA) antibodies which have been

Correspondence: E. Brouwer, MD, Department of Clinical Immunology, University Hospital, Oostersingel 59, 9713 EZ Groningen, The Netherlands.

demonstrated in idiopathic and/or vasculitis-associated crescentic glomerulonephritis (Falk *et al.*, 1988; Cohen Tervaert *et al.*, 1990a; Lee, Adu & Thompson, 1990) and conditions clinically related to WG such as polyarteritis and the Churg–Strauss syndrome (CSS) (Cohen Tervaert *et al.*, 1990b).

No clear concept concerning the induction of ANCA in patients with WG and clinically related disorders has been evolved so far. In view of the elevated IgE levels and eosinophilia and the supposed hypersensitivity in WG and related conditions we studied the IgG subclass distribution of 29-kD ANCA and MPO ANCA in patients with biopsy-proven WG and clinically related disorders, both in the extended and localized forms of disease, during initial presentation and relapses. We compared the IgG subclass distribution of ANCA with that of antibodies to cytomegalovirus (CMV), a latent herpes virus, and antibodies to tetanus toxoid as an exogenous recall antigen. Our findings demonstrate that 29-kD ANCA and MPO ANCA belong predominantly to the IgG1 and IgG4 subclasses.

PATIENTS AND METHODS

Patients and sera

Sera were studied from 22 consecutive patients with 29-kD ANCA and from 19 consecutive patients with MPO ANCA. All 22 patients with 29-kD ANCA had a diagnosis of WG according to the classification of Fauci *et al.* (1983): seven had limited WG and 15 patients had extended WG. The patients with MPO ANCA consisted of two groups: nine patients had a diagnosis of crescentic glomerulonephritis (CGN), either idiopathic or vasculitis-associated, and 10 patients were classified as systemic necrotizing vasculitis (SNV) without renal involvement; the latter group included seven patients with polyarteritis and three patients with the CSS. The total group of 41 patients consisted of 34 men and seven women, with a mean age of 59·9 years (range 31–78).

Blood was drawn at the time of diagnosis before immunosuppressive therapy was instituted. Sera were stored at −20°C until assayed. As control sera we used sera from six normal healthy volunteers and 24 patients with a diagnosis of rheumatoid arthritis and 16 patients with SLE.

Monoclonal antibodies for the detection of IgG subclasses

For the detection of IgG subclass distribution the following monoclonals were used: anti-human IgG1 (clone MH161M), anti-human IgG2 F(ab′)$_2$ fragments (clone MH162M), anti-human IgG3 F(ab′)$_2$ fragments (clone MH163M), and anti-human IgG4 Fc (clone MH164M), all derived from the Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands. The specificities of these monoclonals have been described by Vlug *et al.* (1989). Their affinities have been tested by S. Ruths (personal communication). Comparable affinities were found for the anti-IgG1, IgG3 and IgG4 monoclonals, whereas that of anti IgG2 was considerably lower. For this reason the monoclonal antibodies were used, both in the fluorescence assays and in the ELISAs, in concentrations higher than those used by S. Ruths in order to obtain positive recordings for all monoclonals used.

IgG subclass detection of ANCA by indirect immunofluorescence assay (IIF)

Detection of ANCA was performed as previously described (van der Woude *et al.*, 1985) with minor modifications (van der Giessen *et al.*, 1989). Briefly, granulocytes from a healthy donor were obtained by Isopaque–Ficoll and dextran sedimentation of heparinized blood. After washing the cells, 25 μl of a suspension of 1×10^6 granulocytes/ml were put into each well of 12-well Cooke slides at 37°C for 30 min. After attachment to the wells, cells were ethanol-fixed for 10 min at 4°C. Test or control sera were overlaid in a dilution of 1/16 on the slides and incubated for 1 h. After washing three times with phosphate-buffered saline (PBS) for 5 min, bound antibody was detected with FITC-conjugated 1/400 diluted goat anti-human IgG (Kallestad, Chaska, MN).

Finally, slides were washed again in PBS and mounted in glycerin–PBS for immunofluorescence microscopy. Samples were scored as positive for cANCA if the majority of neutrophils showed bright fluorescence of the cytoplasm in the surrounding of the segments of the nucleus at a serum dilution of at least 1/16, and positive for pANCA if they showed a (peri)nuclear staining pattern. If the test was positive, serum titration was done in two-fold dilutions from 1/16 to 1/512. Slides were read independently by two observers. None of the sera contained anti-nuclear antibodies (ANA) on human fibroblasts.

For the detection of IgG subclass distribution of ANCA by IIF, IgG subclass-specific monoclonal antibodies were used. The monoclonals were added in a dilution of 1/400, followed by rabbit anti-mouse IgG FITC (1/30) (Dakopatts, Copenhagen, Denmark) as a second step. Slides were read independently by two observers and scored positive according to the criteria mentioned above.

ANCA detection by antigen capture ELISA

The specificity of ANCA for either the 29-kD antigen or MPO was detected as previously described (Cohen Tervaert *et al.*, 1990a). Briefly, microtitre plates (Inotech) were incubated with goat anti-mouse IgG (Jackson, West Grove, PA). After washing, the plates were incubated with a mouse monoclonal antibody against the 29-kD antigen or MPO (Moab 12·8 and Moab 7.17, Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands) (Goldschmeding *et al.*, 1989). After washing, an extract of azurophilic granules of normal human neutrophils (2 mg/ml) was added. The extract of the azurophilic granules was isolated according to the method of Borregaard *et al.* (1983), in the presence of protease inhibitors. Subsequently, different dilutions of test serum in the incubation buffer were added for 1 h at 37°C. After washing bound antibody was detected by affinity purified F(ab′)$_2$ goat anti-human IgG linked to alkaline phosphatase (Jackson) and *p*-nitrophenyl-phosphate disodium as a substrate. The optical densities (OD) were read at 405 nm. As a control we used a nonsense monoclonal antibody. The OD values obtained with this antibody were subtracted from the OD values of the 29-kD ANCA or MPO ANCA ELISA. Sera were considered positive when their extinctions exceeded the mean + 2 s.d. of normal and disease controls.

