IgG subclasses of antineutrophil cytoplasm autoantibodies (ANCA)

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Abstract. Sera that had been positive in routine ELISA for ANCA were studied retrospectively for the IgG subclass distribution of these autoantibodies. An ELISA previously developed for measurement of IgG subclasses of anti-GBM antibodies was modified for this purpose. Of a total of 247 sera, 114 were found to be positive in at least one of the assays for IgG subclasses of anti-proteinase 3, 72 of these patients were men and 42 were women, giving a ratio of 1.8. Also 134 sera were positive in at least one of the IgG subclass assays for antimonyeloperoxidase (MPO), with a male/female ratio of 0.97. The ANCA seem to consist mainly of IgG1 and IgG4 autoantibodies. Among the anti-MPO group, IgG2 is relatively common and IgG3 is scarce. Contrasting with this, IgG3 is relatively common in the antiproteinase 3 group. In this group high IgG2 titres are rare. Twelve sera were found to be positive for both autoantigens. Clinical data were studied for 44 patients. Prognosis for old patients was found to be poor. Patients with inactive disease were often positive in only one subclass assay, while patients with active disease were positive in two or more subclass assays (P<0.01).

Key words: IgG subclass; systemic vasculitis; autoantibodies; ANCA glomerulonephritis; Wegener's granulomatosis

Introduction

Autoantibodies directed towards constituents of the cytoplasm of normal human granulocytes and monocytes (antineutrophil cytoplasm antibody (ANCA)) are now established as markers for vasculitides in the kidney with or without signs of systemic disease [1]. The phenomenon of autoantibodies that selectively stain granulocytes and monocytes was first described in 1964 as granulocyte-specific antinuclear antibodies (GS-ANA) [2]. It took until 1982, however, before the first report was published about an association between ANCA and glomerulonephritis [3]. In 1985 the association of ANCA and Wegener's granulomatosis was established [4]. Further research has extended the earlier observations and has demonstrated that ANCA is not limited to the classical form of Wegener's granulomatosis. It can also be found in other forms of systemic vasculitides such as periarteritis nodosa and Churg–Strauss syndrome and in crescentic glomerulonephritis without evidence of extra renal disease [1,5]. Two forms of ANCA can be differentiated by immunofluorescence, one with cytoplasmic staining named c-ANCA and one with perinuclear staining named p-ANCA. Biochemical analyses have shown that in most instances c-ANCA is directed to a serine protease called proteinase 3 (or myeloblastin) [6]. P-ANCA is, at least in renal disease, mostly directed to myeloperoxidase (MPO) [5,7]. Both antigens are present in the alpha granules of the leukocytes. These proteins can be used as antigens in enzyme linked immunosorbent assay (ELISA) [7].

ANCA might not only be a marker of disease but may also take part in the pathogenic process. Two recent reports show that ANCA can change the behaviour of leukocytes, at least in vitro [8,9]. ANCA is usually of the IgG isotype even though reports exist of occasional IgA and IgM ANCA [10,11]. This focuses interest on the Fc portion of the heavy chain of the immunoglobulin molecule, which mediates most of its biological functions. The genes for the heavy chains of human immunoglobulins reside on chromosome 14 and at least nine different genes are expressed in vivo. The genes appear in the following order on the chromosome: IgM, IgD, IgG3, IgG1, IgA1, IgG2, IgG4, IgE, IgA2 [12]. Which chain is produced in response to a certain antigen is dependent upon how and where the antigen is exposed to the immune system, and the chemical composition of the antigen. This process is regulated by different cytokines [13]. Four of the heavy-chain gene products are grouped together, because of structural similarities, and are collectively called IgG. The four different forms of IgG differ in biological properties like complement activation and cell-binding ability [12]. We have previously shown that IgG subclass distribution can help in grouping patients with reactivity towards different epitopes in anti-GBM disease [14].

