Wegener’s granulomatosis (WG) is an inflammatory disorder of presumed autoimmune origin characterized by chronic inflammation of the respiratory tract, vasculitis and glomerulonephritis. WG is strongly associated with antineutrophil cytoplasmic antibodies (ANCA). ANCA in patients with WG are directed against proteinase 3 (Pr3) in most of the cases. In vitro, upon neutrophil priming, ANCA antigens are expressed on the cell surface, thereby becoming available for interaction with ANCA. Subsequently, these neutrophils become activated. Since ANCA can only interact with leucocytes when the ANCA antigens are present on the cell surface, we questioned whether Pr3 is already expressed on the membranes of circulating granulocytes and monocytes of patients with WG, and whether Pr3 expression is related to disease activity, so explaining the systemic nature and severity of the disease. The expression of Pr3, and other ANCA antigens, i.e. myeloperoxidase (MPO) and human leucocyte elastase (HLE), was analysed on circulating granulocytes and monocytes by flow cytometry, using a non-activating whole-blood method. Disease activity was quantitated using the Birmingham Vasculitis Activity Score (BVAS). Seventeen patients with active WG and anti-Pr3 antibodies were included in this study. Nine of these patients were also analysed at the time of remission. Twelve patients with sepsis served as positive controls, and 10 healthy volunteers served as negative controls for granulocyte/monocyte activation. Pr3 expression on neutrophils was increased in patients with active WG compared to patients with quiescent disease and healthy controls. On monocytes, no differences in Pr3 expression were found between those groups. Furthermore, the expression of MPO and HLE did not differ between patient groups and healthy controls. Upon follow-up, the expression of Pr3 on neutrophils from patients with active WG decreased when patients went into remission. Pr3 expression on neutrophils correlated with the BVAS score ($r = 0.40, P < 0.05$). In conclusion, circulating neutrophils from patients with active WG have increased expression of Pr3. In addition, the expression of Pr3 correlates with disease activity, suggesting that the availability of Pr3 for interaction with ANCA plays a central role in the disease process.

**Key words:** ANCA, Pr3 membrane expression, Wegener’s granulomatosis, Disease activity, Flow cytometry.
for anti-proteinase 3 antibodies. Twelve consecutive patients with sepsis served as positive controls for cell activation and 10 healthy volunteers served as normal controls.

**Diagnostic criteria**

The diagnosis of WG was established according to clinical and histological criteria [12], and all patients fulfilled the American College of Rheumatology (ACR) criteria for WG [13]. Patients had active or inactive disease. Patients with active vasculitis had either newly developed disease or relapsing disease. Relapsing disease was defined as previously described [4, 14]. Criteria for relapsing disease are given in Table I. Complete remission (quiescent disease) was defined as the absence of signs or symptoms attributable to active vasculitic disease. As a consequence, patients with quiescent disease had a Birmingham Vasculitis Activity Score (BVAS) of 0. Disease activity was measured according to the BVAS [15].

**ANCA detection**

ANCA were detected by indirect immunofluorescence on ethanol-fixed granulocytes as previously described [2]. Patient or control sera were tested in a dilution of 1:20, and further at 2-fold dilutions. Slides were read by two independent observers, and a titre of 1:≥40 was considered positive.

The specificity of ANCA for either Pr3, MPO or HLE was detected by capture ELISA as previously described [16]. Sera were considered positive for one of the aforementioned specificities when values exceeded the mean + 2 s.d. of normal controls (n = 50).

**C-Reactive protein detection**

C-Reactive protein (CRP) concentrations were measured by using a particle-enhanced nephelometric method and NA latex CRP reagents (Behring, Marburg, Germany).

