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Are circulating neutrophils intravascularly activated in patients with anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitides?

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SUMMARY

Vascular injury in vasculitis may be due to activation of circulating neutrophils resulting in their increased adhesiveness to locally activated endothelium (Shwartzman phenomenon). Previously, we demonstrated up-regulation of endothelial intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in biopsies from patients with ANCA-associated vasculitis. In the present study, we investigated the expression of adhesion molecules (CD11b, ICAM-1, VLA-4, L-selectin) and activation markers (CD66b, CD64, CD63) on circulating neutrophils from patients with ANCA-associated vasculitis in comparison with their expression on cells from healthy volunteers and patients with sepsis. We related these findings to parameters of disease activity. Surface marker expression was determined by using a non-activating whole blood flow cytometric assay. The expression of activation markers, but not the expression of adhesion molecules, was increased on neutrophils from patients with active vasculitis. The expression of CD63 and CD66b on neutrophils correlated with disease activity as determined by the Birmingham Vasculitis Activity Score (BVAS). In contrast to patients with active vasculitis, patients with sepsis showed up-regulation of all markers, including adhesion molecules, suggesting that circulating neutrophils are fully activated in sepsis. We conclude that in ANCA-associated vasculitis, circulating neutrophils are not fully activated, since they do not express increased levels of adhesion molecules as sepsis or in the Shwartzman reaction. These findings are compatible with the concept that *in vivo* vascular damage in ANCA-associated vasculitides does not occur due to a Shwarzman-like reaction but only after ANCA-induced neutrophil activation at the endothelial cell surface.

Keywords ANCA neutrophil activation vasculitis sepsis adhesion molecules flow cytometry

INTRODUCTION

Primary or idiopathic vasculitic syndromes form a group of inflammatory disorders of presumed autoimmune origin characterized by inflammation and necrosis of blood vessels, frequently in combination with granuloma formation. Within the spectrum of vasculitides, small vessel vasculitides, such as Wegener's granulomatosis, microscopic polyangiitis, and Churg–Strauss syndrome, are strongly associated with ANCA. ANCA in those disorders are generally directed against proteinase 3 (PR3) and myeloperoxidase (MPO) [1,2] and, occasionally, against human leucocyte elastase (HLE) [3].

The pathophysiology of idiopathic small vessel vasculitis is not completely understood. *In vitro* experiments have shown that ANCA have the potential to activate pre-activated neutrophils to the production of reactive oxygen species and the release of lytic enzymes [4–8]. Furthermore, *in vitro*, ANCA induce increased expression of CD11b on neutrophils [9].

During an inflammatory response *in vivo*, adhesion molecules mediate the interaction of neutrophils with endothelial cells. Pre-activated neutrophils roll over the endothelial monolayer, where these cells become further activated by locally presented inflammatory mediators. Subsequently, these cells firmly adhere to and transmigrate through the endothelium [10]. In general, during the process of adhesion and transmigration, endothelial cells are not or only minimally damaged.

In vasculitis, various mechanisms of vascular injury, i.e. endothelial cell damage, may be operative. In secondary vasculitic syndromes activation of neutrophils occurs via the activation of the complement cascade after local deposition of immune complexes. However, in ANCA-associated vasculitides immune complexes can not be demonstrated. One of the possible mechanisms of vascular damage in these syndromes is a Shwartzman-like phenomenon in

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which activation of neutrophils occurs intravascularly [11,12]. This intravascular activation results in up-regulation of CD11b/CD18 expression [13,14], followed by degranulation and production of oxygen radicals once these cells adhere to locally activated endothelium. Previously it has been suggested that this mechanism is operative in lesional tissue of patients with systemic lupus erythematosus (SLE) that lack immune complex depositions [15]. Indeed, in these patients up-regulation of CD11b/CD18 has been demonstrated on circulating neutrophils [13,16].

In the present study, we tested the hypothesis that a Shwartzmann-like phenomenon might also be operative in ANCA-associated vasculitides by analysing the expression of CD11b, other adhesion molecules, and markers of activation on circulating neutrophils in both patients with active ANCA-associated vasculitis and patients in remission. We compared neutrophil activation in patients with vasculitis with that in patients with sepsis, a condition in which intravascular activation of circulating neutrophils has already been demonstrated [17]. In addition, we correlated the extent of neutrophil activation in patients with vasculitis with parameters of disease activity.