IgG subclass detection of ANCA by solid-phase ELISA

A crude granule extract was isolated by nitrogen cavitation and, subsequently, by ureum extraction of human neutrophils in the

presence of the necessary protease inhibitors (Borregaard *et al.*, 1983). The antigens were gathered by centrifugation of the extract. Labstar (Greiner) microtiter plates were coated overnight with the extract at a protein concentration of 20 μg/ml in 0·1 M carbonate buffer, pH 9·6. The plates were incubated with human sera for 1 h at a dilution of 1/100 and subsequently with the subclass-specific monoclonal antibodies mentioned above in a dilution of 1/250 (400 ng/well) for 1 h. All dilutions were made in 0·05 M Tris, 0·05% Tween, 2% BSA and 0·3 M NaCl, pH 8·0.

Antibody binding was detected with a goat anti-mouse IgG linked to alkaline phosphatase (Sigma) at a dilution of 1/1000 in 0·01 M Tris, 0·15 M NaCl, pH 8·0 and *p*-nitrophenyl phosphate disodium as a substrate. The OD was read at 405 nm. Values were expressed in OD units after subtraction of blanks. In order to evaluate the specificity of this ELISA for ANCA, sera from six normal controls, 16 patients with SLE and 24 patients with rheumatoid arthritis were tested. Extinctions from all these sera were below 290 OD units (mean + 2 s.d.) for all subclasses. In order to study the relevance of this neutrophil extract ELISA for measuring MPO ANCA, an MPO ANCA-positive serum was absorbed in MPO isolated according to Merril (1980) and coupled to concanavalin A (Con A)/sepharose with excess MPO. MPO ANCA-positive serum samples could be inhibited in the neutrophil extract ELISA by MPO coupled to Sepharose whereas a 29-kD ANCA-positive serum was not inhibited by MPO (data not shown). As for anti-29-kD ANCA detected by neutrophil extract ELISA, we compared the results of this assay with that of the capture ELISA for measuring IgG-class ANCA in all the individual sera. A significant correlation ($P < 0·05$) was found.

Quantification of total serum levels of IgG subclasses and IgE

Total serum levels of IgG subclasses were measured with a radial immunodiffusion technique according to Mancini, Carbonara & Heremans (1965), using polyclonal antibodies (van der Giessen *et al.*, 1976).

IgE was measured with the IgE RIACT 100 Kit from Pharmacia (S-751 82).

Anti-tetanus toxoid antibodies

These were measured by ELISA as previously described (Houtman *et al.*, 1986). Briefly, microtitre plates were coated with tetanus toxoid and incubated with human sera. IgG subclasses of anti-tetanus toxoid antibodies were detected with the antibodies mentioned in the previous sections. Values were expressed as OD units.

Anti-cytomegalovirus antibodies

IgG class specific antibodies to the late antigen of CMV were measured according to van der Giessen *et al.* (1990). IgG subclasses were determined with the use of Greiner plates coated with 100 μl of a solution of CMV La antigens in 0·1 M carbonate buffer, pH 9·6. The sera were incubated in one dilution (1/100). After washing, the plates were incubated at 37°C with the IgG subclass-specific mouse monoclonal antibodies mentioned before in a dilution of 1/250. Bound antibodies were detected by HRPO-conjugated rabbit anti-mouse IgG (Dako 260; Dakopatts) in a dilution of 1/1000 and OPD as a substrate. The ODs were read at 492 nm.

Statistical analysis

Spearman's rank sum test was applied for detecting correlations between the different study parameters. Differences in paired parameters between the groups were evaluated with the Wilcoxon test for paired observations. Multiple regression analysis was performed with Systat statistical package for personal computers. A P value of $< 0·05$ was considered significant.

RESULTS

Subclass distribution of 29-kD ANCA

All 22 sera positive for cANCA by IIF were positive also by ELISA using the antibody 12.8 (directed against the 29-kD serine protease) as a catching antibody and by solid-phase neutrophil extract ELISA. These sera were all negative for MPO ANCA by capture ELISA. The distribution of the IgG subclasses was evaluated by IIF and solid-phase neutrophil extract ELISA. When tested by IIF, we found that all of the 22 sera were positive for IgG1 class 29-kD ANCA (100%), 18% for IgG2 class ANCA, 45% for IgG3 class ANCA, and 91% for IgG4 class ANCA (Fig. 1). When tested by neutrophil extract ELISA IgG subclass distribution of ANCA was comparable to that assayed by IIF (Fig. 2). Percentages of positive results for the several IgG subclasses of ANCA by solid-phase ELISA were 100% for IgG1, 35% for IgG2, 70% for IgG3, and 96% for IgG4. Titres of IgG subclasses of cANCA by IIF correlated well with levels of ANCA by solid phase ELISA for IgG1, IgG3, and IgG4 ($P < 0·05$), with the following correlation coefficients: 0·69 for IgG1; 0·28 for IgG2; 0·61 for IgG3; and 0·52 for IgG4.