IgG subclass distributions of ANCA have previously...
been studied by other investigators, using different methods and by using antigens with varying degrees of purity [15–17]. These studies have shown that there is no subclass restriction, that IgG1 dominates and that IgG4 is over represented. Previous investigations have been limited to rather small patient populations. Studies of the total level of IgG subclasses in serum have shown increased levels of IgG4 in patients with Wegener’s granulomatosis [15,18]. Our goal was to analyse IgG subclasses in a large number of unselected ANCA-positive sera, using highly purified antigens in ELISA, and to see whether IgG subclass patterns correlate with clinical findings.

Subjects and methods

Sera

Sera that had been positive in a routine ELISA for ANCA (alpha granule or MPO), were obtained from the archives of Wieslab AB, Lund, from the period January 1987 to October 1990. Of a total of 247 sera, 44 had been referred from the University Hospital of Lund or the General Hospital of Malmö, the rest had been referred from other hospitals in Sweden. Case records of these 44 patients were studied retrospectively. Sera from 18 healthy blood donors were used as a control.

The patients were classified as either ‘active’ or ‘inactive’, based on present signs of activity in their inflammatory disease. Findings such as rapidly progressive glomerulonephritis, elevated C-reactive protein (CRP) in the absence of infection, malaise, fresh synovitis, skin vasculitis, and lung bleedings were used to classify patients as ‘active’. Eighty-eight patients were considered to have an ‘acute’ vasculitic disease. The patients with exacerbation of a chronic disease or long prodromal symptoms were considered as ‘non-acute’.

Antigens

Myeloperoxidase (MPO) was prepared as described by Olsson et al. [19]. Proteinase 3 was prepared from the alpha fraction of human neutrophils as described before [20]. Briefly, the alpha fraction was extracted with 1% Triton X100. The extract was applied to a column of DEAE-cellulose at pH 5.5 in 0.05 M NaAc buffer to remove acidic proteins. The unbound material was applied to a Mono-S column and bound material eluted off in a gradient from 0-1 M NaCl. The peak containing proteinase 3 was collected and concentrated and then applied to a TSK SW-3000 column. A major peak eluted, corresponding to a molecular weight of 30 kDa, that contained the proteinase 3.

Antibodies

Monoclonal subclass specific antibodies to human IgG1 (NL 16), IgG2 (HP 6014), IgG3 (ZG 4), and IgG4 (RJ 4) were purchased from Unipath, Birmingham, UK.

Buffers

Coating buffer = 0.05 M carbonate pH 9.6. Incubation buffer = 10 mM sodium phosphate, pH 7.5, 0.15 M NaCl, 4 mM KCl, Bovine serum albumin 2 mg/ml, 0.05% Tween 20. Washing buffer = 0.9% NaCl, 0.05% Tween 20. Substrate buffer = p-nitro-phenylphosphate 1 mg/ml in 1 M diethanolamine, pH 9.8 containing 5 mM MgCl₂.

ELISA

MPO was coated onto microtitre plates (NUNC, Roskilde, Denmark) overnight at room temperature, using 200 µl/well at an antigen dilution of 10 µg/ml in coating buffer. Plates were rinsed three times in washing buffer; this was repeated between each step. Sera from patients and healthy blood donors were diluted 1/100 in incubation buffer and 200 µl per well were incubated for 1 h. All samples were analysed in triplicate. Monoclonal antibodies were diluted 1/2000 in incubation buffer and 200 µl per well were incubated for 1 h. 200 µl of Goat-anti-Mouse IgG alkaline phosphatase conjugate (Sigma, St Louis, USA) diluted 1/1000 in incubation buffer, was added to each well and incubated for 1 h. 200 µl of substrate buffer was added to each well and absorbance at 405 nm was measured after 22.5 min for monoclonal antibody R4, 25 min for ZG4, 30 min for NL16 and 32.5 min for HP6014 using a Titertek Multiskan spectrophotometer (Flow, Helsinki, Finland). The different reaction times were chosen to standardize the assays to each other. This was based on experiments using myeloma proteins as coating [14 and unpublished results]. 200 µl of alkaline-phosphatase-conjugated swine anti-human IgG (Orion, Helsinki, Finland) diluted 1/250 were used in the assay for total IgG.

Absorbances for these plates were measured after 30 min.

