Anca associated vasculitis
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Native and recombinant proteins to analyze auto-antibodies to myeloperoxidase in pauci-immune crescentic glomerulonephritis

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Abstract

The prevalence of Anti Neutrophil Cytoplasmic Antibodies (ANCA) directed against myeloperoxidase (MPO) in pauci-immune necrotizing crescentic glomerulonephritis (NCGN) is dependent on the assay(s) used. We investigated the frequency of MPO-ANCA as detected by different assays for MPO-ANCA in a large cohort of patients with biopsy-proven pauci-immune NCGN. Sera from 121 consecutive untreated patients presenting with pauci-immune NCGN were tested for ANCA directed to proteinase-3 (PR3) at diagnosis. PR3-ANCA negative sera were tested by direct ELISA using recombinant or native MPO and by capture ELISA using 2 different specific monoclonal antibodies directed to MPO and 3 different antigenic sources. Sera from 80 relevant disease controls were tested to explore the specificity of the different assays. Thirty-eight out of 121 patients (31%) with pauci-immune NCGN did not have PR3-ANCA. Sufficient amounts of serum from 30 of these 38 PR3-ANCA negative patients were available for further testing. Recombinant and native MPO were recognized by similar numbers of sera in a direct ELISA (recombinant MPO: 93%, native MPO: 93%) and a capture ELISA (recombinant MPO: 77-87%, native MPO: 93%). Sera of patients with PR3-ANCA positive pauci-immune NCGN and disease controls were less frequently positive for MPO-ANCA in a capture ELISA (recombinant MPO: 3-7%, native MPO: 6-7%) than in a direct ELISA (recombinant MPO: 25%, native MPO: 13%). Both direct and capture ELISA assays using either native or recombinant MPO are sensitive techniques to detect MPO-ANCA in patients with pauci-immune NCGN. A capture ELISA performs better than a direct ELISA because it combines a higher specificity with a comparable sensitivity. Recombinant MPO is a good alternative for native MPO when used as antigen in a capture ELISA, but not when used in a direct ELISA because of lower specificity in this latter assay.

Introduction

Rapidly progressive glomerulonephritis is characterized by rapid deterioration of renal function occurring within days or weeks. In the majority of cases renal biopsies show fibrinoid necrosis of the glomerular capillary wall with extracapillary proliferation and crescent formation, findings designated as necrotizing crescentic glomerulonephritis (NCGN) (Couser, 1988). In the majority of patients only few or no immune deposits are found. This pauci-immune form of NCGN is associated with Wegener’s granulomatosis (WG), microscopic polyangiitis (MPA), and Churg-Strauss syndrome (CSS), or occurs as an idiopathic variant (idiopathic NCGN) (Couser, 1988). Pauci-immune NCGN is strongly associated with the presence of Anti Neutrophil Cytoplasmic Antibodies (ANCA) directed against proteinase-3 (PR3) or myeloperoxidase (MPO) (Cohen Tervaert et al., 1990; Niles et al., 1991).

Previously, we reported that 91% of our patients with pauci-immune NCGN had either PR3-ANCA or MPO-ANCA (Cohen Tervaert et al., 1990). Some studies confirmed this finding...
Native and recombinant proteins

(Niles et al., 1991; Pettersson et al., 1995), while others found a lower prevalence of ANCA in these patients (Harris et al., 1998; Bindi et al., 1993; Saxena et al., 1991). A possible explanation for the observed differences may be the way in which ANCA were detected in these studies. ANCA may be detected by capture ELISA (Cohen Tervaert 1990) or by direct ELISA (Niles et al., 1991; Pettersson et al., 1995; Harris et al., 1998; Bindi et al., 1993; Saxena et al., 1991). In a direct ELISA, recognition of the antigen by antibodies could be hampered by conformational changes in the antigenic proteins induced by denaturation during coating. In a capture ELISA, in which antibodies present the antigen, conformational changes might be circumvented. However, capturing antibodies may block relevant epitopes. For the detection of PR3-ANCA it has been demonstrated that the use of a capture ELISA is to be preferred (Basland et al., 1995; Westman et al., 1998).