PATIENTS, MATERIALS AND METHODS

Patients and controls

The patient group consisted of consecutive patients admitted to our hospital or seen at the out-patient clinic with a diagnosis of WG, Churg–Strauss syndrome (CSS) or microscopic polyangiitis (MPA). All patients were positive for ANCA. Twenty patients with sepsis served as positive controls for leucocyte activation. All patients with sepsis entered the study within hours after admittance to the intensive care unit. Healthy laboratory personel served as normal controls. This study was approved by the Institutional Review Board.

Diagnostic criteria

The diagnoses of WG, CSS and MPA were established according to clinical and histological criteria [2]. All patients fulfilled the Chapel Hill Consensus Conference definitions for WG, CSS and/or MPA [18]. Patients had either active or inactive disease. Patients with active disease had newly developed disease (N) or relapsing disease (R). Relapsing disease was defined as previously described [19,20]. Criteria for relapsing disease are given in Table 1. Patients with newly developed disease were studied before treatment was started. Patients with relapsing disease were studied before treatment was instituted or intensified. Complete remission was defined as the absence of signs or symptoms attributable to active vasculitic disease. Sepsis was defined as previously described [21].

Disease activity

Vasculitis disease activity was measured according to the Birmingham Vasculitis Activity Scoring index (BVAS) [22], whereas vasculitic damage was measured according to the Vascular Damage Index [23]. Severity of sepsis was scored with the Acute Physiology and Chronic Health Evaluation (APACHE) II scoring system [24].

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

ANCA detection

ANCA were detected by indirect immunofluorescence on ethanol-fixed granulocytes as previously described [3] using FITC-labelled goat anti-human IgG (Dako, Glostrup, Denmark) in a 1:50. dilution. Test or control sera were tested at a dilution of 1:20, and further at two-fold dilutions. Slides were read by two independent observers, and a titre ≥ 40 was considered positive.

The specificity of ANCA for either PR3, MPO or HLE was detected by capture ELISA as previously described [25]. Anti-lactoferrin antibodies were detected by ELISA on plates directly coated with lactoferrin (5 μg/ml; Sigma, St Louis, MO) as described [26]. Sera were considered positive for one of the above mentioned specificities when values exceeded the mean + 2 s.d. of normal controls ($n = 50$).

Surface marker analysis of activation markers by flow cytometry

To avoid *in vitro* activation of granulocytes we used a whole blood method [27–29]. EDTA anti-coagulated blood was kept on ice until sample preparation. Sample preparation was started always within 5 min after blood sampling. All steps were performed in Hanks' balanced balt solution (HBSS) without calcium and magnesium (GIBCO, Life Technologies Ltd, Paisley, UK), supplemented with 1% bovine serum albumin (BSA; Boseral, Organon Teknika, Boxtel, The Netherlands). Cells were fixed with 1% paraformaldehyde in PBS for 10 min on ice, washed, followed by two times erythrocyte lysis with lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0·1 mM $Na_2EDTA.H_2O$) for 5 min at 37°C. A panel of MoAbs to leucocyte surface antigens was used for the analysis of leucocyte activation (Table 2). The first antibody was incubated for 1 h at 4°C. After washing, the cells were incubated with a goat anti-mouse immunoglobulin polyclonal antibody conjugated with PE (Southern Biotechnology Associates Inc., Birmingham, AL), 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

Table 1. Criteria for relapsing disease

Manifestations have to be new or recurrent findings:

(i)	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.
(ii)	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.
(iii)	Sinusitis, otitis, ulceration of nasal mucosa, or a nasal proliferative mass, in combination with necrotizing granulomatous inflammation on biopsy.
(iv)	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.

Table 2. Activation markers and adhesion molecules on neutrophils

Marker	Cellular distribution	Remarks	Monoclonal antibody	Source
Adhesion molecules:				
CD11b	Monocytes macrophages granulocytes NK cells	Subunit of β_2-integrin CD18/CD11b (Mac-1), ligand for ICAM-1, 2, 3, β-glucan, fibronectin, C3bi	2LPM19c	Dako
L-selectin	Most leucocytes	Shed upon activation, constitutively expressed	SK11	Becton Dickinson
ICAM-1	Most leucocytes endothelial cells, other cells	Ligand for β_2-integrins	84H10	Immunotech
CD49d	Monocytes lymphocytes eosinophils	Subunit of β_1-integrin CD29/CD49d (VLA-4)	HP2/1	Immunotech
Activation markers:				
CD66b	Granulocytes	CEA member 6, increased expression after activation and degranulation	CLB-B13.9	CLB
CD63	Monocytes granulocytes activated platelets	GP53, increased expression after activation and degranulation	CLB-gran/12 435	CLB
FcγRI (CD64)	Monocytes granulocytes	Increased expression after activation	22	Medarex

CLB, Central Laboratory for the Blood Transfusion Service, Amsterdam, The Netherlands; Dako, Dakopatts, Glostrup, Denmark; Becton Dickinson, Mountain View, CA; Immunotech, Marseille, France; Medarex, Annandale, NJ.