Subclass distribution of MPO ANCA

MPO ANCA were assayed by IIF and antigen capture ELISA. The sera were all negative for 29-kD ANCA as tested in the 29-kD ANCA capture ELISA. Because of difficulties in determination of the titration end-point, probably caused by the artefactual staining pattern of MPO ANCA which is nuclear instead of cytoplasmic as a result of a fixation artefact (Falk & Jennette, 1988), we measured the subclass distribution for MPO ANCA by solid-phase ELISA only (Fig. 3). For MPO ANCA we found that all (100%) of the 19 sera contained IgG1 class MPO ANCA; 35% of the sera contained IgG2 class ANCA; 35% IgG3 class ANCA; and 85% contained IgG4 class ANCA.

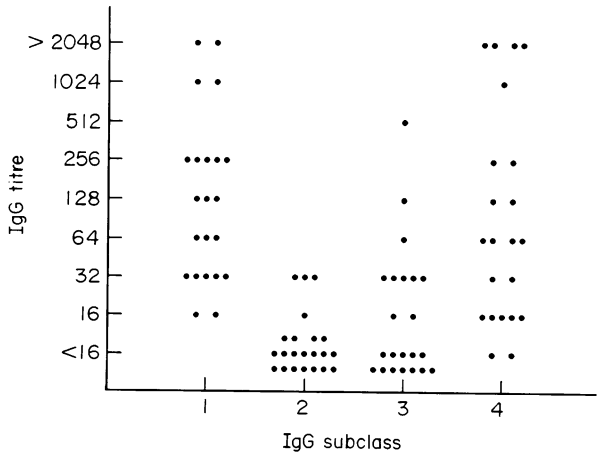

Fig. 1. Titres of cANCA for the different IgG subclasses as detected by indirect immunofluorescence.

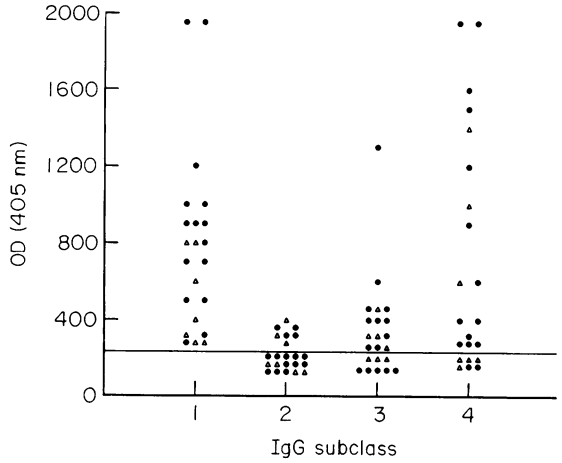

Fig. 2. IgG subclasses of 29-kD cANCA as detected by solid-phase ELISA with a crude granule extract. Values are given in OD units. ●, patients with extended Wegener's granulomatosis (WG); △, patients with limited WG Horizontal bar denotes the upper limit of control sera (mean + 2 s.d.).

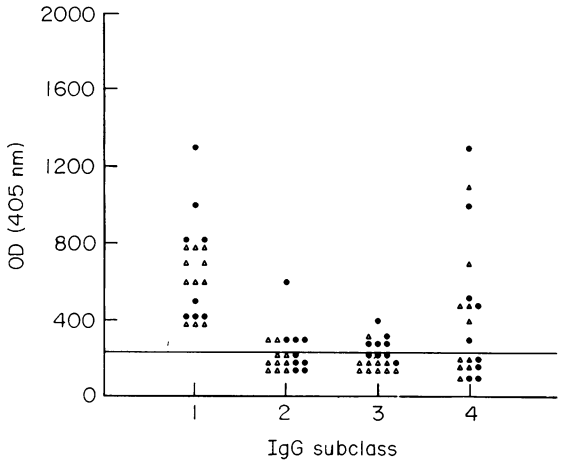

Fig. 3. IgG subclasses of MPO ANCA as detected by solid-phase ELISA with a crude granule extract. Values are given in OD units. ● patients with crescentic glomerulonephritis; △, patients with systemic necrotizing vasculitis. Horizontal bar denotes the upper limit of control sera (mean + 2 s.d.).

Association between subclass distribution of ANCA and disease patterns

Levels of IgG1 and IgG3 subclass 29-kD ANCA were significantly higher ($P < 0{\cdot}05$) in extended WG (with renal involvement) than in limited WG (without renal involvement) (Fig. 2). No significant differences were found for IgG2 and IgG4 subclass ANCA.

Also for MPO ANCA, there was a tendency ($P < 0{\cdot}07$) towards higher levels of IgG3 class ANCA in patients with renal involvement (CGN) compared with patients without renal involvement (SNV) (Fig. 3).

No significant differences were found in levels of 29-kD and MPO IgG4 subclass ANCA between patients with respiratory tract involvement and patients without.

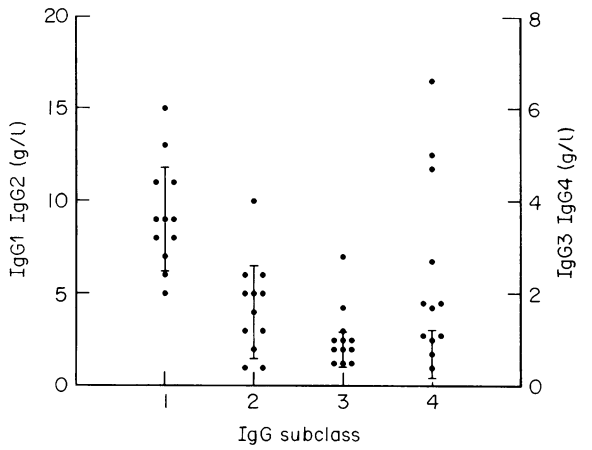

Fig. 4. Levels of total IgG subclasses in 29-kD ANCA-positive patients. Vertical bars denote the normal range for men.

