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Up-regulation of the granulocyte adhesion molecule Mac-1 by autoantibodies in autoimmune vasculitis

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SUMMARY

The characteristic finding of autoantibodies in patients with vasculitis has raised the possibility that these antibodies play a role in the pathogenesis of the disease. The expression of adhesion molecules (AM) on leucocytes and endothelial cells is believed to be integral to the development of vasculitis. We therefore investigated the effect of sera, positive for anti-neutrophil cytoplasmic antibodies (ANCA) or anti-nuclear antibodies (ANA) from patients with vasculitis, on granulocyte expression of the adhesion molecule Mac-1 (CD11b). Autoantibody-positive sera from 15 out of 35 patients with vasculitis stimulated an up-regulation of Mac-1 on granulocytes. In most cases this effect was reproduced by the autoantibody-positive purified IgG fraction. Autoantibody-negative samples did not stimulate AM up-regulation. Of interest, preincubation of sera with purified antigens did not inhibit AM up-regulation by the autoantibody samples. Blocking the Fc receptors on granulocytes did result in a decrease of Mac-1 up-regulation, but this trend was not statistically significant. These results suggest that both ANCA and ANA have the capacity to up-regulate granulocyte AM expression, and that while Fc interaction with granulocyte Fc receptors is important, it is not the only mechanism whereby such autoantibodies activate cells.

Keywords vasculitis autoantibodies ANCA adhesion molecules

INTRODUCTION

Antibodies directed against neutrophil cytoplasmic antigens (ANCA) are associated with vasculitis syndromes such as Wegener's granulomatosis (WG), microscopic polyarteritis (MPA) and polyarteritis nodosa (PAN) [1–7]. Recently these autoantibodies have been implicated in the pathogenesis of the disease [8–17]. Two types of ANCA have been described, based on the characteristic immunofluorescence staining patterns on alcohol-fixed neutrophils incubated with sera from patients: antibodies exhibiting a cytoplasmic pattern (C-ANCA) and antibodies exhibiting a perinuclear staining pattern (P-ANCA) [5]. Under normal non-inflammatory conditions, neutrophils are not adherent to the vascular endothelium. However, in the presence of inflammatory mediators neutrophils adhere to the endothelial cell surface and migrate through the vessel wall to the site of tissue damage. In some cases, the neutrophil may not migrate into the tissue but degranulate onto the endothelium, causing direct vessel wall injury. This neutrophil-mediated injury of human endothelial cells is considered to be an important mechanism in the pathogenesis of WG and related vasculitidies [9]. Close association or adhesion of neutrophils to vascular endothelium is an essential prerequisite for this phenomenon to occur.

The aim of these investigations was to examine the effect of ANCA-positive sera and purified IgG fractions from patients with vasculitis upon granulocyte expression of the adhesion molecule Mac-1. *In vitro* studies have shown that ANCA can activate primed polymorphonuclear leucocytes (PMN), induce degranulation, and produce oxygen radicals [18–20]. Other studies have shown that ANCA are involved in functional interactions between neutrophils and endothelium, as suggested by observations of increased neutrophil adhesion to cultured human umbilical vein endothelial cells (HUVEC) [20,21]. In contrast, the studies reported in this paper aimed to assess the direct effects of ANCA-positive sera and purified IgG fractions from patients with vasculitis upon granulocyte expression of the adhesion molecule Mac-1.

The mechanisms by which ANCA may activate neutrophils have yet to be clearly established. In order to investigate ANCA-induced neutrophil activation further, purified ANCA antigens proteinase 3 (PR3) and myeloperoxidase (MPO) were added to sera and to purified IgG fractions from patients in an attempt to block any cellular activation by ANCA. Also, MoAbs to PR3 and MPO were added to investigate whether reactivity to PR3 or MPO alone is sufficient to cause neutrophil activation. Finally, Fc receptors were blocked using rabbit serum in an effort to establish

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which portion of the immunoglobulin molecule was critical for neutrophil up-regulation of Mac-1.