Recently, several studies reported great variability between kits for MPO-ANCA detection by direct ELISA (Pollock et al., 1999; Wang et al., 1997). This may be due to contamination of purified MPO with lactoferrin (Audrain et al., 1999). To avoid contamination with other myeloid proteins, the availability of recombinant MPO (rMPO) would be of great help.

Recently, the cDNA coding for human MPO has been cloned and expressed in Chinese hamster ovary cells (Moguilevsky et al., 1991). In this system, rMPO is secreted as a single chain protein of 84-kD, equivalent to a molecule of hemiperoxidase with the promolecule retained at the N-terminus. This rMPO has similar cytotoxic activity, glycosylation, and physical properties as native MPO (Moguilevsky et al., 1991). Short et al. suggested that rMPO is useful for the detection of MPO-ANCA, since serum samples of 95% of patients with antibodies directed against native MPO also recognized rMPO (Short et al., 1995). In contrast, Audrain et al. reported that rMPO was recognized by only 70% of patient sera that were selected based on the presence of antibodies directed against native MPO (Audrain et al., 1997).

To test the clinical relevance of different methods to detect antibodies to MPO, we tested serum samples from a cohort of consecutive PR3-ANCA negative patients with pauci-immune NCGN for the presence of MPO-ANCA using different MPO-ANCA tests. Serum samples were tested for the presence of MPO-ANCA by direct ELISA using either native MPO or rMPO as antigens and capture ELISA using two different monoclonal antibodies (MoAb) and purified native MPO, rMPO, or a crude neutrophil extract as antigenic source, respectively.
Chapter 5

Methods

Patients and serum samples

All consecutive patients who were diagnosed in our hospital between March 1984 and April 1999 with pauci-immune NCGN entered the study. A diagnosis of pauci-immune NCGN was based on a renal biopsy showing focal or diffuse segmental crescentic glomerulonephritis and a paucity of immune deposits by immunofluorescence studies (Cohen Tervaert et al., 1990; Hogan et al., 1996).

Serum samples from these patients, which were all obtained within one week of renal biopsy and before the start of treatment, had been routinely tested for the presence of PR3-ANCA by our standard capture ELISA using an extract of azurophilic granules and the PR3-specific MoAb 12.8 (CLB, Amsterdam, NL)(Cohen Tervaert et al., 1990).

To compare the different methods (see below) for detection of MPO-ANCA, all available sera from patients with PR3-ANCA negative pauci-immune NCGN were tested for the presence of MPO-ANCA. As disease controls sera from patients with various diseases, in which ANCA of known or unknown specificity are frequently detected, were used (Kallenberg et al., 1992).

For this, 27 serum samples from patients with PR3-ANCA positive pauci-immune NCGN, 20 serum samples from patients with systemic lupus erythematosus (SLE), 20 serum samples from patients with autoimmune hepatitis (AIH), and 20 serum samples from patients with ulcerative colitis (UC) were randomly selected. Patients with SLE fulfilled at least four of the ACR criteria (Tan et al., 1982), patients with AIH fulfilled the International Autoimmune Hepatitis Study Group criteria (Johnson et al., 1993), and the diagnosis UC was based on clinical, endoscopic, and radiological criteria supported by histopathology (O'morain et al., 1989). In addition, serum samples from 20 patients with dialysis dependent end stage renal failure (ESRF) without evidence for ANCA associated vasculitis (renovascular n = 1, polycystic kidney disease n = 5, IgA nephropathy n = 5, tubulo-interstitial disease n = 5, unknown n = 4) were tested. Sera from 32 age and sex-matched healthy volunteers were used to establish normal values for the assays. Serum samples were stored at −20°C until analyzed. The sera were tested in triplicate in all ELISAs.