The antiproteinase 3 ELISAs were performed in the same manner, but because of scarcity of antigen the coating concentrations were reduced to 0.3 µg/ml. Volumes per well were reduced to 100 µl in each step. Serum dilution was 1/100 in incubation buffer ± 0.025% gelatin. Reaction times for individual monoclonal antibodies were increased to 60 min for NL16, 65 min for HP6014, 50 min for ZG4, and 45 min for R4. The total IgG assay for antiproteinase 3 was read after 60 min.

Calculations

Crude absorbance values were reduced with the absorbance of ‘blank wells’ on each plate. The blank wells were treated identically to the test well except that incubation buffer without serum was added in the second step. Results were considered positive when the mean value of a triplicate of an analysed sample exceeded the results of the mean value of normal sera ± 2 standard deviations. Eighteen normal sera were used for anti-MPO and 10 normal sera were used for antiproteinase 3.

Statistical evaluation was by Student’s t test or Fisher’s test.

Results

Of the 247 sera available for this study, 141 had been positive in routine ELISA for MPO and 123 had been positive in alpha granule ELISA. Seventeen of the sera had been positive in both assays.
IgG subclasses of autoantibodies to proteinase 3

Sera that were positive in the ELISA for alpha granule directed to the proteinase 3 antigen, and 114 of 123 were positive in at least one of the assays for IgG1, IgG2, IgG3, or IgG4. The remaining nine all had low titres in the routine assay. Of these 114 patients, 72 were men and 42 women, giving a male/female ratio of 1.8. The average age of the men was 58.4 years and of the women 62.6 years (P < 0.001). Figure 1 presents the age and sex distribution. In mid-life (45–64 years of age), men outnumber women 36 to 10; while in the elderly (above 65 years of age) the distribution is more even (26 versus 27).

The absorbance values for the 114 sera are presented in Figure 2. This figure shows that IgG1 is the dominating subclass and that IgG4 is relatively overrepresented.

IgG subclasses of autoantibodies to MPO

Sera that had been positive in routine ELISA for anti-MPO were analysed for IgG subclass distribution of the autoantibodies, and 134 of 141 were positive in at least one of the subclass assays. The remaining seven all had low activity in the routine assay. The mean age of the 134 patients was 62.9 years. Figure 3 shows the age distribution and sex distribution of the anti-MPO sera. The sex distribution was even, with 66 men and 68 women. The mean age for men was 63.7 years while for women it was 62.4 years.

The absorbance values for the 134 sera are presented in Figure 4. IgG1 is found to be the dominating subclass, 126 sera (94%) were positive. IgG1 showed the highest absorbance value in 109 cases and it was the only positive in eight instances. The mean absorbance value for IgG1 was 1.129. IgG2 was found in most sera, the analysis was positive in 103 (77%) cases, but the mean absorbance was only 0.326.
Fig. 3. Age distribution of 134 Swedish patients positive in at least one ELISA for IgG subclasses of autoantibodies to MPO. Among the patients 66 are men and 68 women. Sex distribution seems to be equal in all age groups.

Fig. 4. Results of IgG subclass analysis for 134 anti-MPO-positive sera. Results for normal sera + 2 SD are indicated by dashed lines.

cases IgG2 had the highest values, and in two of these IgG2 was the only positive subclass. IgG3 showed the lowest mean absorbance value, but was found positive in as many as 88 (66%) sera. In three cases the IgG3 assay yielded the highest rating and in one case it was the only positive assay. The IgG4 assay was positive in 96 (72%) sera, with a mean absorbance value of 0.450. In 16 sera IgG4 had the highest absorbance value, and in three of these it was the only positive subclass found.

IgG subclasses of autoantibodies to both proteinase 3 and MPO

Twelve sera were positive in assays for both of the antigens. This means that 10.6% of the antiproteinase 3 positive sera also were positive for anti-MPO and that 9.0% of the anti-MPO positive sera were positive for antiproteinase 3. In six of these the IgG subclass distribution patterns were similar. In five sera IgG1 and in four of these both IgG1 and IgG3 were negative for one of the antigens. Among the eight IgG1 negative of 134 anti-MPO sera, three were found to be positive for antiproteinase 3.