SUBJECTS AND METHODS

Patients and sera

Sera were obtained from healthy normal volunteers ($n = 45$) and from patients with autoimmune vasculitis ($n = 37$) (11 patients with MPA, seven patients with WG, two patients with PAN, three patients with systemic lupus erythematosus (SLE), three patients with rheumatoid arthritis (RA) and 11 patients with miscellaneous forms of vasculitis (MV): Sjögren's syndrome ($n = 3$), Churg–Strauss syndrome ($n = 1$), cutaneous vasculitis ($n = 2$), and five patients without a definitive clinical or histological diagnosis who had clinical features of vasculitis (skin and/or respiratory tract involvement)). Three of these five patients responded clinically to steroid immunosuppression. Two patients had C-ANCA-positive sera and two patients had P-ANCA-positive sera. In addition, ANCA-negative serum samples were obtained from 10 patients with septicaemia, a condition known to be associated with vascular injury. Serum samples were immediately stored in endotoxin-free vials at 4°C, filter sterilized, aliquoted and stored at −20°C within 1 week of venepuncture. Samples were heated to 56°C for 30 min before testing to inactivate complement.

Characterization of serum samples

Standard indirect immunofluorescence (IIF) assays ([22–24], V. Broomhead and co workers, Regional Immunology Laboratory, Newcastle-upon-Tyne, personal communication) were employed to identify the presence of ANCA and/or anti-nuclear antibodies (ANA) in the serum samples. The molecular specificity of the ANCA antibodies (MPO, PR3, lactoferrin (L), elastase (E) and cathepsin G (CG)) was determined by ELISA kits (Shield Diagnostics Ltd, Dundee, UK). With the exception of samples from patients with septicaemia, sera were free of endotoxin as measured by E-TOXATE (Limulus Amebocyte Lysate (LAL) assay; Sigma Chemical Co, Poole, UK) and proinflammatory cytokines tumour necrosis factor-alpha (TNF-α) and IL-1β, as measured by cytokine assay kits (R & D Systems, Minneapolis, MN).

Purified IgG preparations

Purified IgG was obtained from the sera of patients with autoimmune vasculitis, septicaemia and from healthy control volunteers by affinity chromatography using a protein G column (Pharmacia Biotec Ltd, St Albans, UK). Purified IgG was obtained from the serum samples and the purity assessed by SDS–PAGE.

Incubation of cell populations with patient and control serum and purified IgG fractions

Heparinized blood was obtained from healthy volunteers and aliquoted into 200-μl amounts. Serum and purified IgG fractions (100 μl) were added within 10 min of venepuncture. Preparations were mixed gently and incubated at 37°C for 1 h. Following incubation, samples were gently mixed again and examined for granulocyte expression of Mac-1 by flow cytometry.

Flow cytometry

Following incubation with serum and purified IgG, 100 μl of each blood sample were added to 20 ml of FITC-conjugated murine MoAb to human Mac-1 (CD11b) (Becton Dickinson, San Jose, CA). As negative control, samples were added to 20 μl of FITC-conjugated murine MoAbs to keyhole limpet haemocyanin (KLH) (Becton Dickinson). The preparations were mixed and allowed to stand at room temperature for 15 min. The erythrocytes were then lysed with 2 ml of a 1:10 dilution of FACS lysing solution (Becton Dickinson) for 10 min at room temperature. Leucocytes were separated from the lysed erythrocytes by centrifugation at 70 ***g*** for 5 min. The leucocyte pellet was mixed and washed twice in PBS. Following the final centrifugation, the leucocyte pellet was mixed and resuspended in Cell Fix (Becton Dickinson) at a dilution of 1:10.

Reproducibility and dose response

The baseline values of neutrophil adhesion molecule (AM) expression did vary among the four normal individuals whose blood was assayed in this study. However, for each individual at least 10 normal control sera were analysed to establish if variation also existed in response to incubation with serum. A total of 45 normal controls were assayed and samples were considered positive for Mac-1 up-regulation when a mean channel fluorescence (MCF) of > 2 s.d. above the mean of the control sera was recorded. Three serum samples from patients and three samples from normal healthy individuals were assayed 10 times to establish the intra-assay variation, and in triplicate over 3 days to assess the interassay variation. Two positive samples were assayed at dilutions ranging from 1:2 to 1:1280 to establish a dose response.

Cytokine stimulation of granulocytes

Heparinized blood from two healthy volunteers was incubated for 1 h at 37°C with human recombinant TNF-α and IL-1β (National Institute for Biological Standards and Controls, Potter's Bar, UK) at concentrations of 0·1, 1 and 10 U of TNF-α per ml and 1, 10 and 100 U IL-1β per ml. Granulocyte Mac-1 expression was then examined using flow cytometry.

