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Up-regulation of the endothelial cell adhesion molecule intercellular adhesion molecule-1 (ICAM-1) by autoantibodies in autoimmune vasculitis

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SUMMARY

Autoimmune vasculitis is characterized by the presence of autoantibodies, particularly anti-neutrophil cytoplasmic antibodies (ANCA) and anti-nuclear antibodies (ANA), in patient sera. These autoantibodies have an incompletely understood role in development of vascular injury. The expression or up-regulation of cell adhesion molecules is an early phase in the development of an inflammatory vascular lesion. Autoantibody-positive sera from patients with vasculitis were assessed for their ability to modulate adhesion molecule expression by human umbilical vein endothelial cells (HUVEC). Autoantibody-positive serum samples from 11 out of 21 patients with primary vasculitis produced substantial up-regulation of ICAM-1 on HUVEC. Autoantibody-negative samples did not produce adhesion molecule up-regulation. Up-regulation of adhesion molecules on HUVEC was observed with samples positive for ANA, a phenomenon not previously reported. Preincubation of the sera with purified antigens recognized by ANCA failed to block this activation. In addition, MoAbs to ANCA antigens were ineffective at inducing ICAM-1 up-regulation, suggesting that activation is independent of the molecular specificity of the antibody. This capacity of ANCA- and ANA-positive sera to up-regulate adhesion molecules on endothelial cells may be a factor in the vessel wall inflammation seen in ANCA-associated vasculitis.

Keywords vasculitis autoantibodies anti-neutrophil cytoplasmic antibodies endothelial cells adhesion molecules

INTRODUCTION

Primary vasculitis syndromes such as Wegener's granulomatosis (WG), microscopic polyarteritis (MPA) and polyarteritis nodosa (PAN) are associated with anti-neutrophil cytoplasmic antibodies (ANCA), which represent a heterogeneous group of antibodies directed against components of the neutrophil cytoplasm [1–10]. Although ANCA have been intensively studied, their exact role in the development of vasculitis syndromes remains to be elucidated. Two types of ANCA have been described, based on the immunofluorescence staining patterns on alcohol-fixed neutrophils: antibodies exhibiting a cytoplasmic pattern (C-ANCA), and antibodies exhibiting a perinuclear staining pattern (P-ANCA) [7].

Research to date has concentrated on the effect of ANCA on neutrophils, which possess the target antigens of ANCA (proteinase 3 (PR-3), myeloperoxidase (MPO), elastase (EL), lactoferrin (LF), and cathepsin G (CG)). However, as ANCA also come into direct contact with the endothelium *in vivo*, the potential effects of ANCA-containing sera on endothelial cell function are relevant to determining their role in the pathogenesis of this disease. Adhesion molecules are up-regulated on the surface of endothelium in biopsies from patients with ANCA-positive vasculitis [12–15]. It is therefore of interest to establish if ANCA-containing sera can directly stimulate endothelial cell expression of adhesion molecules.

This study reports the effect of ANCA-positive sera and purified IgG from patients with vasculitis on endothelial cell expression of the adhesion molecule ICAM-1. Recent studies have demonstrated that MPO binds to the endothelium and in turn is bound by antibodies to MPO (P-ANCA) [16,17], thus providing another scenario in which ANCA may react with the endothelium *in vivo*. In order to examine further the nature of the endothelial–ANCA interactions, human umbilical vein endothelial cell (HUVEC) monolayers were coated with ANCA antigens MPO and PR-3. The monolayers were then examined for evidence of ICAM-1 up-regulation in response to sera and purified IgG from patients with vasculitis.

In addition, to investigate the importance of antibody molecular specificity, attempts were made to block the interaction by preincubation of the serum and IgG samples with purified PR-3 and MPO. Furthermore, murine MoAbs directed against ANCA

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antigens were examined for their ability to up-regulate ICAM-1 expression on HUVEC.