Coulter Epics ELITE flow cytometer (Coulter, Hialeah, FL), the same day or occasionally the next day (within 18 h). When the cell pellet contained erythrocytes, the intercalating dye, LDS751 (Exiton Chemical, Dayton, OH) 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 life gate. Neutrophils were identified by forward and sideways scatter. Eosinophils were excluded from the neutrophil population by their high autofluorescence. Data were analysed using Immuno-4 software [30].

QC3 beads (Flow Cytometry Standards, Leiden, The Netherlands) were used to calibrate the flow cytometer [31]. However, batch to batch quality of those beads varied remarkably, and within one batch fluorescence intensity diminished over time. Therefore, we decided to compare the results obtained in patients with the results obtained in healthy age-matched volunteers who were analysed in parallel [29].

The expression of surface markers was calculated as mean fluorescence intensity (MFI), corrected for non-specific binding of an irrelevant antibody and the conjugate (NSB), in combination with a percentage of positive cells (pos percentage). The percentage of positive cells was defined as the percentage of cells of the cell population gated in the forward/sideways scatter dot blot with a higher MFI than the NSB stained population. Data were expressed as a percentage of the value obtained from the healthy control who was tested in parallel, according to the following formula:

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

In order to assess the variability of the normal population, 10 healthy controls, with the same age distribution as the patient population, were analysed simultaneously. Their individual data were expressed as a percentage of the mean of the healthy control population.

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 by using GraphPad Instat2 Software. A two-tailed P value $< 0{\cdot}05$ was considered to indicate statistical significance.

RESULTS

Patients

Twenty-eight patients (15 female, 13 male, mean age 53 years, range 22–85 years) with active ANCA-associated vasculitis, i.e. either newly developed disease ($n = 14$) or relapsing disease ($n = 14$), were included in this study. At the time of admittance,

Table 3. Clinical and serological findings in 28 patients with systemic vasculitis in different stages of disease activity

Patient no.	Age	Sex	Diagnosis	State	Titre	Spec.	BVAS	CRP mg/*l*	VDI	Leucocyte count 10^9/*l*	Immune suppressive treatment at time of analysis
1	28	F	WG	N	>640	PR3	33	190	0	14·4	No
				Q	40	PR3	0	<3	2	6·9	Aza 150 mg+ pred 10 mg
2	63	M	WG	N	>640	PR3	39	149	2	11·5	No
				Q	80	PR3	0	<3	2	6·5	Pred 12·5 mg+ CP 75 mg
3	85	M	WG	N	160	PR3, HLE	27	99	1	8·0	No
4	36	F	WG	N	80	PR3	8	5	0	7·6	No
5	38	F	WG	R	>640	PR3	17	32	5	6·0	CP 75 mg+ pred 20 mg
6	56	M	WG	R	160	PR3	7	13	3	6·0	No
7	74	M	CSS	R	80	MPO	13	85	5	10·0	No
8	63	M	WG	R	320	PR3	11	47	2	4·4	No
				Q	20	PR3	0	44	3	8·7	Pred 10 mg
9	35	M	WG	R	160	PR3	17	166	0	15·7	Pred 20 mg
				Q	20	PR3	0	11	1	7·5	Pred 2·5 mg+ aza 150 mg
10	76	F	WG	R	160	MPO	9	108	7	7·5	No
11	49	M	WG	R	80	MPO	6	4	1	6·8	No
				Q	40	MPO	0	4	2	6·8	No
12	56	F	WG	N	320	PR3	19	55	0	18·6	No
				Q	20	PR3	0	3	1	8·0	Pred 10 mg+ CP 100 mg
13	65	M	CSS	N	640	MPO	20	117	0	14·6	No
				Q	20	MPO	0	<3	1	13·8	Pred 12·5 mg
14	75	M	WG	R	160	PR3	7	17	2	5·8	No
				Q	40	PR3	0	8	2	10·5	Pred 7·5 mg
15	47	F	MPA	R	>640	MPO	9	96	1	6·5	No
				Q	160	MPO	0	47	1	9·0	Pred 5 mg+ aza 100 mg
16	63	F	WG	R	80	HLE	3	8	2	5·2	Pred 5 mg+ aza 100 mg
17	48	F	WG	N	320	PR3	20	118	0	13·4	No
				Q	0	–	0	<3	0	4·1	Aza 25 mg+ pred 20 mg
18	22	F	MPA	N	320	MPO	13	70	0	5·6	No
19	54	F	WG	R	80	PR3	1	92	3	12·4	No
20	51	F	WG	N	>640	MPO, HLE	19	22	0	3·6	No
21	27	M	WG	N	320	PR3	15	40	0	9·4	No
				Q	40	PR3	0	52	1	9·7	Pred 20 mg+ aza 150 mg
22	44	M	WG	N	320	LF	11	5	0	5·2	No
				Q	80	LF	0	5	2	8·1	Pred 32·5 mg+ CP 50 mg
23	79	F	WG	N	320	PR3	31	81	0	8·9	No
24	23	F	WG	R	320	MPO	10	<3	2	4·9	Aza 75 mg
25	72	F	WG	R	160	MPO	12	<3	2	7·8	No
26	25	F	WG	N	40	PR3	20	105	0	13·1	No
27	48	M	WG	R	80	PR3	13	20	2	5·1	No
28	69	M	WG	N	320	PR3	28	NT	0	NT	No