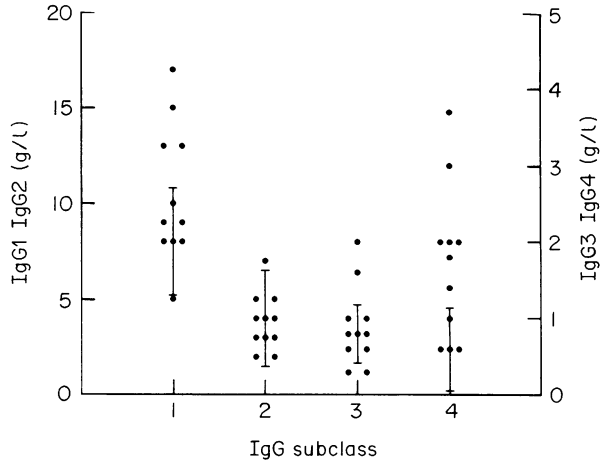

Fig. 5. Levels of total IgG subclasses in MPO ANCA-positive patients. Vertical bars denote the normal range for men.

No differences in levels of subclasses of ANCA were found between sera drawn during the first presentation and those during a relapse.

Subclass distribution of total IgG levels

Due to shortage of sera we could test only 12 out of the 22 sera positive for 29-kD ANCA (seven from patients with renal involvement and five from patients without renal involvement) and 11 out of the 19 MPO ANCA-positive sera (six from patients with renal involvement and five from patients without renal involvement) for total levels of IgG subclasses.

The distribution of total IgG subclass levels is shown in Figs 4 and 5: levels of healthy men are given as controls, because 80% of our group of patients were male patients, and men are known to have higher levels of IgG4 (French & Harrison, 1984). Increased levels of IgG4 were found in seven out of 12 (58%) 29-kD ANCA-positive sera and in seven out of 11 (64%) MPO ANCA-positive sera, whereas increased levels of IgG1, IgG2, and IgG3 were observed in 17%, 17%, and 8% for 29-kD ANCA-positive sera, and in 46%, 9%, and 9%, respectively, for MPO ANCA-positive sera.

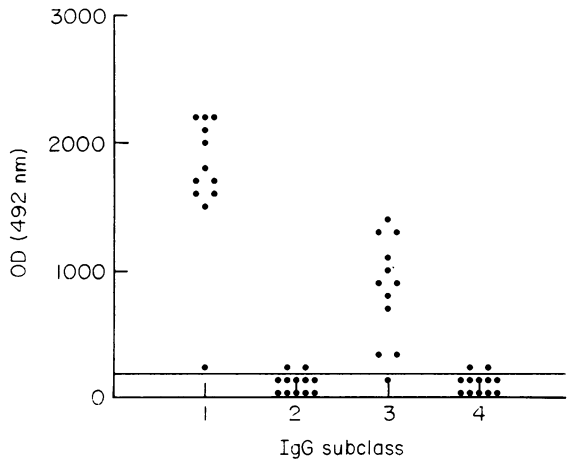

Fig. 6. Levels of IgG subclasses of cytomegalovirus antibodies in patients with ANCA. Values are given in OD units. Horizontal bar denotes the upper limit of control sera (mean + 2 s.d.).

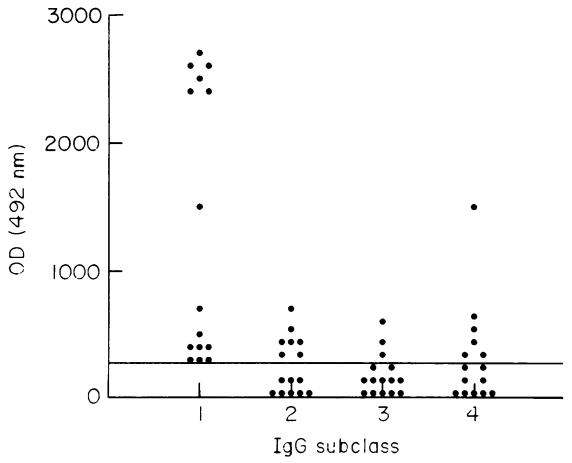

Fig. 7. Levels of IgG subclasses of anti-tetanus toxoid antibodies in patients with ANCA. Values are given in OD units. Horizontal bar denotes the upper limit of control sera (mean + 2 s.d.).

Multiple regression analysis revealed a significant relation between levels of IgG4 subclasses of 29-kD ANCA and MPO ANCA and levels of total IgG4 ($P < 0.05$).

Total IgE levels

Levels of IgE (kU/*l*) were increased in three out of 21 29-kD ANCA-positive patients and in six out of 18 MPO ANCA-positive patients. Three of the latter six patients had a diagnosis of CSS. We found no significant relation between levels of IgG4 class ANCA and levels of IgE.

When we performed multiple regression analyses between levels of IgG4 subclass ANCA, IgG1 subclass ANCA, IgE, total IgG4, and nose and/or lung involvement, we found only a dependent relation between IgG4 subclass ANCA and total levels of IgG4.