Correlation with clinical data

Records from all the 44 patients treated at the University Hospital of Lund and the General Hospital of Malmö were studied retrospectively. Eighteen of these were positive for antiproteinase 3 and 27 were positive for anti-MPO; one patient was double-positive. The patients were divided into groups based on the clinical status at the time when the serum sample was drawn. Table 1 shows the number of patients, for both autoantibodies, referred to each clinical group. After 1 year nine patients of 44 had died; after 2 years 14 had died. Two patients were already on renal replacement therapy when the index sample was taken. Two years later four more patients had started renal replacement therapy. For those with 'inactive disease' the prognosis was good, after 2 years none had died and none had lost renal function. Treatment in patients with active disease mostly consisted of prednisone and cyclophosphamide. Plasmapheresis was sometimes added. For those with active disease and age above 65, prognosis was poor. Only seven of 19 (37%) were alive after 2 years, and a further two patients required chronic haemodialysis treatment. Prognosis for patients <65 years of age and with active disease is good with current therapy; 14 of 16 patients (88%) were alive, 12 of these had preserved renal function with serum creatinine below 300 μmol/l.

Of 247 patients, 33 were positive in only one IgG subclass assay; four of these had been treated in Lund or Malmö. None of these four was among the 36 patients considered to have an active disease. In contrast, four of eight patients with inactive disease were positive in one single subclass assay (P<0.01). Long prodromal symptoms or previous autoimmune disease were typical in anti-MPO positive patients. Only in one case was there no record of such a history. However, even in this case there was a history of
Monoclonal antibodies is a relatively simple technique with suspected systemic vasculitis and positive ANCA. Other places differ in clinical picture or subclass distribution. Consequently, we consider our data to be representative of the Swedish population of patients since the whole clinical spectrum of ANCA is still not known. Recently, a new form of ANCA has been found only in one of the other seven patients with RPGN. Such a pattern (IgG3/(IgG2 + IgG4) > 1) was found only in one of the other seven patients with RPGN ($P < 0.05$).

### Discussion

The IgG subclass distribution of autoantibodies in the two nephrologically most important forms of ANCA, antiproteinase 3 and anti-MPO, were studied in 247 sera. Sera were admitted from most parts of Sweden. We estimate the proportion of the cases in Sweden being analysed at other laboratories during the period of collection to be about 50%. Incidence and prevalence, however, cannot be calculated from these data since the whole clinical spectrum of ANCA is still not known. Recently, a new form of ANCA has been identified in ulcerative colitis [21]. ANCA is, of course, only found when it has been suspected. There is little reason, however, to believe that sera diagnosed at other places differ in clinical picture or subclass distribution. Consequently, we consider our data to be representative of the Swedish population of patients with suspected systemic vasculitis and positive ANCA.

ELISA with purified antigens and subclass-specific monoclonal antibodies is a relatively simple technique to perform; therefore we could study a large number of unselected patient sera. The results could then be used in studies to correlate clinical parameters to subclass findings. However, the technique also has shortcomings that must be kept in mind when results are analysed. The assays are performed in vitro, and the relevance in vivo can be questioned. A major difficulty in measuring IgG subclasses of autoantibodies is that the standardization of the four individual assays is indirect [14,22] and different kinetic properties of the monoclonal antibodies make indirect standardization procedures unreliable. Antibodies of different IgG subclasses can have different affinities [23]. This means that a population of antibodies of one subclass with high affinity can mask binding sites for antibodies of other subclasses, if they are directed to the same epitope. The absorbance values obtained in our assays depend not only on the amount of antibody but also on affinity. Comparison between individual assays must hence be considered as only semiquantitative.

In 247 sera that previously had been positive in the 'routine assay', we found positive results in 236 using purified proteinase 3 and MPO as antigens; 102 were only positive for antiproteinase 3, 122 were only positive for anti-MPO, while 12 were positive for both antigens. Of 123 c-ANCA positive in the routine alpha granule ELISA, nine were negative in the subclass analysis for proteinase 3 antibodies. All had weak reactivity in the routine test. Five of these cases were also positive in anti-MPO in the routine ELISA and remained positive in subclass analysis for MPO autoantibodies.
In these cases residual MPO in the alpha granule extract can account for the reactivity. This means that other antigens than proteinase 3 play an unimportant role, if any, in alpha granule ELISA reactivity, at least in these patients.