**Surface marker analysis by flow cytometry**

To avoid *in vitro* activation of granulocytes, we used a whole-blood method [17, 18], EDTA-anticoagulated blood was kept on ice until sample preparation. Sample preparation was always started within 5 min after blood sampling. All steps were performed in Hanks Balanced Salt Solution (HBSS) without calcium and magnesium (Gibco, Life Technologies Ltd, Paisley), supplemented with 1% bovine serum albumin (BSA; Boseral, Organon Teknika, Boxtel, The Netherlands). Cells were fixed with 1% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min on ice, washed, followed by erythrocyte lysis twice with lysis buffer (155 mM NaHCl, 10 mM KHCO₃, 0.1 mM Na₂EDTA·H₂O) for 5 min at 37°C. A panel of monoclonal antibodies was used for the analysis of ANCA antigen expression (anti-Pr3, 12.8, and anti-MPO, 4.15, Central Laboratory for Bloodtransfusion Service, Amsterdam, The Netherlands; anti-HLE, NP57, Dakopatts, Glostrup, Denmark). The first antibody was incubated for 1 h at 4°C. After washing, the cells were incubated with a goat anti-mouse Ig polyclonal antibody conjugated with phycoerythrin (Southern Biotechnology Associates Inc., Birmingham, USA), 1:20 diluted, supplemented with 5% normal goat serum and 5% normal human serum, for 30 min at 4°C in the dark. Subsequently, cells were washed and stored until flow cytometric analysis was performed.

Analysis of surface marker expression was performed on a Coulter Epics ELITE flow cytometer (Coulter, Hialea, FL, USA), the same day or, in some cases, the next day (always within 18 h). When the cell pellet contained erythrocytes, the intercalating dye LDS751 (Exiton Chemical, Dayton, OH, USA) was added before flow cytometry measurement. Erythrocytes could successfully be excluded from the leucocyte population in the LDS751/forward scatter dot plot, when combined with a lifegate. Neutrophils and monocytes were identified by forward and side-ward scatter. Eosinophils were excluded from the neutrophil population by their high autofluorescence. Data were analysed using Immuno-4 software [19].

In the first experiments, QC3 beads (Flow Cytometry Standards, Leiden, The Netherlands) were used to calibrate the flow cytometer. However, these beads appeared not to be stable during the study period. In addition, batch-to-batch quality varied remarkably. Therefore, we decided to compare the results obtained in patients with those obtained in samples from healthy, age-matched volunteers who were measured simultaneously.

The expression of surface markers was calculated as a mean fluorescence intensity (MFI) in combination

**TABLE I**

Criteria for relapsing disease [12]

<table>
<thead>
<tr>
<th>Manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Progressive glomerulonephritis, i.e. a decrease in renal function of 30% or more within 3 months in combination with (microscopic) haematuria or evidence of acute necrotizing lesions on renal biopsy</td>
</tr>
<tr>
<td>b. Pulmonary infiltrates with or without cavitation with rising C-reactive protein levels either with necrotizing granulomatous inflammation or necrotizing vasculitis on biopsy, or—when no histological proof can be obtained—after exclusion of infection and malignancy</td>
</tr>
<tr>
<td>c. Sinusitis, otitis, ulceration of nasal mucosa, or a nasal proliferative mass, in combination with necrotizing granulomatous inflammation on biopsy</td>
</tr>
<tr>
<td>d. Miscellaneous: progressive mononeuritis multiplex, cranial nerve palsy, cerebral vasculitis, necrotizing scleritis, orbital pseudotumour, progressive tracheal stenosis with active disease on biopsy, peripheral gangrene, necrotizing vasculitis of medium-sized or small muscular arteries</td>
</tr>
</tbody>
</table>
with the percentage of positive cells (pos%). Data were expressed as a percentage of the expression (MFI) on neutrophils and monocytes from healthy controls, corrected for non-specific binding of an irrelevant antibody and the conjugate (NSB), and the percentage of positive cells, according to the following formula:

\[
\text{expression index} = \frac{(\text{MFI-NSB})_{\text{patient}} \times \text{pos\%}_{\text{patient}}}{(\text{MFI-NSB})_{\text{control}} \times \text{pos\%}_{\text{control}}} \times 100\%
\]

**Statistical analysis**

Groups were analysed for differences in surface expression by means of the Kruskal–Wallis test. Subsequently, differences between groups were analysed by the Mann–Whitney test. Correlation between parameters was analysed by the Spearman rank correlation test. The paired Wilcoxon test was used to test differences between paired observations. These tests were performed using GraphPad Instat2 Software.