IgG subclass distribution of antibodies to CMV and tetanus toxoid

Twelve out of 24 patients randomly selected out of the four clinical groups (29-kD ANCA-positive patients with and without renal involvement, and MPO ANCA-positive patients with and without renal involvement) had antibodies against the late antigens of CMV. Anti-CMV antibodies were mainly of the IgG1 and IgG3 subclasses. No antibodies of the IgG4 subclass were found (Fig. 6). The subclass distribution of anti-CMV antibodies differed significantly ($P < 0.05$) from the subclass distribution of ANCA in the same patients. The subclass distribution was comparable to that of normal CMV-positive controls (Gilljam & Wahren, 1989; and our unpublished observations).

Fifteen out of 30 patients randomly selected out of the four different groups were positive for anti-tetanus toxoid antibodies. The anti-tetanus toxoid antibodies were mainly restricted to the IgG1 subclass (Fig. 7). Comparable to anti-CMV antibodies, the subclass distribution of anti-tetanus toxoid antibodies was significantly different from the subclass distribution of ANCA.

DISCUSSION

We found that in patients with 29-kD ANCA and MPO ANCA, the autoantibodies predominantly belong to the IgG1 and IgG4 subclass. In addition, levels of total IgG4 and IgE were elevated, and levels of IgG4 subclass ANCA related significantly with levels of total IgG4. In contrast, IgG subclasses of antibodies to CMV and tetanus toxoid were normally distributed. Although levels of IgG3 subclass ANCA were low in all groups of patients, patients with WG and renal involvement had higher levels of IgG1 and IgG3 subclass 29-kD ANCA than patients with WG without renal involvement. For MPO ANCA, we found a trend towards higher levels of IgG3 subclass ANCA in patients with renal involvement.

To assess levels of IgG subclasses of ANCA we used an ELISA with a crude extract of neutrophil granules as a substrate and IgG subclass-specific mouse monoclonal antibodies as reagents. This ELISA does not specifically measure antibodies to the 29-kD serine protease and/or MPO. The specificity of the sera, however, was determined by antigen capture ELISA using antibodies to the 29-kD antigen and MPO as specific reagents. However, we can not exclude that antibodies in our patients' sera reacting with different neutrophil granule constituents contributed to the results of the neutrophil extract ELISA. To approach this question, we compared the results of the 29-kD ANCA capture ELISA with that of the neutrophil extract ELISA, and found a significant correlation. More importantly, the reactivity of MPO ANCA in the neutrophil extract ELISA could be inhibited by MPO, whereas 29-kD ANCA could not be inhibited by MPO. In addition, none of the 29-kD ANCA sera was positive for MPO ANCA. Since proteinase 3, the target antigen of 29-kD ANCA, was not available, these inhibition experiments could not be performed for 29-kD ANCA. Thus, we consider the neutrophil extract ELISA as used in this study relevant for the assessment of the IgG subclasses of the respective ANCA. The ELISA results are further influenced by the affinities of the IgG subclass-specific monoclonal antibodies used in this study. Although we applied a relative excess of monoclonal antibodies in the ELISAs, we may have underestimated the relative amounts of IgG2 subclass antibodies due to the rather low affinity of the IgG2 subclass monoclonal antibodies, as compared with the affinities of the other monoclonal antibodies directed to IgG1, IgG3 and IgG4.

The predominance of IgG1 and IgG4 subclasses for 29-kD ANCA and MPO ANCA is different from the distribution of IgG subclasses of ANA but resembles that of anti-GBM autoantibodies (Bowman *et al.*, 1987). IgG4 subclass predominance has also been observed for autoantibodies to thyroglobulin and microsomes in Hashimoto's thyroiditis (Parkes *et al.*, 1984), for autoantibodies to factor VIII (Andersen & Terry, 1985; Shapiro & Carrol, 1986), and for antibodies to grass pollen in atopic allergic individuals (van der Giessen *et al.*, 1976).

Why do ANCA, in contrast to antibodies to dsDNA, show a predominance of IgG4 class antibodies? The shift from IgG1 to IgG4 is known to be dependent both on the antigen involved and on T cell regulatory factors. Genes coding for IgG4 are located at the most down-stream switch site of the genes coding for immunoglobulin (Flanagan & Rabbitts, 1982; Mongini, Paul & Metcalf, 1982). IgG4 is produced after recurrent stimulation with antigen (Aalberse, van der Gaag & van Leeuwen, 1983; Shakib, 1987). The predominance of IgG4 subclass ANCA may therefore suggest a chronic antigenic stimulation of the immune system and an antigen driven B cell stimulation underlying the development and production of ANCA. It is not clear why the autoimmune response develops in certain patients, but cross-reactivity with microbial antigens, possibly derived from the upper respiratory tract, may play a role. The long period between the first signs and symptoms in WG, often related to the upper respiratory tract, and the time of extensive disease activity (Cohen Tervaert *et al.*, 1987) could be responsible for the formation of IgG4 ANCA. As we found no difference in distribution and levels of IgG subclasses between patients at initial presentation and in relapse, the induction of ANCA probably occurs long before disease symptoms are present.

As mentioned already, isotype switching to IgG4 is dependent not only on repeated antigenic stimulation but also on regulatory factors produced by T cells. TH2 cells in mice producing interleukin-4 (IL-4) and B cell growth factor (BCGF) are capable of stimulating B cells to produce IgG1 and IgE (Dekruyff *et al.*, 1989) and the shift from IgG1 to IgG4 is also regulated by IL-4 (Spiegelberg, 1990). The autoantigens involved in WG and clinically related disorders are located in the azurophilic granules of the polymorphonuclear granulocytes (PMN). The presence of chronic/recurrent infections in the upper respiratory tract in combination with degranulating PMN might be the triggering stimulus for the production of autoantibodies against lysosomal enzymes. The local presence of IL-4-producing T cells may induce a switch to IgG4. IL-4 may also play a role in the activation of granulocytes by stimulating phagocytosis and potentiating degranulation and the respiratory burst (Boey *et al.*, 1989). In addition, ANCA may directly activate PMN (Falk *et al.*, 1989).