F, Female; M, male; WG, Wegener's granulomatosis; CSS, Churg–Stauss syndrome; MPA, microsopic polyangiitis; N, newly developed disease; R, relapse; Q, quiescent disease; titre, ANCA titre; spec., ANCA specificity; PR3, proteinase 3; MPO, myeloperoxidase; HLE, human leucocyte elastase; CRP, C-reactive protein; CP, cyclophosphamide; pred, prednisolone; aza, azathioprine; NT, not tested.

patients with active disease had a median BVAS score of 13, range 1–39; of these, patients with newly diagnosed disease had a median BVAS score of 20, range 8–39, whereas patients with relapsing disease had a median BVAS score of 9·5, range 1–17 ($P < 0{\cdot}001$). Additionally, 12 of these patients were analysed also at times of quiescent disease. Twenty patients with sepsis (12 male, eight female, median age 69 years, range 29–87 years) were included as positive controls for leucocyte activation. At the time of diagnosis, patients had a median APACHE II score of 26, range 8–42. Patient characteristics are given in Table 3 for patients with vasculitis and Table 4 for patients with sepsis

The expression of adhesion molecules on neutrophils

On neutrophils, the expression of CD11b, the α subunit of the Mac-1 integrin, did not differ between the total population of patients with active vasculitis and healthy controls. Expression during active disease did not differ from that during quiescent disease. However, in patients with newly diagnosed vasculitis an increased expression of CD11b ($P = 0{\cdot}04$) was found, whereas in patients with relapsing disease the expression of CD11b was not higher than that of healthy controls. CD11b expression on neutrophils from patients with sepsis was increased compared with patients with active vasculitis ($P < 0{\cdot}01$) (Fig. 1a).

In vitro, during cell activation, L-selectin is shed from the cell surface [32,33]. We did not find differences in expression of L-selectin on neutrophils between patients with active disease, sepsis, and healthy controls (Fig. 1b). However, the expression of L-selectin correlated inversely with the BVAS score ($r = -0{\cdot}34$,

Table 4. Clinical and serological findings in 20 patients with sepsis

Patient no.	Age	Sex	Diagnosis: sepsis due to:	CRP mg/*l*	Leucocyte count 10^9/*l*	APACHE II score
1	64	M	Skin infection	201	4·9	12
2	48	F	Cholangitis	19	12·3	22
3	74	M	Pneumonia	193	17·2	22
4	71	M	Cholangitis	244	23·7	22
5	29	F	Peritonitis due to endometritis	181	22·4	21
6	77	M	Urinary tract infection	193	14·8	34
7	61	M	Pneumonia	203	7·8	31
8	46	M	Peritonitis, alcohol hepatitis	121	8·5	34
9	36	F	Urinary tract infection	239	24·8	8
10	80	M	Faecal peritonitis	14	4·2	14
11	47	F	Pancreatitis	141	11·7	15
12	67	F	Pneumonia	208	33·1	42
13	68	M	Urinary tract infection	NT	27·8	28
14	87	F	Pneumonia	234	17·4	25
15	70	M	Urinary tract infection	130	3·1	31
16	79	F	Urinary tract infection	206	6·6	29
17	73	F	Cholangitis	148	35·0	35
18	76	M	Pneumonia	292	22·4	23
19	78	M	Septic arthritis	216	5·3	28
20	38	M	Aspiration pneumonia	181	15·5	32

M, Male; F, female; CRP, C-reactive protein; APACHE II, Acute Physiology and Chronic Health Evaluation II Score; NT, not tested;

$P = 0{\cdot}04$). Moreover, analysis of paired data showed that the L-selectin expression in patients at the time of active disease tended to be decreased compared with its expression during remission ($P = 0{\cdot}09$).