The study was carried out in accordance with the 1997 Declaration of Helsinki of the World Medical Association (World Medical Association, 1997).

Detection of ANCA by Indirect Immunofluorescence

ANCA detection by indirect immunofluorescence (IIF) was performed on ethanol-fixed granulocytes as described previously (Cohen Tervaert et al., 1993). Staining patterns were described as ‘C-ANCA’ when granular staining of the cytoplasm with accentuation between the nuclear lobes was present, as ‘P-ANCA’ when a (peri)nuclear pattern was observed, and as ‘atypical’ when a non-granular cytoplasmic pattern was observed. A titer ≥ 1:40 was considered positive.
**Native MPO**

Isolation of native MPO was performed as described previously (Boomsma et al., 2000). Briefly, enriched leukocyte populations were collected by centrifugation, cells were washed and subsequently disrupted using nitrogen cavitation. Alpha granules were isolated by centrifugation on a discontinuous Percoll gradient (Pharmacia, Fine Chemicals AB, Uppsala, Sweden). Antigens were extracted from the alpha granules using PBS containing 2% Triton X-100 (Sigma Chemicals Co., St Louis, MO). After centrifugation, supernatants were dialyzed and applied to a Bio-rex column (Bio-Rad Laboratories, Richmond, CA). Native MPO was eluted from the Bio-rex column using 1M NaCl, dialyzed against sodium acetate buffer, absorbed to a concanavalin A Sepharose gel (Pharmacia), and eluted with 1M α-methyl-D-mannoside (Sigma). Samples containing MPO were finally purified using a Superdex-100 column (Pharmacia).

The purity of native MPO was checked by electrophoresis. Specific ELISAs were used to exclude contamination with PR3, elastase and lactoferrin (Brouwer et al., 1993).

**Recombinant MPO**

rMPO was purified from culture supernatants of Chinese hamster ovary cells transfected with the cDNA coding for human MPO using a two step chromatography procedure as described previously (Short et al., 1995). In brief, the culture medium was passed through a Q-Sepharose column in phosphate buffer and the flow-through fraction was loaded onto a carboxymethyl-Sepharose fast-flow column. rMPO was eluted with a NaCl gradient. All steps were carried out at 40C. All fractions were run on SDS-PAGE and by Western blotting. The pooled fractions containing the rMPO were characterized in terms of enzymatic activity using O-dianisidine and monochlorodimedon as substrates. The purified rMPO was also run on SDS-PAGE to confirm purity and was tested in an MPO-capture ELISA to confirm recognition of rMPO (Short et al., 1995). rMPO had a ratio of absorbance at optical density (OD) 430 nm / 280 nm of 0.427, a concentration of 1.6 mg/ml and a chlorinating activity of 93.8 U/ml.

**Detection of MPO-ANCA by direct ELISA**

Detection of MPO-ANCA by direct ELISA was performed as previously described (Franssen et al., 1998). Ninety-six well microtitre plates (Maxisorp; NUNC NS, Roskilde, Denmark) were coated for 1 hour at room temperature with native MPO at a concentration of 1.6 µg/ml in coating buffer [0.03M Na2CO3, 0.07M NaHCO3, pH 9.6]. Subsequently, coating buffer with 1% BSA (blocking buffer) was added and incubated for 0.5 hour at room temperature. Sera were diluted 1:50 using incubation buffer [PBS, 0.2% BSA , 0.05% Tween 20 (Sigma)] and were incubated for 1.5 hours at room temperature. The direct ELISA in which
recombinant MPO was used was identical to the ELISA in which native MPO was used, except that rMPO was coated at a concentration of 10 µg/ml. Criteria for positivity were established for the assays based on the results from the sera of 32 healthy volunteers. An extinction (OD 405 nm) of 0.35 or more resulted in 97% negative results in these sera and was considered to be the cut-off value for a positive / negative test result for both antigens.