Monoclonal antibodies

Four murine antibodies raised against PR3 (1A3, 2A3 and 6A3, a generous gift from Professor Jorgen Wieslander, Staten Serum-institut, Copenhagen, Denmark) and 7H1 (a gift from Dr E. Dermott, Royal Victoria Hospital, Belfast, UK) and one MoAb reacting with MPO (AMPO; Dako Ltd, High Wycombe, UK) were each diluted in PBS or normal sera to titres of 1:80, 1:320 and 1:640 and added to heparinized blood from healthy individuals as before (serum and purified IgG samples) and analysed for AM expression by flow cytometry.

Inhibition studies with purified ANCA antigens

Purified human PR3 (a gift from Dr T. W Johnston, Department of Biochemistry, The Queen's University, Belfast, UK) and MPO antigens were diluted in ANCA-positive serum or purified IgG samples derived from patients with vasculitis to a concentration of 1 μg/ml of PR3 and 3 μg/ml of MPO and incubated for 2 h at 37°C and added to heparinized blood preparations as before. Granulocyte AM expression was assessed by flow cytometry.

Blocking of Fc receptors

Granulocyte Fc receptors were blocked using rabbit serum [25]. Blood was obtained from a vein in the ear of a healthy control rabbit and placed in sterile tubes. The blood was centrifuged (200 ***g***) and the serum harvested. Within 10 min of venepuncture, 5 ml of heparinized blood from a healthy volunteer were added to 1 ml of rabbit serum and placed at 37°C for 30 min in the dark. The

blood was then aliquoted and incubated with ANCA-positive serum from patients with vasculitis as before.

RESULTS

Purification of IgG

The ANCA/ANA-positive IgG samples and their molecular specificities are detailed in Table 1a. From the measurement of IgG in serum and purified fractions and allowing for dilution factors, the yield of IgG from each sample was calculated to be between 75% and 90%. In several cases, titres dropped to approximately half the level recorded for the original serum sample, resulting in some instances in a loss of antigen specificity (data not shown). The decrease in samples testing positive for PR3 following IgG purification was probably due to the dilution factor. Dilution of the sample was necessary for ease of purification. It is widely recognized that immunofluorescence is more sensitive than ELISA in ANCA studies, and this may explain why some samples appeared to retain ANCA staining while losing reactivity to PR3. For comparsion, purified IgG samples were analysed in the same dilution ratio as serum samples. Purity of five IgG-containing elutions was assessed by SDS–PAGE. IgG was estimated to comprise 70–85% of the fractions analysed (data not shown)

Granulocyte expression of adhesion molecules

Samples were considered positive for Mac-1 up-regulation on granulocytes when the MCF values exceeded 2 s.d. above the mean value for 45 normal controls (i.e. samples which recorded a MCF value of >1702 were considered positive). Samples treated with purified IgG were considered positive for Mac-1 up-regulation when an MCF of >3 s.d. above the mean of 10 normal controls was recorded, i.e. an MCF value of >1317.

MPA, WG and PAN

Serum samples from six out of nine patients with MPA, three out of seven patients with WG and one out of two patients with PAN produced an up-regulation of Mac-1 on granulocytes. The samples were either positive for C-ANCA, P-ANCA or ANA (Table 1b). In all cases the effect was reproduced with the ANCA- or ANA-positive IgG purified fractions. Washes and autoantibody-negative elutions did not stimulate Mac-1 up-regulation. The histogram plots for granulocytes incubated with control samples, and serum and purified IgG from a patient with MPA, are detailed in Fig. 1, demonstrating the rise in Mac-1 in the presence of ANCA-positive IgG.

MV, SLE and RA

Sera from five out of 11 patients with MV produced an up-regulation of Mac-1 on granulocytes (Table 1b). The samples were positive either for P-ANCA, C-ANCA or ANA. Up-regulation also occurred with the purified IgG component of three of these serum samples. Serum samples which were negative for autoantibodies did not induce Mac-1 up-regulation. None of the sera and purified IgG samples from patients with RA or SLE produced an up-regulation of Mac-1 on granulocytes, despite having high-titre ANA.

Control group

Serum samples from patients with septicaemia did not induce an up-regulation of Mac-1 on granulocytes, despite the presence of cytokines or endotoxin in some samples.

Reproducibility and dose response

The intra-assay variation was calculated to be between 2% and 5% ($n=10$) and the interassay variation was calculated to be between 6% and 12·5% ($n=3$). Granulocyte expression of Mac-1 continued to be up-regulated with dilutions of sera up to 1:320, but declined thereafter (data not shown).