**RESULTS**

**Patients**

Seventeen patients (nine male, eight female; mean age 50.5 yr, range 27–85 yr) with WG were studied. All had active disease, i.e. either newly developed disease \((n = 10)\) or relapsing disease \((n = 7)\). Additionally, nine of these patients were also analysed at times of quiescent disease. Patient data are given in Table II for patients with vasculitis and in Table III for patients with sepsis.

**Expression of ANCA antigens on neutrophils**

The expression of Pr3 on neutrophils from patients with active disease was higher compared to patients with quiescent disease \((P < 0.05)\) and healthy controls \((P < 0.05)\). In paired observations, the expression of Pr3 on neutrophils from patients with active disease was higher than that on neutrophils from the same patients during quiescent disease \((P < 0.05)\). Data are represented in Figs 1 and 2, respectively.

Bimodal expression of Pr3 on granulocytes, as described by Halbwachs-Mecarelli et al. [20], was observed in both patients and controls. In our study, the bimodal distribution of Pr3 was found in 10 out of 17 patients and in eight out of 53 healthy controls who were analysed during the study \((P < 0.01)\). Representative data are shown in Fig. 3.

In contrast to the expression of Pr3, the expression of MPO and HLE on neutrophils did not differ between the various stages of disease activity, nor did it differ from controls, whereas the expression of MPO \((P < 0.001)\) and Pr3 \((P = 0.05)\), but not the expression of HLE on neutrophils from patients with sepsis, was increased compared to healthy controls. The expression of MPO on neutrophils from patients and controls is shown in Fig. 4.

**Expression of ANCA antigens on monocytes**

The expression of Pr3, MPO and HLE on monocytes did not differ between patients and healthy controls.
**TABLE III**
Clinical and serological findings in 12 patients with sepsis

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age</th>
<th>Sex</th>
<th>Diagnosis: sepsis due to</th>
<th>CRP (mg/l)</th>
<th>WBC (10^9/l)</th>
<th>APACHE II score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>64</td>
<td>m</td>
<td>Skin infection</td>
<td>201</td>
<td>4.9</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>48</td>
<td>f</td>
<td>Cholangitis</td>
<td>19</td>
<td>12.3</td>
<td>22</td>
</tr>
<tr>
<td>3</td>
<td>74</td>
<td>m</td>
<td>Pneumonia</td>
<td>193</td>
<td>17.2</td>
<td>22</td>
</tr>
<tr>
<td>4</td>
<td>71</td>
<td>m</td>
<td>Cholangitis</td>
<td>244</td>
<td>23.7</td>
<td>22</td>
</tr>
<tr>
<td>5</td>
<td>29</td>
<td>f</td>
<td>Peritonitis due to endometritis</td>
<td>181</td>
<td>22.4</td>
<td>21</td>
</tr>
<tr>
<td>6</td>
<td>77</td>
<td>m</td>
<td>Urinary tract infection</td>
<td>193</td>
<td>14.8</td>
<td>34</td>
</tr>
<tr>
<td>7</td>
<td>61</td>
<td>m</td>
<td>Pneumonia</td>
<td>203</td>
<td>7.8</td>
<td>31</td>
</tr>
<tr>
<td>8</td>
<td>46</td>
<td>m</td>
<td>Peritonitis, alcohol hepatitis</td>
<td>121</td>
<td>8.5</td>
<td>34</td>
</tr>
<tr>
<td>9</td>
<td>36</td>
<td>f</td>
<td>Urinary tract infection</td>
<td>239</td>
<td>24.8</td>
<td>8</td>
</tr>
<tr>
<td>10</td>
<td>80</td>
<td>m</td>
<td>Faecal peritonitis</td>
<td>14</td>
<td>4.2</td>
<td>14</td>
</tr>
<tr>
<td>11</td>
<td>47</td>
<td>f</td>
<td>Pancreatitis</td>
<td>141</td>
<td>11.7</td>
<td>15</td>
</tr>
<tr>
<td>12</td>
<td>67</td>
<td>f</td>
<td>Pneumonia</td>
<td>208</td>
<td>33.1</td>
<td>42</td>
</tr>
</tbody>
</table>