Detection of MPO-ANCA by capture ELISA
Detection of MPO-ANCA by capture ELISA was performed as previously described with minor modifications (Cohen Tervaert et al., 1990). Greiner microtitre plates were coated for 48 hours at 40°C with 3.2 µg/ml of goat anti-mouse IgG (Jackson Immunoresearch, Westgrove, PA), in coating buffer. The plates were incubated with 0.34 µg/ml of MPO-specific MoAb 4.15 (CLB, Amsterdam, NL) or with 1.0 µg/ml of MPO-specific MoAb 266 6K2 (IQ Products, Groningen, NL) in incubation buffer [100 mM Tris-HCL pH 8.0 containing 0.5% Tween-20 (Sigma), 0.3M NaCl, 1% BSA and 1% NGS (CLB, Amsterdam, NL)] for 2 hours at 37°C. rMPO was incubated at a concentration of 10 µg/ml, native MPO at a concentration of 1.6 µg/ml and a crude extract of azurophilic granules (2 mg/ml), isolated as described previously (Cohen Tervaert et al., 1990), was incubated at 4 µg/ml. Sera were diluted 1:50 using incubation buffer and incubated for 2 hours at 37°C. Results were corrected for background staining by testing each serum additionally by an ELISA identical to that described, with the exception that a non-related MoAb directed against a small cell lung carcinoma cell epithelial glycoprotein was used (De Leij et al., 1994).
Criteria for positivity were established for the assays based on the results from the sera of 32 healthy volunteers. An extinction (OD 405 nm) of 0.15 or more resulted in 97% negative results in these sera and was considered to be the cut-off value for a positive / negative test result for the various anti-MPO specific capture ELISAs.

Statistical analysis
Demographic and clinical characteristics were compared using the Fischer’s exact test for categorical quantities, and the Mann-Whitney test for continuous quantities. Scatterplots of extinctions as measured by ELISA using rMPO and native MPO were generated using GraphPad Prism™ software. Correlation was evaluated using Spearman correlation coefficients. A two-sided p-value of <0.05 was used to indicate statistical significance.
Results

Clinical Findings

Between March 1984 and April 1999, 121 patients (67 male / 54 female) were diagnosed with pauci-immune NCGN. The mean age at the time of the renal biopsy was 58 years (range: 17 to 87 years). Renal function was impaired in all patients. Thirty-nine patients (32%) required dialysis at the time of the diagnosis. The median plasma creatinine level of the remaining patients was 305 µmol/l (range: 90 to 780 µmol/l). Microscopic hematuria and proteinuria were present in all patients.

Table 1 Clinical findings and organ system involvement in 121 patients with pauci-immune NCGN

<table>
<thead>
<tr>
<th></th>
<th>PR3-ANCA positive</th>
<th>PR3-ANCA negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pauci-immune NCGN</td>
<td>pauci-immune NCGN</td>
</tr>
<tr>
<td>n.</td>
<td>83</td>
<td>38</td>
</tr>
<tr>
<td>Mean age, y</td>
<td>59</td>
<td>54</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>52 / 31</td>
<td>15 / 23</td>
</tr>
<tr>
<td>Organ involvement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>83 (100)</td>
<td>38 (100)</td>
</tr>
<tr>
<td>-dialysis required</td>
<td>29 (35)</td>
<td>10 (26)</td>
</tr>
<tr>
<td>Lung</td>
<td>50 (60)</td>
<td>18 (47)</td>
</tr>
<tr>
<td>-Haemoptysis</td>
<td>27 (54)</td>
<td>14 (78)</td>
</tr>
<tr>
<td>Paranasal sinuses</td>
<td>38 (46)</td>
<td>10 (26)</td>
</tr>
<tr>
<td>Nose or nasopharynx</td>
<td>67 (81)</td>
<td>16 (42)</td>
</tr>
<tr>
<td>Ear</td>
<td>35 (42)</td>
<td>8 (21)</td>
</tr>
<tr>
<td>Joint</td>
<td>66 (80)</td>
<td>17 (45)</td>
</tr>
<tr>
<td>Eye</td>
<td>37 (45)</td>
<td>6 (16)</td>
</tr>
<tr>
<td>Skin</td>
<td>34 (42)</td>
<td>8 (21)</td>
</tr>
<tr>
<td>Nervous system</td>
<td>41 (49)</td>
<td>6 (16)</td>
</tr>
<tr>
<td>Heart</td>
<td>12 (14)</td>
<td>2 (5)</td>
</tr>
</tbody>
</table>