VLA-4, a β_1-integrin, is expressed mainly on monocytes, lymphocytes and eosinophils [34], but can also be demonstrated on neutrophils [35]. VLA-4 expression on neutrophils did not differ between patients with various stages of disease activity. Neutrophils from patients with sepsis, however, had an increased expression of VLA-4 compared with neutrophils from patients with active vasculitis ($P = 0{\cdot}01$) and healthy controls ($P = 0{\cdot}02$) (Fig. 1c).

ICAM-1 is expressed on a wide variety of cells, including monocytes and neutrophils [36,37]. ICAM-1 expression on neutrophils did not differ between patients with vasculitis and healthy controls. In patients with sepsis, however, the expression of ICAM-1 was higher than that in patients with active vasculitis ($P = 0{\cdot}02$) and healthy controls ($P = 0{\cdot}03$) (Fig. 1d). No differences were found in the expression of L-selectin, VLA-4 and ICAM-1 between patients with newly diagnosed disease or relapsing disease and healthy controls. No correlations were found between the extent of expression of CD11b, L-selectin, ICAM-1 and VLA-4 and parameters of disease activity.

Other neutrophil activation markers

The expression of degranulation markers, such as CD66b and CD63, is increased upon activation. CD66b and CD63 are stored in the specific and the azurophilic granules, respectively [38–40]. Patients with active disease showed a higher expression of CD66b on neutrophils than patients with quiescent disease ($P = 0{\cdot}03$) and healthy controls ($P < 0{\cdot}01$). In patients with sepsis the expression was even higher compared with patients with active vasculitis ($P < 0{\cdot}0001$) (Fig. 2a). Analysis of paired data showed that the expression of CD66b during active disease was higher than during remission ($P < 0{\cdot}01$) (Fig. 2b). Furthermore, in patients with active vasculitis the expression of CD66b correlated with the BVAS score ($r = 0{\cdot}64$ and $P < 0{\cdot}001$; Fig. 2c) and CRP values ($r = 0{\cdot}37$, $P = 0{\cdot}02$), but not with the ANCA titre ($P < 0{\cdot}05$).

Patients with active disease demonstrated a higher expression of CD63 on neutrophils than healthy controls ($P < 0{\cdot}01$), but not compared with patients in remission. In patients with sepsis the expression of CD63 was higher than in patients with active vasculitis ($P < 0{\cdot}01$) (Fig. 3a). Analysis of paired data showed that the expression of CD63 during active disease was higher than during remission, but this difference did not reach statistical significance ($P = 0{\cdot}11$). In patients with active vasculitis the expression of CD63 correlated with the BVAS score ($r = 0{\cdot}40$, $P = 0{\cdot}04$, Fig. 3b) and CRP values ($r = 0{\cdot}37$, $P = 0{\cdot}02$), but not with the ANCA titre ($P < 0{\cdot}05$).

Expression of CD64, the first Fcγ receptor, is increased upon activation by interferon-gamma (IFN-γ) or granulocyte colony-stimulating factor (G-CSF) [41]. Patients with active disease had an increased expression of CD64 on neutrophils compared with healthy controls ($P = 0{\cdot}0062$), but not compared with patients with quiescent disease. In patients with sepsis the expression of CD64 was higher than in patients with active vasculitis ($P < 0{\cdot}0001$) (Fig. 4). The expression of CD64 did not correlate with parameters of disease activity or the ANCA titre.

DISCUSSION

In the present study we tested the hypothesis that a Shwarzman-like reaction underlies vascular damage in patients with ANCA-associated vasculitides. We analysed the expression of different