IL-4 may also be involved in granuloma formation since it activates monocytes and macrophages to form giant cells (Hassan *et al.*, 1989). The relative absence of interferon-gamma (IFN-γ) in WG (Grau *et al.*, 1989), which has antagonistic effects to IL-4 on B cell functions such as the production of IgE (Peleman *et al.*, 1989), is also compatible with a dominant role for IL-4 in the production of ANCA of the IgG4 subclass.

To assess whether the predominance of IgG4 is due to a more generalized disturbance in immune regulation in WG, we studied the IgG subclass distribution of antibodies to CMV and tetanus toxoid, antibodies that are present also in normal controls. Antibodies to CMV, positive in 12 out of the 24 ANCA-positive patients tested for these antibodies, were predominantly of the IgG1 and IgG3 subclasses, which compares with the distribution found in normal persons (Gilljam and Wahren, 1989; and our own observations). For anti-tetanus toxoid antibodies, we found a predominance of the IgG1 subclass with a relative increase in IgG2 and IgG3 antibodies, a pattern also seen in patients with active SLE and contrasting with the distribution in healthy individuals (Rubin *et al.*, 1986b). Thus, the predominance of IgG4 subclass in ANCA-positive patients is not a general phenomenon.

What can be hypothesized on the pathophysiological relevance of these findings? In our group of patients, both IgG1 and IgG4 ANCA were present in high titres at the time of diagnosis. In addition, IgG3 subclass ANCA was present almost exclusively in patients with renal involvement. Recent studies showed a strong correlation between ANCA titre and disease activity (Cohen Tervaert *et al.*, 1989). Relapses were preceded by a rise in titre (Cohen Tervaert *et al.*, 1989). IgG1 and IgG3 are complement-fixing antibodies in contrast to IgG4 which has previously been reported to be a blocking or protective antibody because of its poor effector functions *in vitro* (van der Giessen *et al.*, 1976). Thus, IgG3 could play a role as a complement-fixing antibody in the immunopathogenesis of renal involvement in WG. Recently, however, IgG4 class autoantibodies in sera from patients with endemic pemphigus foliaceus were also found to be pathogenetic. Two possible mechanisms were proposed for IgG4 pathogenicity: one by IgG4 activation of proteases, and the other by their binding to a cell adhesion molecule (Rock *et al.*, 1989; Rock, Labib & Diaz, 1990). A role for IgG4 in WG could be binding to and activation of granulocytes resulting in extensive tissue necrosis.

Although the role of ANCA in the pathogenesis of WG and related disorders has not been fully elucidated, the present data may suggest that their induction is due to antigenic stimulation and are compatible with their possible role in the pathophysiological processes underlying the disease symptoms.

ACKNOWLEDGMENTS

This study was supported by grant C88.733 from the Dutch Kidney Foundation. We thank Mr S. Postma for his excellent analytical assistance, and Prof. J. J. Weening for advice and critical reading of this manuscript.

REFERENCES

Aalberse, R.C., van der Gaag, R. & van Leeuwen, J. (1983) Serologic aspects of IgG4 antibodies. *J. Immunol.* **130,** 722.

Andersen, B.R. & Terry, W.D. (1985) Gamma G4-globulin antibody causing inhibition of clotting factor VIII. *Nature,* **217,** 174.

Bird, P., Calvert, J.E. & Amlot, P.L. (1990) Distinctive development of IgG4 subclass antibodies in primary and secondary responses to Keyhole limpet haemocyanin in man. *Immunology,* **69,** 355.

Boey, H., Rosenbaum, R., Castracane, J. & Borish, L. (1989) Interleukin 4 is a neutrophil activator. *J. Allergy clin. Immunol.* **83,** 978.

Borregaard, N., Heiple, J.M., Simons, F.R. & Clark R.A. (1983) Subcellular localisation of the b-cytochrome component of the human neutrophil microbicidal oxidase translocation during activation. *J. cell. Biol.* **97,** 52.

BOWMAN, C., AMBRUS, K. & LOCKWOOD, C.M. (1987) Restriction of human IgG subclass expression in the population of auto-antibodies to glomerular basement membrane. *Clin. exp. Immunol.* **69,** 341.

CARRINGTON, C.B. & LIEBOW, A.A. (1966) Limited forms of angiitis and granulomatosis of Wegener's type. *Am. J. Med.* **42,** 497.

COHEN TERVAERT, J.W., VAN DER WOUDE, F.J. & KALLENBERG, C.G.M. (1987) Analyse van symptomen voorafgaand aan de diagnose ziekte van Wegener. *Ned. Tijdschr. Geneesk.* **131,** 1391.

COHEN TERVAERT, J.W., GOLDSCHMEDING, R., ELEMA, J.D., VAN DER GIESSEN, M., HUITEMA, M.G., VAN DER HEM, G.K., THE, T.H., VON DEM BORNE, A.E.G.K. & KALLENBERG, C.G.M. (1990a) Autoantibodies against myeloid lysosomal enzymes in crescentric glomerulonephritis. *Kidney Int.* **37,** 799.