*a All patients had renal involvement because of the inclusion criteria
b Haemoptysis data refer to patients with lung involvement only
PR3 = Proteinase 3.

Serum samples from these patients were tested at the time of diagnosis for the presence of PR3-ANCA by routine capture ELISA (Cohen Tervaert et al., 1990). Overall, 83 patients (69%) were PR3-ANCA-positive. Of the remaining 38 patients without PR3-ANCA, 35 had vasculitis-associated or idiopathic NCGN and 3 patients were clinically suspected for post-infectious NCGN. The paranasal sinuses, ears, nose, nasopharynx, joints, eyes, skin and
nervous system were significantly more often affected in PR3-ANCA positive patients than in PR3-ANCA negative patients (Table 1).

Of 38 PR3-ANCA negative patients with pauci-immune NCGN 30 serum samples obtained at the time of diagnosis were available for further testing. These 30 serum samples, 27 serum samples of PR3-ANCA positive patients with pauci-immune NCGN, and 80 serum samples from other disease controls were tested for the presence of MPO-ANCA by direct and capture ELISA. Furthermore, we examined whether rMPO could be used as an alternative for native MPO in both the direct and capture ELISA.

**Indirect immunofluorescence**

All tested sera of the patients with PR3-ANCA negative pauci-immune NCGN had a positive IIF test. In 29 out of 30 sera (97%) the staining pattern was perinuclear and in the remaining serum (3%) the staining pattern was atypical (Table 2).

All 27 sera of PR3-ANCA positive patients with pauci-immune NCGN (100%), and 49 of 80 samples from disease controls (61%) had a positive IIF test (Table 2).

**Table 2** Patients characteristics at measurement (mean [range])

<table>
<thead>
<tr>
<th>n.</th>
<th>Age, y</th>
<th>Gender (M/F)</th>
<th>Negative</th>
<th>P-ANCA</th>
<th>C-ANCA</th>
<th>Atypical</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR3-ANCA negative pauci-immune NCGN</td>
<td>30</td>
<td>54 (20–84)</td>
<td>12 / 18</td>
<td>0</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td>PR3-ANCA positive pauci-immune NCGN</td>
<td>27</td>
<td>59 (56–75)</td>
<td>16 / 11</td>
<td>0</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>SLE</td>
<td>20</td>
<td>34 (19–50)</td>
<td>2 / 18</td>
<td>2</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>AIH</td>
<td>20</td>
<td>42 (17–70)</td>
<td>6 / 14</td>
<td>6</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>UC</td>
<td>20</td>
<td>38 (18–71)</td>
<td>10 / 10</td>
<td>8</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>ESRF</td>
<td>20</td>
<td>43 (22–57)</td>
<td>14 / 6</td>
<td>15</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

NCGN = necrotizing crescentic glomerulonephritis; SLE = systemic lupus erythematosus; AIH = autoimmune hepatitis; UC = ulcerative colitis; ESRF = end stage renal failure; IIF = indirect immunofluorescence; P-ANCA = perinuclear staining in IIF; C-ANCA = cytoplasmatic staining in IIF.
**Direct MPO ELISA**

Twenty-eight out of 30 sera from patients with PR3-ANCA negative pauci-immune NCGN (93%) tested positive for MPO-ANCA by direct ELISA in which native MPO or rMPO was used. There is a highly significant correlation ($r = 0.75$, $P < 0.0001$) between MPO-ANCA levels as tested by direct ELISA in which either native MPO or rMPO was used (Figure 1A). One patient with a post-infectious GN and one patient with WG were ANCA-negative in both assays.