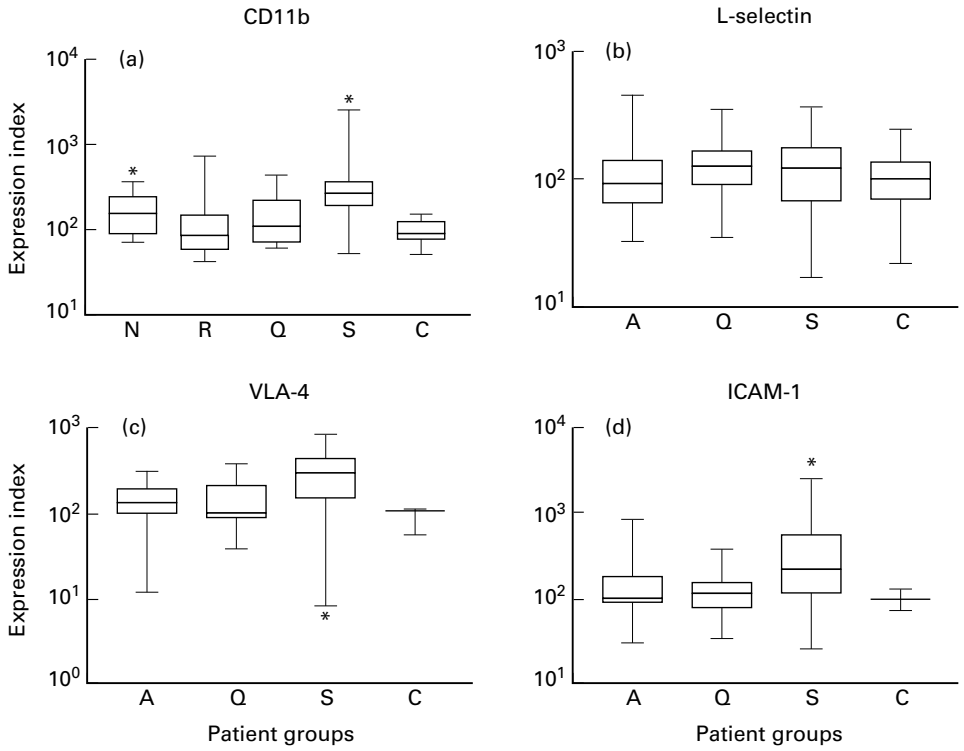

Fig. 1. Box and whisker plots indicating the overall range (error bars), 25–75% range (boxes), and median value (horizontal lines) of the surface expression of adhesion molecules (CD11b, VLA-4, intercellular adhesion molecule-1 (ICAM-1) and L-selectin) on neutrophils from patients with active (A), newly diagnosed (N), relapsing (R), and quiescent (Q) ANCA-associated vasculitis, patients with sepsis (S) and healthy controls (C). *$P < 0{\cdot}05$ compared with healthy controls.

activation markers and adhesion molecules on circulating neutrophils. Furthermore, we compared neutrophil activation in patients with vasculitis with that in sepsis, a condition in which it has been demonstrated that circulating neutrophils are intravascularly activated [17].

In the classical Shwartzman reaction an initial intradermal injection of endotoxin or a comparable stimulant results in local activation of endothelial cells. A subsequent intravenous injection of endotoxin results in complement activation and intravascular neutrophil activation, as demonstrated by the increased expression of CD11/CD18 [14]. Intravascular neutrophil activation eventually leads to endothelial damage as a result of degranulation and production of oxygen radicals once these cells adhere to locally activated endothelium.

In this study, the expression of CD11b and other adhesion molecules on circulating neutrophils was investigated. In inflammation, adhesion molecules play an important role in the interaction between neutrophils and the endothelial monolayer. This interaction is thought to be important for the development of endothelial damage [42]. The expression of CD11b, L-selectin

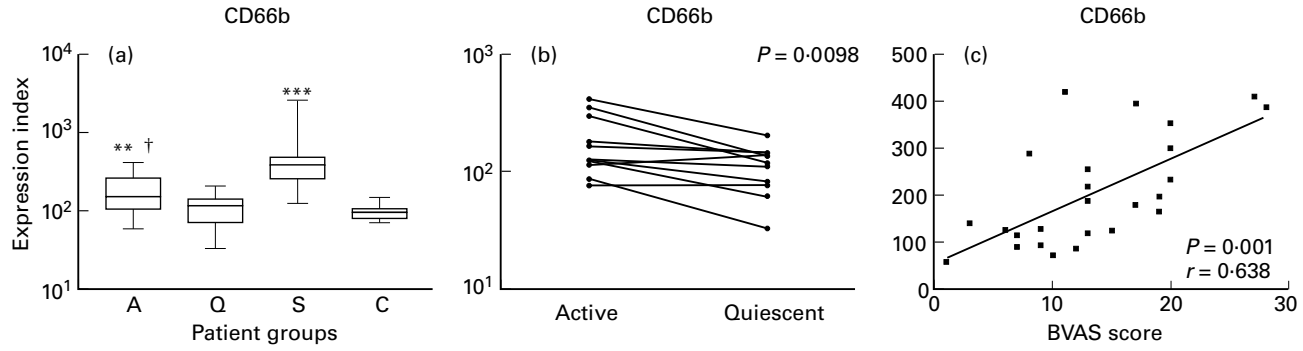

Fig. 2. (a) Box and whisker plots indicating the overall range (error bars), 25–75% range (boxes), and median value (horizontal lines) of the surface expression of CD66b on neutrophils from patients with active (A), and quiescent (Q) ANCA-associated vasculitis, patients with sepsis (S) and healthy controls (C). **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$ compared with healthy controls; †$P < 0{\cdot}05$ compared with patients with quiescent disease. (b) Paired analysis of the expression of CD66b on neutrophils from patients with active and quiescent disease. (c) Correlation between the expression of CD66b on neutrophils and disease activity as expressed by the Birmingham Vasculitis Activity Score (BVAS).