Monoclonal antibodies

MoAbs to ANCA antigens did not up-regulate Mac-1 on the surface of granulocytes when added in PBS or control serum (data not shown).

Purified antigens

Purified ANCA antigens PR3 and MPO did not interfere with the up-regulation of Mac-1 by sera or purified IgG from patients with ANCA (data not shown).

Blocking Fc receptors

Although blocking the Fc receptors resulted in almost all cases in a decrease in Mac-1 up-regulation, reduction to within the normal range was observed with only one (denoted by *) of the seven samples (Table 2). However, when the percentage blocking is considered it is apparent that several of the activating samples were inhibited by Fc receptor blocking outside the batch variation (i.e. $\pm$ 12·5%). Down-regulation of Mac-1 did not occur with Fc-blocked granulocytes incubated with normal serum or rabbit serum alone, and the reduction in MCF for these samples cannot be attributed to a lowering in activation potential or Mac-1 expression by rabbit serum alone.

Cytokines

Cytokines TNF-α and IL-1β up-regulated Mac-1 on granulocytes in a dose-dependent manner. The more potent cytokine was TNF-α, producing a five-fold increase in expression at a concentration of 10 U/ml (Table 3).

DISCUSSION

It has been suggested that vasculitic lesions develop as a result of cytotoxic products released by neutrophils, and that when these products are released adjacent to endothelial or other vascular cells, injury to the cells occurs [26]. Thus the adhesion of neutrophils to the endothelium is important in the development of vasculitis. This study demonstrates that ANCA-positive sera and purified IgG up-regulate the granulocyte adhesion molecule Mac-1. Up-regulation was not observed by autoantibody-negative washes (IgG-free serum) or elutions. These findings are in keeping with other studies which demonstrate that ANCA-positive IgG and $F(ab)_2$ fragments increase the adhesion of normal neutrophils to cultured HUVEC [20,21]. Furthermore, up-regulation is also induced by samples positive for ANA, although not all autoantibody-positive samples cause AM up-regulation. The effect of cytokines on AM expression was also examined. Up-regulation of Mac-1 was observed with TNF-α and to a considerably lesser extent with IL-1β. An interesting finding was that even high concentrations of TNF-α did not induce the same degree of Mac-1 up-regulation as some of the autoantibody-positive samples, suggesting that, at least *in vitro*, these autoantibodies are more potent cellular activators than TNF-α. Also of interest is the finding that serum samples diluted up to 1:320 retained their capacity to up-regulate this AM. This may have important physiological implications.

Table 1a. Characterization of patient and control serum for the presence of cytokines (tumour necrosis factor-alpha (TNF-α), IL-1β), endotoxin and autoantibodies

Patient group	Number of patients	Total number of samples tested		Number TNF-α-positive	Number IL-1β-positive	Number LPS-positive	Number P-ANCA-positive	Number anti-MPO-positive	Number C-ANCA-positive	Number anti-PR3-positive	Number anti-EL,LF,CG-positive	Number ANA-positive
MPA	9	Sera	19	0	0	0	0	0	15	11	0	3
		IgG	17	–		–	0	0	11	4	–	–
WG	7	Sera	11	0	0	0	1	1	7	7	0	0
		IgG	11	–		–	1	1	6	7	–	–
PAN	2	Sera	3	0	0	0	3	3	0	0	1(LF)	0
		IgG	3	–		–	–	–		–	–	–
MV	11	Sera	12	0	0	0	2	2	2	0	0	4
		IgG	6	–		–	1	1	0	0	–	4
SLE and RA	6	Sera	6	0	0	0	0	0	0	0	0	6
		IgG	1	–		–	0	0	0	0	–	1
Septicaemia	10	Sera	10	2	1	3	0	0	0	0	0	0
		IgG	8	–		–	–	–	–	–	–	–
Normal Control	45	Sera	45	0	0	0	0	0	0	0	0	0
		IgG	10	–	–	–	–	–	–	–	–	–

MPA, Microscopic polyarteritis; WG, Wegener's granulomatosis; PAN, polyarteritis nodosa; RA, rheumatoid arthritis; SLE, systemic lupus erythythematosus; LPS, endotoxin, PR3, proteinase 3; MPO, myeloperoxidase; LF, lactoferrin, CG, cathepsin G; EL, elastase; –, not tested.