None of the 27 sera from PR3-ANCA positive patients with pauci-immune NCGN (0%), and 14 out of 80 samples from disease controls (18%) were positive when tested on native MPO. When tested on rMPO, one of the sera from PR3-ANCA positive patients with pauci-immune NCGN (4%) and 26 sera from disease controls (34%) were positive (Figure 2A). Overall specificity using the disease controls in this study was 87% (93 out of 107) and 75% (80 out of 107) for direct MPO ELISA using native MPO and rMPO, respectively. The concordance between direct ELISA with rMPO and native MPO was 122 of 137 samples (89%): 41 with a positive test and 81 with a negative test.

**Figure 1** The binding to recombinant MPO (rMPO) as compared to native MPO and an extract of azurophilic granules of 30 sera from PR3-ANCA negative patients with pauci-immune NCGN are shown in extinctions corrected for background staining. The results are displayed for a direct ELISA (A), and a capture ELISA in which monoclonal antibody 266 6K2 is used (B&C). The dotted line indicates the cut-off value for a positive / negative test.
Chapter 5

Figure 2 MPO-ANCA levels as detected in sera from PR3-ANCA-negative patients with pauci-immune NCGN (PR3-; N = 30), patients with PR3-ANCA-positive patients with pauci-immune NCGN (PR3+; N = 27), patients with systemic lupus erythematosus (SLE; N = 20), autoimmune hepatitis (AIH; N = 20), ulcerative colitis (UC; N = 20) and end stage renal failure (ESRF; N = 20). The results are displayed for a direct ELISA (A) in which native MPO (○), or recombinant MPO (rMPO) (●) was used, and a capture ELISA using monoclonal antibody (MoAb) 266 6K2 (B) or MoAb 4.15 (C) in which an extract of azurophilic granules (▲), native MPO (○) or rMPO (●) was used. The dotted line indicates the cut-off value for a positive / negative test.

Capture MPO ELISA

Using MoAb 266 6K2 as capture antibody and different antigen sources, 27 out of 30 (90%) serum samples from patients with PR3-ANCA negative pauci-immune NCGN tested positive with an extract from granulocytes, 28 of 30 (93%) serum samples tested positive with native MPO, and 26 of 30 (87%) serum samples tested positive with rMPO, respectively (Figure 2B, 3A, 4A-C). With MoAb 4.15 as the capturing antibody, 27 of 30 (90%) serum samples from patients with PR3-ANCA negative pauci-immune NCGN tested positive with an extract from granulocytes, 28 of 30 (93%) patients tested positive with native MPO, whereas only 23 out of 30 patients (77%) tested positive with rMPO (Figures 2C, 3B, 4A-C). Although a highly significant correlation was found between the tests, extinctions were usually lower when rMPO instead of purified native MPO (Figure 1B: r = 0.77, P < 0.0001) or an extract of azurophilic granules (Figure 1C: r = 0.80, P < 0.0001) was used as antigenic source. This was
also true for the capture ELISAs in which MoAb 4.15 (data not shown) was used as capture antibody.

The results of the 27 sera from PR3-ANCA positive patients with pauci-immune NCGN and 80 sera from disease controls in a capture ELISA for MPO-ANCA in which either an extract of granulocytes, native MPO, or rMPO was used are presented in Figure 2B (MoAb 266 6K2) and Figure 2C (MoAb 4.15). For capture MPO ELISA with the different antigenic preparations, specificity based on the 27 sera of patients with PR3-ANCA positive pauci-immune NCGN and 80 sera of disease controls ranged from 94% to 97% using MoAb 266 6K2, and from 93% to 95% using MoAb 4.15.