IgG subclasses of autoantibodies against proteinase 3 and MPO were measured with slightly different methods, using different coating concentrations and different volumes. The main reason for this was relative scarcity of sera and the proteinase 3 antigen. These differences in the assays have little effect on the results of the subclass distributions (results not shown). Attempts could have been made to make the assays look more similar, but since we do not know the number of relevant epitopes on each molecule, the effect would be mainly cosmetic.

The results show that IgG1 is the dominating subclass in both the proteinase 3 and the MPO group. This is in accordance with previous reports [15-17]. Autoantibodies of the IgG2 subclass are rare in the proteinase 3 group. Only a small proportion of the sera have results above the level of normal sera and most of these have low absorbance values. In only one serum did IgG2 dominate over IgG3 and IgG4. In the MPO group, on the contrary, a substantial part of the reactivity is derived from the IgG2 subclass. In 25 sera IgG2 dominated over IgG3 and IgG4, in six of these IgG2 yielded higher absorbance values than IgG1. This difference may be due to the higher carbohydrate content of the MPO molecule compared to proteinase 3, since an IgG2 response is evoked mainly by carbohydrate-rich antigens [24].

Autoantibodies of the subclass IgG3 contribute to a smaller proportion of the anti-MPO reactivity compared to the antiproteinase 3 reactivity. Substantial elevations of IgG3 titres were found only in a few MPO-positive patients, while this was rather common in the proteinase 3-positive group. The gene for IgG3 is located most upstream on the chromosome, indicating that it is produced early in an immune response. This has been demonstrated for antigens of some infectious diseases [25]. Esnault et al. found that IgG3 anti-MPO was lower in 12 follow-up sera compared to the levels found in the initial sera analysed [17]. In this study all four antiproteinase-3-positive patients with short prodromal symptoms and without evidence of prior exacerbation of vasculitic disease had a relatively elevated titre of IgG3. A part of the difference of IgG3 distribution can thus be due to a more chronic nature of anti-MPO disease and a more subacute nature of antiproteinase 3 disease. Brouwer et al. found that IgG1 and IgG3 titres against alpha granule extracts were greater in patients with vasculitis with renal involvement than in those without renal involvement. For anti-MPO they found the same correlation for IgG3 [15]. Their finding that high IgG3 is found in a more severe setting does not contradict our view that low IgG3/(IgG4 + IgG2) ratio could indicate a chronic type of disease.

In these patients the two autoantibodies were equally common among men (72 antiproteinase 3 and 66 anti-MPO), while MPO antibodies were more common among women (68 versus 42). The mean ages for MPO women were the same as for proteinase 3 women (62.4 versus 62.7); the men with anti-MPO were older than the men with antiproteinase 3 (63.6 versus 58.4). Exogenous factors are believed to play a role in systemic vasculitis [26]; if so, assuming that both sexes are genetically equally susceptible, exposure to the factors inducing MPO antibodies are equally encountered among men and women in Sweden; and men encounter the factors inducing proteinase 3 antibodies more often than women. Falk [1] reported seasonal variation, with more cases being found in the winter, at his centre in North Carolina, USA. In this material of Swedish patients no such correlation could be found, either for anti-MPO or for antiproteinase 3 (data not shown).

In most of the sera two or more subclasses together make up the ANCA reactivity. However, in a minor portion of the sera the immune response is limited to one subclass. Our study indicates that such restricted immune response is preferentially seen in patients in remission. This observation is based on a small number of cases, measured at one time. To confirm this finding, longitudinal studies are necessary. Most sera were positive for at least one of the complement-binding subclasses IgG1 or IgG3. When these subclasses were negative, the sera often belonged to the little group of 12 sera that were positive for both autoantibodies. IgG1 or IgG3 were then positive for the other autoantibody. We speculate that sera with elevation of only IgG2 and/or IgG4 of these autoantibodies are more common than indicated by this study; however, they are not detected because of absence of active disease. If these findings can be confirmed it can be of clinical importance when monitoring patients with positive ANCA and remission.

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