COHEN TERVAERT, J.W., GOLDSCHMEDING, R., ELEMA, J.D., LIMBURG, P.C., VAN DER GIESSEN, M., HUITEMA, M.G., KOOLEN, M.I., HENÉ, R.J., THE, T.H., VAN DER HEM, G.K., VON DEM BORNE, A.E.G.K. & KALLENBERG, C.G.M. (1990b) Autoantibodies to myeloperoxidase are associated with different forms of vasculitis. *Arthritis Rheum.* **33,** 1264.

COHEN TERVAERT, J.W., VAN DER WOUDE, F.J., FAUCI, A.S., AMBRUS, J.L., VELOSA, J., KEANE, W.F., MEIJER, S., VAN DER GIESSEN, M., THE, T.H., VAN DER HEM, G.K. & KALLENBERG, C.G.M. (1989) Association between active Wegener's granulomatosis and anti-cytoplasmatic antibodies. *Arch. intern. Med.* **149,** 2461.

CONN, D.L., MCDUFFIE, F.C., HOLLEY, K.E. & SCHROETER, A.L. (1976) Immunologic mechanisms in systemic vasculitis. Mayo Clin. Proc. **51,** 511.

DEKRUYFF, R.H., TURNER, T., ABRAMS, J.S., PALLADINO, M.A. & UMETSO, D.T. (1989) Induction of human IgE synthesis by $CD4^+$ T cell clones. *J. exp. Med.* **170,** 1477.

FAGAN, D.L., SLAUGHTER, C.A., CAPRA, J.D. & SULLIVAN, T.S. (1982) Monoclonal antibodies immunoglobulin G4 induce histamine release from human basophils in vitro. *J. Allergy clin. Immunol.* **70,** 399.

FALK, R.J. & JENNETTE, J.C. (1988) Anti-neutrophil cytoplasmatic antibodies specific for myeloperoxidase in patients with systemic vasculitis and idiopathic necrotizing and crescentic glomerulonephritis. *N. Engl. J. Med.* **318,** 1651.

FALK, R.J., TERRELL, R., HUNEYCUTT-CALDER, L. & JENNETTE, J.C. (1989) Anti-neutrophil cytoplasmatic autoantibodies (ANCA) stimulate neutrophil activation in vitro. *Kidney Int.* **35,** 346 (Abstract).

FAUCI, A.S., HAYNES, B.F., KATZ, P. & WOLFF, S.M. (1983) Wegener's granulomatosis: prospective clinical and therapeutic experience with 85 patients for 21 years. *Ann. intern. Med.* **98,** 76.

FIENBERG, R. (1981) The protracted superficial phenomenon in pathergic Wegener's granulomatosis. *Hum. Pathol.* **12,** 458.

FLANAGAN, J.G. & RABBITTS, T.H. (1982) Arrangement of human immunoglobulin heavy chain constant region genes implies evolutionary duplication of a segment containing γ, ε and α genes. *Nature,* **300,** 709.

FRENCH, M.A.H. & HARRISON, G. (1984) Serum IgG subclass concentrations in healthy adults. A study using monoclonal antisera. *Clin. exp. Immunol.* **56,** 473.

GILLJAM, G. & WAHREN, B. (1989) Properties of IgG subclasses to human cytomegalovirus. *J. virol. Methods,* **25,** 139.

GOLDSCHMEDING, R., VAN DER SCHOOT, C.E., TEN BOKKEL HUININK, D., HACK, C.E., VAN DEN ENDE, M.E., KALLENBERG, C.G.M. & VON DEM BORNE, A.E.G.K. (1989) Wegener's granulomatosis autoantibodies identify a novel DFP-binding protein in the lysosomes of normal human neutrophils. *J. clin. Invest.* **84,** 1577.

GRAU, G.E., ROUX-LOMBARD, P., GYSLER, C., LAMBERT, C., LAMBERT, P.H., DAYER, J.M. & GUILLEVIN, L. (1989) Serum cytokine changes in vasculitis. *Immunology,* **68,** 196.

HASSAN, N.F., KAMANI, N., MESZAROS, M.M. & DOUGLAS, S.D. (1989) Induction of multinucleated giant cell formation from blood-derived monocytes by phorbol myristate acetate in in vitro culture. *J. Immunol.* **143,** 2179.

HEINER, D.C. (1984) Significance of immunoglobulin G subclasses. *Am. J. Med.* **30,** 1.

HOUTMAN, P.M., KALLENBERG, C.G.M., LIMBURG, P.C., VAN LEEUWEN, M.A., VAN RIJSWIJK, M.H. & THE, T.H. (1986) Fluctuations in anti-nRNP levels in patients with mixed connective tissue disease are related to disease activity as part of a polyclonal B cell response. *Ann. rheum. Dis.* **45,** 800.

JOHNSON, R.J., COUSER, W.G., CHI, E.Y., ADLER, S. & KLEBANOFF, S.J. (1987) New mechanisms for glomerular injury. *J. clin. Invest,* **79,** 1379.

LEE, S.S., ADU, D. & THOMPSON, R. (1990) Anti-myeloperoxidase antibodies in systemic vasculitis. *Clin. exp. Immunol.* **79,** 41.

MANCINI, G., CARBONARA, A.O. & HEREMANS, J.F. (1965) Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunol Chem.* **2,** 235.

MERRIL, D.P. (1980) Purification of human myeloperoxidase by concanavalin A-sepharose affinity chromotography. *Prep. Biochem.* **10,** 133.