Concordance for either positivity or negativity between capture ELISAs with an extract of azurophilic granules, native MPO, and rMPO occurred in 127 out of 137 samples (93%) (MoAb 266 6K2), and in 124 out of 137 samples (91%) (MoAb 4.15).

Comparison of direct and capture MPO ELISA

Overall concordance between results obtained by direct ELISA and capture ELISA was noted in 94 out of the 137 samples (69%). Twenty-two out of 30 sera of PR3-ANCA negative patients with pauci-immune NCGN (73%) were consistently positive in all MPO-ANCA assays and 2 sera (7%) tested consistently negative in all anti-MPO assays. Six patients (20%) that were positive in a direct ELISA with native MPO as well as rMPO tested negative in one or more (maximum 3) out of 6 different MPO-ANCA capture ELISAs.

Using native MPO resulted in comparable sensitivity in both a direct ELISA (93%) and capture ELISA (93%), whereas the use of rMPO led to a slightly lower sensitivity in capture ELISA (MoAb 4.15: 77%, MoAb 266 6K2: 87%) than in direct ELISA (93%).

Nine out of 27 PR3-ANCA positive patients with pauci-immune NCGN (33%) and 28 out of 80 other disease controls (29%) tested positive in 1 to 5 out of 6 different MPO-ANCA assays. A large difference between the results of direct versus capture ELISA was noted in this respect: PR3-ANCA positive patients with pauci-immune NCGN more often tested...
positive in a capture ELISA than in a direct ELISA for MPO-ANCA, 8 versus 1, respectively, whereas other disease controls less often tested positive in a capture ELISA than in a direct ELISA for MPO-ANCA, 5 versus 27, respectively. None of the 27 sera from patients with PR3-ANCA positive pauci-immune NCGN and 80 sera from disease controls was consistently positive in all MPO-ANCA assays.

Based on the analysis of 27 sera from patients with PR3-ANCA positive pauci-immune NCGN and 80 sera from disease controls, the use of native MPO resulted in a slightly lower overall specificity in a direct ELISA (87%) than in a capture ELISA (MoAb 4.15: 93%, MoAb 266 6K2: 94%), whereas the use of rMPO led to a marked decrease in specificity in a direct ELISA (75%) as compared to a capture ELISA (MoAb 4.15: 93%, MoAb 266 6K2: 97%).

**Figure 4** MPO-ANCA levels in PR3-ANCA negative patients with pauci-immune NCGN (n = 30) obtained by capture ELISA using an extract of azurophilic granules (A), native MPO (B), or recombinant MPO (C), as captured by either monoclonal antibody (MoAb) 266 6K2 or MoAb 4.15. The dotted line indicates the cut-off value for a positive / negative test.

**Discussion**

In the present study, we found that 83 of our 121 patients with pauci-immune NCGN (69%) had PR3-ANCA. In addition, of the 38 patients without PR3-ANCA only 4 (11%) tested negative for MPO-ANCA. Three of these 4 patients were clinically suspected of post-infectious NCGN. These findings of more than 95% ANCA positivity in a large patient cohort of patients with NCGN are in accordance with a previous study from our group in a smaller patient cohort (Cohen Tervaert et al., 1990) and with other studies in which a direct ELISA was used to detect ANCA (Niles et al., 1991; Pettersson et al., 1995). However, other groups using a direct ELISA found a lower prevalence of ANCA in pauci-immune NCGN patients (Harris et al., 1998; Bindi et al., 1993; Saxena et al., 1991). Since these discrepancies might be due to differences in the methodology of ANCA testing, we compared the sensitivity of different MPO-ANCA assays. We used both rMPO and native MPO in a direct ELISA and a capture ELISA. We found that 93% of the serum samples from patients with pauci-immune NCGN that tested negative for PR3-ANCA had MPO-ANCA as detected by capture ELISA.
using native MPO, and 87% (MoAb 266 6K2) of the serum samples had MPO-ANCA as detected by capture ELISA using rMPO. In the direct ELISA, 93% of the sera were found to be positive using either native MPO or rMPO. From these findings, we conclude that the sensitivity of both assays and both antigens are comparable and that it is unlikely that the lower prevalence of ANCA in some studies (Harris et al., 1998; Bindi et al., 1993; Saxena et al., 1991) can be explained by technical differences. When, however, MoAb 4.15 instead of MoAb 266 6K2 was used in the capture ELISA, sensitivity of the assay dropped to 77% when rMPO was used as antigen demonstrating that it is crucial to select adequate MoAb(s) for the capture ELISA. In the case of MoAb 4.15, epitopes on rMPO recognized by MPO-ANCA may have been blocked.