m, male; f, female; neg, negative; CRP, C-reactive protein.

**Fig. 1.**—Box and whisker plots indicating the overall range (error bars), 25–75% range (boxes) and median value (horizontal lines) of the surface expression of proteinase 3 on neutrophils (PMN) from patients with ANCA-associated vasculitis (A, active disease; Q, quiescent disease) compared to the expression on cells from patients with sepsis (S) and healthy controls (C). *P < 0.05, &P = 0.05 compared to healthy controls; &P < 0.05 compared to patients with quiescent disease.

**Fig. 2.**—Paired observations of proteinase 3 expression on neutrophils from patients with active and quiescent disease (n = 9).

**Fig. 3.**—Flow cytometry histogram representing the bimodal distribution (light grey) and a normal distribution (dark grey) of proteinase 3 surface expression and the negative control staining (black) on neutrophils from a patient with active ANCA-associated vasculitis and a healthy control. Absolute cell count and the mean fluorescence intensity (MFI) are depicted on the y and x axes, respectively.

**Fig. 4.**—Box and whisker plots indicating the overall range (error bars), 25–75% range (boxes) and median value (horizontal lines) of the surface expression of myeloperoxidase (MPO) on neutrophils (PMN) from patients with ANCA-associated vasculitis (A, active disease; Q, quiescent disease) compared to the expression on cells from patients with sepsis (S) and healthy controls (C). *P < 0.05 compared to healthy controls.
In contrast, in patients with sepsis, the expression of Pr3 \((P < 0.05)\) was increased, whereas the expression of MPO \((P = 0.06)\) tended to be increased compared to the expression on cells from healthy controls (Figs 5 and 6).

In addition, we found that monocytes from both patients and controls exhibited a higher surface expression of MPO, whereas neutrophils exhibited a higher surface expression of Pr3 (Fig. 7).

**Correlations with markers of disease activity**

A statistically significant correlation was found between Pr3 expression on neutrophils and the activity of the disease as measured by the BVAS score \((P < 0.05, r = 0.40)\). Furthermore, neutrophil Pr3 expression tended to correlate with CRP values \((P = 0.08, r = 0.36)\). In addition, Pr3 expression correlated with white blood cell counts \((P < 0.01, r = 0.51)\). Pr3 expression tended to correlate with the expression of MPO on neutrophils \((P = 0.09, r = 0.34)\).

## DISCUSSION

This study demonstrates that in patients with WG, the expression on neutrophils of Pr3, the main target antigen for ANCA in these patients, is increased during active disease. During remission, the expression of Pr3 decreases to values comparable to healthy controls. Furthermore, the expression of Pr3 on neutrophils correlates with disease activity as measured by the BVAS score.

The mechanism by which ANCA antigens are expressed at the cell surface is not fully elucidated. ANCA antigens are stored intracellularly in granules in both neutrophils and monocytes. From *in vitro* data, it is known that, after priming, ANCA antigens can be detected at the cell surface of these cells. Priming of these cells results from stimulation by pro-inflammatory cytokines, which *in vivo* may be released locally during infections. Indeed, in patients with WG, infections play an initiating role in the disease process [21].