b. Up-regulation of Mac-1 on granulocytes following incubation with serum and purified IgG from patients with vasculitis, patients with septicaemia and normal healthy volunteers

Patient group	Number of patients	Total number of samples tested		Serum and purified IgG samples which stimulated an up-regulation of Mac-1 on granulocytes to >2 s.d. above the mean of 45 normal controls (OD)					
				Number P-ANCA-positive	Number anti-MPO-positive	Number C-ANCA-positive	Number anti-PR3-positive	Number ANA-positive	Number autoantibody-negative
MPA	9	Sera	19	0	0	10	7	1	0
		IgG	17	0	0	9	3	0	0
WG	7	Sera	11	0	0	3	3	0	0
		IgG	11	0	0	3	3	0	0
PAN	2	Sera	3	1	1	0	0	0	0
		IgG	3	1	1	0	0	0	0
MV	11	Sera	12	1	1	1	0	3	0
		IgG	6	1	1	0	–	0	0
SLE and RA	6	Sera	6	0	–	0	–	0	0
		IgG	1	0	–	0	–	0	0
Septicaemia	10	Sera	10	0	–	0	–	0	0
		IgG	8	0	–	0	–	0	0
Normal	45	Sera	45	0	–	0	–	0	0
Control		IgG	10	0	–	0	–	0	0

MPA, Microscopic polyarteritis; WG, Wegener's granulomatosis; PAN, polyarteritis nodosa; RA, rheumatoid arthritis; SLE, systemic lupus erythythematosus; PR3, proteinase 3; MPO, myeloperoxidase; –, not tested.

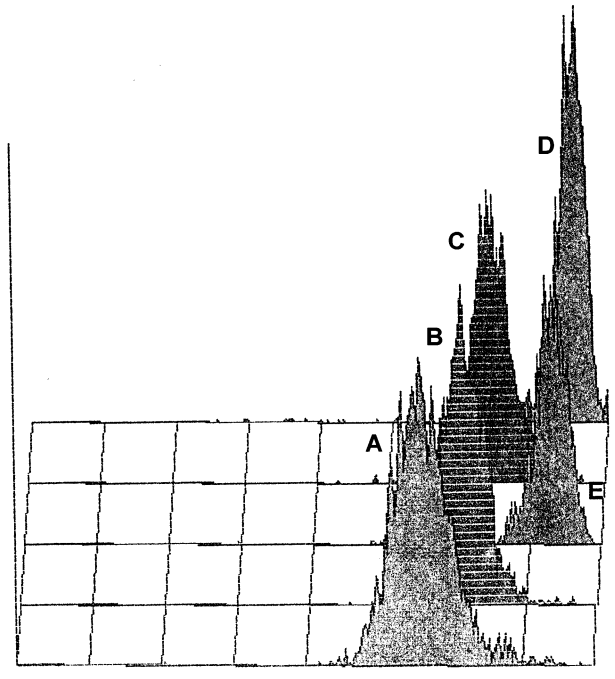

Fig. 1. Histograms of Mac-1 expression on granulocytes stimulated with serum (D), IgG separation wash (C), anti-neutrophil cytoplasmic antibody (ANCA)-positive purified IgG elution (E) and IgG-negative elution (B) from a patient with microscopic polyarteritis (MPA). A histogram of a normal control sample is also included (A). Adhesion molecule up-regulation is observed with ANCA-positive serum (D) and IgG fractions (E) from this patient.

The mechanism of neutrophil activation by ANCA remains unclear. Activation may be due to non-specific interaction of the Fc portion of the ANCA molecule with Fc receptors on the surface of the granulocyte [29]. A recent study by Tse and co-workers suggests that neutrophil FcγRIIA polymorphism is a heritable risk factor for ANCA-positive vasculitis, implicating ANCA–Fc receptor interactions in disease development (Tse *et al*., Renal Association, Autumn Meeting, October 1996). Additionally or independently, ANCA may react with their target antigens on the surface of the neutrophil [17,27]. Cross-linking with these molecules may result in internalization and subsequent neutrophil activation. Alternatively, exogenous antigen may associate with a receptor complex on the neutrophil which, upon reaction with the antibody, results in cellular activation [17]. For this to occur, PR3, MPO or other ANCA antigens must be accessible to the autoantibody. The binding of exogenous antigen is unlikely to be a factor, as such enzymes are probably not present in blood samples from healthy individuals.