In the direct ELISA, all serum samples of our patients with PR3-ANCA negative pauci-immune NCGN that recognized native MPO, also recognized rMPO. Short et al. also found that the great majority of patients with vasculitis with antibodies directed against native MPO recognized rMPO as detected by direct ELISA (Audrain et al., 1997). In contrast, Audrain et al. found that 30% of their consecutive MPO-ANCA positive patients did not recognize rMPO as detected by direct ELISA (Hogan et al., 1996). In both studies (Audrain et al., 1997; Hogan et al., 1996), patient sera were selected based on ANCA positivity by IIF and MPO-ANCA positivity by antigen specific direct ELISA. In contrast, we selected our patients on the presence of biopsy-proven pauci-immune NCGN and found no difference between results for MPO-ANCA when using either native MPO or rMPO in a direct ELISA. Audrain et al. suggested that not all epitopes that are recognized by MPO-ANCA are present on rMPO. Competition assays with 5 purified mouse monoclonals revealed at least four epitopes on native MPO, and only two were conserved on rMPO (Hogan et al., 1996). Since nearly all of our sera from patients with pauci-immune NCGN recognized rMPO, we speculate that these missing epitopes are of minor importance in pauci-immune NCGN.

With regard to specificity, we found that a capture ELISA proved a more specific tool to detect MPO-ANCA than a direct ELISA. In patients without pauci-immune NCGN but with other diseases in which ANCA with known or unknown antigenic specificity are frequently observed (Kallenberg et al., 1992), we found positive results for MPO-ANCA less frequently in a capture ELISA than in a direct ELISA. This high level of specificity of a capture ELISA is in accordance with our previous findings (Cohen Tervaert et al., 1990). The results of the capture ELISA, however, must be evaluated with caution in patients with PR3-ANCA associated NCGN since we frequently observed borderline positive results for MPO-ANCA in these patients. Although double positivity certainly exists in rare cases (Hagen et al., 1998; Dolman et al., 1993), and switch from one specificity to the other has been described (Choi et al., 1999), we do not believe that the borderline positive results in these patients reflect true positivity.
In a direct ELISA we found a lower specificity of MPO-ANCA for pauci-immune NCGN when rMPO was used in comparison to native MPO. Therefore, native MPO should be the first choice antigen when samples are tested using a direct ELISA.

In conclusion, in almost all patients with pauci-immune NCGN MPO-ANCA or PR3-ANCA can be detected. Serum samples from patients with MPO-ANCA associated pauci-immune NCGN nearly always recognize both rMPO and native MPO in a direct and a capture ELISA. In a direct ELISA, however, many sera from disease controls also react with native MPO and rMPO. The use of rMPO more frequently causes false positive results than native MPO in a direct ELISA. When native MPO and rMPO are used in a capture ELISA, false positive results by disease controls without pauci-immune NCGN are obtained less frequently compared to results obtained using a direct ELISA. Thus, in patients with pauci-immune NCGN a capture ELISA is the preferred method to detect MPO-ANCA, and rMPO is an alternative for native MPO for the detection of MPO-ANCA in these patients.

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Literature