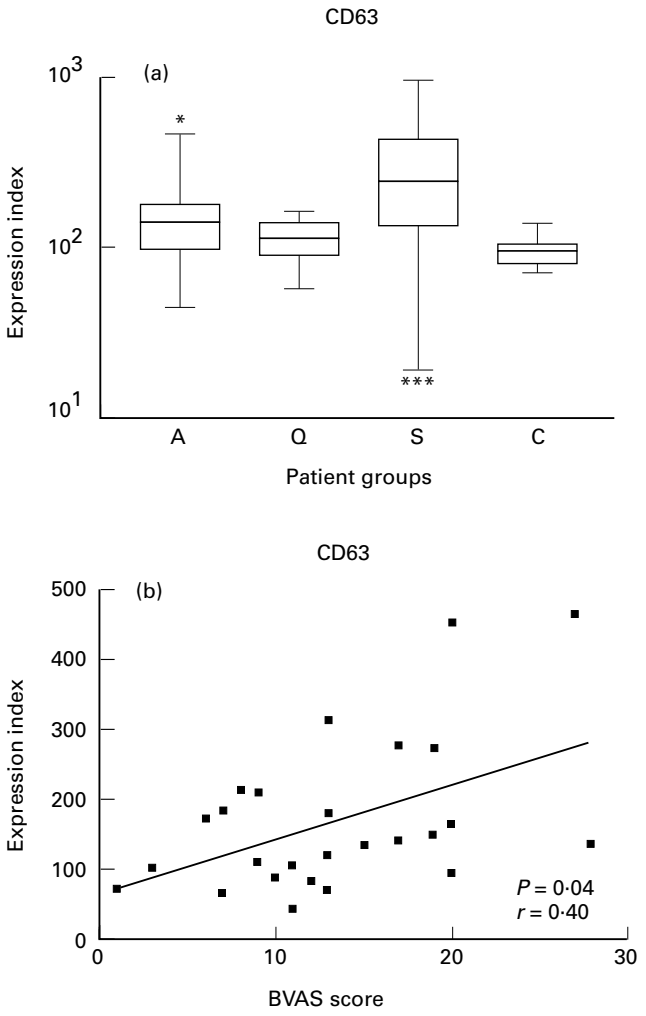

Fig. 3. (a) Box and whisker plots indicating the overall range (error bars), 25–75% range (boxes), and median value (horizontal lines) of the surface expression of CD63 on neutrophils from patients with active (A), and quiescent (Q) ANCA-associated vasculitis, patients with sepsis (S) and healthy controls (C). $*P<0{\cdot}05$; $***P<0{\cdot}001$ compared with healthy controls. (b) Correlation between the expression of CD63 on neutrophils and disease activity as expressed by the Birmingham Vasculitis Activity Score (BVAS).

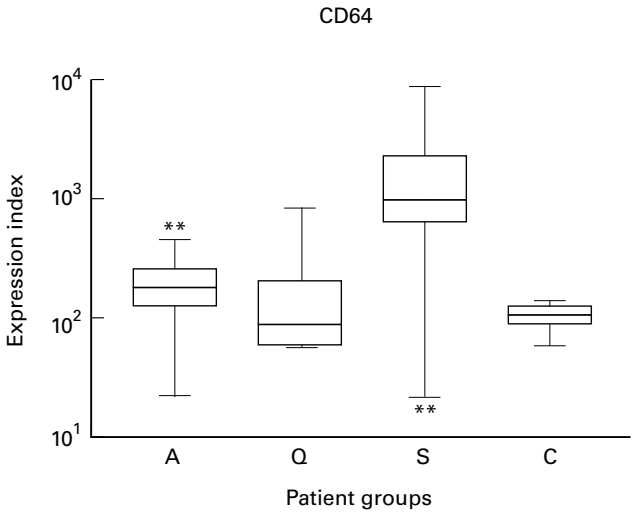

Fig. 4. Box and whisker plots indicating the overall range (error bars), 25–75% range (boxes), and median value (horizontal lines) of the surface expression of CD64 on neutrophils from patients with active (A), and quiescent (Q) ANCA-associated vasculitis, patients with sepsis (S) and healthy controls (C). $**P<0{\cdot}01$ compared with healthy controls.