Recent studies have reported that patients with active WG demonstrated enhanced PR3 expression on the plasma membrane of circulating neutrophils compared with healthy controls [27,28]. They also demonstrate that these antigens may be induced on the surface of normal neutrophils by cytokines, suggesting that some priming of the neutrophil must occur before ANCA react with the neutrophil. In this present study, however, experiments were performed with normal neutrophils without priming of the granulocyte population. As the experiments employed whole blood with no separation of cellular subpopulations, artefactual priming should have been minimized. It is possible that some cellular activation occurs secondary to venepuncture and sample collection, resulting in the translocation of PR3 to the surface. If activation was simply related to the expression of the antigens on the cell surface, it does not explain why only some ANCA- or ANA-positive

Table 2. Granulocyte activation by patients' sera before and after blocking Fc receptors

Patient no.	No blocking (MCF)	Blocking (MCF)	Percent blocking
MPA 4c	3105	2996	3·5
MPA 4e	1806	1928	0
MPA 10e	2414	1439*	40
WG 1a	2519	2049	18
WG 1b	2440	2070	15
MPA 6c	4996	2998	39
MPA 6e	1249	1468	0
MPA 9a	2672	2044	23·5
MPA 9b	1245	1010	18
Normal	1065	1136	0
Normal	1132	1052	7
Normal	1469	1621	0
Normal	1532	1356	11
Normal	935	1019	0
Rab serum	1236	1404	0

*A loss in up-regulation following blocking. MCF, Mean channel fluorescence.

Table 3. Expression of Mac-1 on normal human granulocytes following incubation with cytokines tumour necrosis factor-alpha (TNF-α) or IL-1β

	Mac-1 (MCF)
TNF-α (U/ml)	
0·1	1771
1·0	2931
10	3278
IL-1β (U/ml)	
1	894
10	1036
100	1697
No cytokines	598

MCF, Mean channel fluorescence.

samples were capable of causing AM up-regulation. Furthermore, Csernok and co-workers did not detect enhanced expression of MPO at any time on the surface of neutrophils either from patients with active vasculitis or on normal neutrophils following stimulation with cytokines TNF-α and IL-8 [28]. Thus, how might the activation of neutrophils by P-ANCA anti-MPO be explained? In addition, AM up-regulation could not be blocked by ANCA antigens or induced with monoclonal ANCA. This inability of the purified antigens to block Mac-1 up-regulation is surprising. Although the PR3 was previously shown to inhibit the binding of anti-PR3 in patients' serum in an anti-PR3 ELISA, the possibility that the antigen may not have bound or may have disassociated from the antibody during the experiment cannot be ruled out. However, if activation was due to interaction of the ANCA antibody with its target antigen on the cell surface, then all samples should possess some activating capacity, and this clearly was not the case. Mulder and co-workers also observed that not all ANCA-positive samples activate neutrophils [29]. The presence of ANCA antigens on the surface of the neutrophil may enhance activation, but clearly some other factor is involved in this *in vitro* ANCA stimulation. One explanation might be that these antibodies simply cross-react with a receptor involved in cellular activation.

As already stated, the mechanisms of granulocyte activation by ANCA remain to be identified. Earlier studies suggest that the $F(ab)_2$ mediates neutrophil activation *in vitro* [20,21]. However, a recent study has demonstrated activation via the Fc portion of the ANCA molecule [29]. To investigate this phenomenon further the granulocyte Fc receptors were blocked using rabbit serum. A considerable reduction in expression was noted for most samples. This implicates the Fc portion of the autoantibodies in Mac-1 up-regulation, but a significant decrease was only observed for one of the samples analysed, suggesting that other interactions are involved. This is in keeping with the report by Mulder and co-workers, which demonstrated that neutrophil activation by ANCA is dependent on the expression of the ANCA antigens on the surface of the cell and the presence of FcγRII receptors [29].

It was observed that not all ANCA-positive sera were able to induce AM molecule up-regulation, and that ANCA titres in samples capable of stimulation were comparable to those in samples incapable of stimulation. Mulder and co-workers suggest that the subclass of the antibody may be important [29]. They found that stimulating samples contained high concentrations of IgG3-ANCA, whereas non-stimulating samples did not.

Mac-1 up-regulation by ANCA may explain part of the mechanism underlying neutrophil–endothelial cell interaction in systemic vasculitis. The variability of this adhesion molecule up-regulation in response to ANCA sera *in vitro* may parallel the variability in clinical severity of vasculitis *in vivo* despite comparable ANCA titres.

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