MONGINI, P.K.A., PAUL, W.E. & METCALF, E.S. (1982) T cell regulation of immunoglobulin class expression in the antibody response to trinitrophenyl-Ficoll. *J. exp. Med.* **155,** 884.

NÖLLE, B., SPECKS, V., LÜDEMANN, J., ROHRBACH, M.S., DEREMEE, R.A. & GROSS, W.L. (1989) Anticytoplasmatic antibodies: their immunodiagnostic value in Wegener's granulomatosis. *Ann. intern. Med.* **111,** 28.

PARKES, A.B., MCLACHLAN, S.N., BIRD, P. & REES SMITH, B. (1984) The distribution of microsomal and thyroglobulin antibody activity among the IgG subclasses. *Clin. exp. Immunol.* **57,** 239.

PELEMAN, R., WU, J., FARGEAS, C. & DELESPESSE, G. (1989) Recombinant interleukin 4 suppresses the production of interferon γ by human mononuclear cells. *J. exp. Med.* **170,** 1751.

ROCK, B., LABIB, R.S. & DIAZ, L.A. (1990) Monovalent Fab' immunoglobulin fragments from endemic pemphigus foliacius autoantibodies reproduce the human disease in neonatal Balb/c mice. *J. clin. Invest.* **85,** 296.

ROCK, B., MARTINS, C.R., ARGYRIOS, N.T., BALBEDERAS, R.S., ANHALT, G.J., LABIB, R.S., FUTAMARA, S., RIVITTI, E.A. & DIAZ, L.A. (1989) The pathogenetic effect of IgG4 autoantibodies in endemic pemphigus foliaceus (fogo selvagem). *N. Engl. J. Med.* **320,** 1463.

RUBIN, R.L., TANG, F.L., CHAN, E.K.L., POLLARD, K.M., TSAY, G. & TAN, E.M. (1986a) IgG subclasses of autoantibodies in systemic lupus erythematosus, Sjögren's syndrome, and drug-induced autoimmunity. *J. Immunol.* **137,** 2528.

RUBIN, R.L., TANG, F.L., LUCAS, A.H., SPIEGELBERG, H.L. & TAN, E.M. (1986b) IgG subclasses of anti tetanus toxoid antibodies in adult and newborn normal subjects and in patients with systemic lupus erythematosus, Sjögren's syndrome and drug-induced autoimmunity. *J. Immunol.* **137,** 2522.

SAVAGE, C.O.S., JONES, S., WINEARLS, C.G., MARSHALL, P.D. & LOCKWOOD, C.M. (1987) Prospective study of radioimmunoassay for antibodies against neutrophil cytoplasm in diagnosis of systemic vasculitis. *Lancet,* **i,** 1389.

SCHUR, P.H. (1987) IgG subclasses—a review. *Ann. Allergy,* **58,** 89.

SHAKIB, F. (1987) Aspects of the pathological significance of IgG4. *Exp. clin. Immunogenet.* **4,** 193.

SHAPIRO, S.S. & CARROL, K.S. (1986) Acquired factor VIII antibodies: further immunologic and electrophoretic studies. *Science,* **160,** 786.

SKAVRIL, F. (1986) IgG subclasses in viral infections. In *Basic and Clinical Aspects of IgG Subclasses* (ed. by F. Shakib) p. 134, Karger, Basel.

SPIEGELBERG, H.L. (1990) Biological role of different antibody classes. *Int. Arch. Allergy appl. Immunol.* **90,** 22.

VAN DER GIESSEN, M., VAN DER BERG, A.P., VAN DER BIJ, W., POSTMA, S. VAN SON, W.J. & THE, T.H. (1990) Quantitative measurement of cytomegalovirus-specific IgG and IgM antibodies in relation to cytomegalovirus antigenaemia and disease activity in kidney recipients. *Clin. exp. Immunol.* **80,** 56.

VAN DER GIESSEN, M., HOMAN, W.L., VAN KERNEBEEK, G., AALBERSE, R.C. & DIEGES, P.H. (1976) Subclass typing of IgG antibodies formed by grass pollen allergic patients during immunotherapy. *Int. Arch. Allergy appl. Immunol.* **50,** 625.

VAN DER GIESSEN, M., HUITEMA, M.G., COHEN TERVAERT, J.W. & KALLENBERG, C.G.M. (1989) A technical note on the routinely performed ACPA detection. *APMIS*, **97,** 37.

VAN DER GIESSEN, M., ROSSOUW, E., ALGRA VAN VEEN, T., VAN LOGHEM, E., ZEGERS, B.J.M. & SANDER, P.C. (1975) Quantification of IgG subclasses in sera of normal adults and healthy children between 4 and 12 years of age. *Clin. exp. Immunol.* **21,** 501.

VAN DER WOUDE, F.J., LOBATTO, S., PERMIN, H., VAN DER GIESSEN, M., RASMUSSEN, N., WIIK, A., VAN ES, L.A., VAN DER HEM, G.K. & THE, T.H. (1985) Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker of disease activity in Wegener's granulomatosis. *Lancet*, **i,** 425.

VAN DER ZEE, J.S., VAN SWIETEN, P. & AALBERSE, R.C. (1986) Serologic aspects of IgG4 antibodies. *J. Immunol.* **11,** 3566.

VLUG, A., DE BOT, A.J., BROUWER, H., DE LANGE, G.G., VAN LEEUWEN, A.M., DE RON, E.A. & VAN EIJK, R.V.W. (1989) Monoclonal antibodies against IgG subclass. In *Protides of the Biological Fluids* (ed. by H. Peeters & M. D. Povlik) p. 36.