and other adhesion molecules on circulating neutrophils from patients with active vasculitis was not different from their expression in healthy controls, although a slight up-regulation of CD11b was observed in patients with newly diagnosed disease but not in patients with relapsing disease. In contrast, patients with sepsis had significantly increased expression of adhesion molecules on circulating neutrophils, indicating that in these patients cells are activated in the circulation. Several authors have studied the extent of activation of circulating neutrophils in patients with WG. Riecken and co-workers demonstrated a decreased expression of L-selectin compared with healthy controls [43]. These authors, however, used density gradient-isolated granulocytes, which may result in *in vitro* activation, including shedding of L-selectin, due to purification methods [27,44,45]. In the present study, we used a whole blood method, in which cells were fixed with paraformaldehyde within minutes after collection. All preparation steps were carried out at 4°C. This method minimizes artificial *in vitro* cell activation, when compared with various cell isolation procedures [28]. Using a non-activating whole blood method, Haller and co-workers also found increased expression of CD11b in patients with newly diagnosed, active vasculitis [46]. In our study we found that CD11b expression was moderately increased only in patients with newly diagnosed active disease, who had more active disease than patients with relapsing disease, as demonstrated by BVAS scores, probably due to delayed diagnosis of progressively active vasculitis. A Shwartzman-like phenomenon may marginally contribute to vascular damage in those patients only who present with ongoing and increasing disease activity. Since we observed that CD11b expression was not increased in relapsing patients, despite the presence of vasculitic lesions, we conclude that other mechanisms of vascular damage are probably more relevant in the pathophysiology of ANCA-associated vasculitides. Previously, Reumeax *et al*. showed that *in vitro* ANCA-induced leucocyte activation only occurs when pre-activated cells are bound to a surface, e.g. activated endothelial cells [47]. This mechanism requires pre-activation of circulating neutrophils as well as possibilities for interaction with endothelial cells, e.g. by L-selectin.

In order to investigate whether circulating neutrophils in patients with vasculitis are pre-activated, we assessed the expression of activation markers, such as the degranulation markers CD66b and CD63 and the first Fcγ receptor, CD64, as well as adhesion molecules on those cells. In patients with active vasculitis the expression of CD66b, CD63 and CD64 on neutrophils was increased compared with healthy controls. Patients with active vasculitis lacked, however, increased expression of adhesion molecules on circulating neutrophils. These findings are consistent with a state of pre-activation. Several investigators demonstrated *in vitro* that pre-activation of neutrophils with cytokines leads to PR3 membrane expression [48,49]. A pre-activated state, i.e. availability of ANCA antigens for interaction with ANCA, is a prerequisite for interaction with and subsequent activation by ANCA. Expression of PR3 can only be explained if a certain degree of degranulation of azurophilic granules has occurred. Indeed, we demonstrated that circulating neutrophils from patients with ANCA-associated

vasculitides have an increased expression of CD63, a marker for azurophilic granule degranulation. Furthermore, the expression of these degranulation markers in patients with vasculitis correlated with disease activity, indicating a role for *in vivo* neutrophil pre-activation in the pathophysiology of ANCA-associated vasculitides.

However, no correlations were found between the extent of activation of circulating neutrophils and ANCA titre. As discussed earlier, we believe that activation of primed neutrophils by ANCA occurs when these cells are bound to a surface, i.e. endothelial cells. Since we investigated the extent of activation of circulating cells, activation of these cells by ANCA had probably not yet occurred. This may explain why no correlation between cell activation and ANCA titre was observed.

The differences in neutrophil activation between patients with active and quiescent disease could, in part, be explained by the effects of treatment of patients with quiescent disease. immunosuppressive drugs are known to influence leucocyte activation. Since it is the treatment that induces quiescent disease and since we believe that it is the activated leucocyte that causes damage to endothelial cells, the decreased state of activation of neutrophils during quiescent disease may be a beneficial effect of the treatment given to these patients.

In conclusion, in ANCA-associated vasculitides circulating neutrophils are not fully activated intravascularly, suggesting that a Shwartzman-like phenomenon may not primarily underlie the development of vascular damage. The increased expression of activation markers, such as CD66b, CD63 and CD64, on circulating neutrophils in patients with active vasculitis can be attributed to a state of pre-activation ('priming'). These findings are consistent with the hypothesis that 'primed' neutrophils adhere to the endothelium, are then fully activated by ANCA, and release lytic enzymes and oxygen radicals at the endothelial cell surface, resulting in endothelial cell lysis, and eventually vasculitis.

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