The occurrence of ANCA antigens at the cell surface is possibly the result of a charge interaction between the positively charged ANCA antigens and the negatively charged cell membrane. Another possible explanation could be the presence of an unidentified membrane-bound receptor for these antigens. Finally, Gilligan *et al.* [22] demonstrated ANCA antigen surface expression on apoptotic polymorphonuclear neutrophils (PMN), which was a result of cytoplasmic granular translocation during apoptosis.

*In vivo*, the expression of ANCA antigens on circulating neutrophils was demonstrated by Csernok *et al.* [8]. They observed increased Pr3 expression on neutrophils from patients with active and quiescent disease. These investigators used density-gradient-isolated granulocytes, which may result in increased expression...
of degranulation markers due to this purification method [18, 23]. In the present study, we used a whole-blood method, in which cells were not isolated, but fixed with paraformaldehyde within minutes after collection. In our study, Pr3 expression on neutrophils was only increased in patients with active disease, but not in patients with quiescent disease. Furthermore, Pr3 expression in patients with WG was found to correlate with disease activity.

Previously, Halbwachs-Mecarelli [20] demonstrated in vitro a bimodal distribution of Pr3 surface expression on neutrophils from healthy individuals (see also Fig. 3). Our study shows that this bimodal distribution occurs more frequently in patients with WG than in healthy controls. It is, however, unclear whether this phenomenon results from a genetic background or not.

ANCA antigen surface expression is a prerequisite for ANCA-induced cell activation. In vitro, primed neutrophils and monocytes express the ANCA antigens, Pr3 and MPO. Binding of ANCA to these antigens as well as with Fc receptors on the neutrophil cell surface can lead to neutrophil activation. In addition, ANCA are also capable of activating monocytes. Recently, Ralston et al. [24] demonstrated in vitro that primed monocytes express Pr3. Furthermore, these primed cells could be stimulated with an anti-Pr3 antibody to produce IL-8. In the present study, we show that in vivo monocytes are capable of expressing Pr3 on their cell surface. However, the expression of Pr3 on monocytes from patients with WG compared to healthy controls was not increased.

Interestingly, in the present study, we observed that neutrophils, from both controls and patients, predominantly express Pr3, whereas monocytes predominantly express MPO. As a result, anti-MPO antibodies may be more potent activators of monocytes than anti-Pr3 antibodies, whereas anti-Pr3 antibodies may be more potent activators of neutrophils. These effects may be of importance in explaining the clinical differences between patients with anti-Pr3 antibodies and patients with anti-MPO antibodies [25, 26]. Patients with anti-MPO antibodies have more chronic lesions in renal biopsies and a slower decline in renal function than patients with anti-Pr3 antibodies, who have more active lesions and a much faster decline in renal function [25].

Reumaux et al. [27] showed that ANCA-induced leucocyte activation only occurs when cells are bound to a surface, i.e. at the endothelial surface, and not when they are in suspension, i.e. in the circulation. If ANCA-induced cell activation occurs in close contact with the endothelial lining, this may result in endothelial damage due to the local release of lytic enzymes and oxygen radicals by neutrophils and monocytes. Upon ANCA-induced cell activation, neutrophils express increased levels of adhesion molecules, permitting these cells to adhere to the endothelium [28]. These adherent, fully activated, cells are not detected when analysing peripheral blood-derived cells. Therefore, the expression of Pr3 on circulating neutrophils does not reflect the extent of full activation, but reflects the extent of priming, a state of pre-activation, of these cells. These primed cells can be targets for ANCA once they adhere to (locally activated) endothelium.

In conclusion, circulating neutrophils from patients with active WG have increased expression of Pr3. In addition, the expression of Pr3 correlates with disease activity, suggesting that the availability of Pr3 for interaction with ANCA plays a central role in the disease process.

Acknowledgements

The authors wish to thank M. G. Huitema, W. W. Oost-Kort, I. Bouma and G. Mesander for their technical assistance, Drs J. Zijlstra, C. A. Stegeman and C. F. A. Franssen for their clinical input, and Dr P. C. Limburg for his valuable advice.

References