SUBJECTS AND METHODS

Patients and sera

Serum samples were obtained from 37 patients (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 with clinical features of vasculitis (skin and/or respiratory tract involvement) who did not have a definite clinical or histological diagnosis). 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. As a control group, serum samples were also obtained from 10 patients with ANCA-negative septicaemia, a condition known to be associated with vascular injury. As a further control group, 45 serum samples from healthy volunteers were also examined for their effect on endothelial cell expression of ICAM-1. 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

Serum samples were analysed for the presence of ANCA and antinuclear antibodies (ANA) by standard indirect immunofluorescence (IIF) assays ([18–20], V. Broomhead and co-workers (Regional Immunology Laboratory, Newcastle-upon-Tyne, UK), personal communication) and for the molecular specificity of the ANCA antibodies (MPO, PR-3, LF, EL and CG) 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).

Culture of HUVEC

Endothelial cells were harvested from human umbilical cord veins by collagenase digestion according to the methods described by Jaffe and co-workers [21] and seeded onto gelatin-coated tissue culture flasks. The cultured cells were identified by their characteristic cobblestone appearance and by the expression of Factor VIII-related antigen.

Anti-endothelial cell antibodies

Cultured HUVEC between passages 2 and 4 were seeded onto fibronectin-coated 96-well microtitre plates at a concentration of 2×10^4 cells/well. Cells were allowed to grow to confluence overnight, washed in PBS and fixed with 0·2% glutaraldehyde/PBS on ice for 4 min. Using 10% fetal bovine serum (FBS)/PBS, the plate was blocked for 1 h at 37°C (200 μl/well). The plate was then washed six times with PBS (200 μl/well), tapping the plate to remove excess wash. Serum was diluted 1:100 in 10% FBS and added to the plate at 100 μl/well. All samples were tested in duplicate. The plate was washed as before and 100 μl/well of alkaline phosphatase goat anti-human (AHG) conjugate (Sigma), diluted 1:1000 in 16% v/v new-born goat serum in PBS, were added for 1 h at 37°C. The plate was washed as before and 100 μl/well of alkaline phosphatase substrate solution were added for 1 h at room temperature in the dark. Absorbance values were read at 405 nm on an ELISA reader.

Detection of ICAM-1 on HUVEC by ELISA

Cultured HUVEC between passages 2 and 4 were seeded onto gelatin-coated microtitre plates at a concentration of 2×10^4 cells/well. Confluent monolayers where allowed to form overnight. The monolayers were fixed in 0·2% glutaraldehyde (Sigma) and either stained immediately or stored in 5% FBS/PBS overnight at 4°C. Using 10% FBS/PBS, the plate was blocked for 1 h at 37°C (200 μl/well) and washed six times with PBS (200 μl/well). A 1:20 (0·1 mg/ml) dilution in PBS of mouse MoAb to human endothelial ICAM-1 (Immunotec S.A., Marseille, France) (100 μl/well) was added for 1 h at 37°C. The plate was washed in PBS. Alkaline phosphatase goat anti-mouse conjugate (Sigma) (100 μl/well), diluted 1:1000 in 16% v/v newborn goat serum in PBS, was added for 1 h at 37°C and the plate washed as before. Alkaline phosphatase substrate solution (Sigma) (100 μl/well) was added and the plate was incubated for 1 h at room temperature in the dark. Absorbance values were read at a wavelength of 405 nm by an ELISA reader.

Detection of ICAM-1 on HUVEC by IIF

Cultured HUVEC were seeded onto gelatin-coated glass cover slips at a concentration of 8×10^5 cells per slip. Confluent monolayers were allowed to form overnight. The monolayers were fixed in glutaraldehyde as before and either stained immediately or stored in 5% FBS/PBS overnight at 4°C. Coated glass cover slips were washed once with distilled water and three times in PBS. One hundred microlitres of a 1:20 dilution of mouse MoAb to human endothelial ICAM-1 were added to each coverslip. The samples were incubated at 37°C for 30 min. The monolayers were washed and incubated for a further 30 min at 37°C with FITC-conjugated rabbit immunoglobulin to mouse immunoglobulin (Dako Ltd, High Wycombe, UK). Stained coverslips were mounted in glycerol saline and examined by fluorescence microscopy.

Purification of IgG from human serum

Immunoglobulin was purified from patients by affinity chromatography using a protein G column (Pharmacia Biotec Ltd, St Albans, UK). Immunoglobulin was purified from activating and inactivating serum samples from a total of 24 patients with vasculitis. In ANCA-positive cases, elutions were analysed for the presence of ANCA by IIF and the maintenance of molecular specificity by ELISA. For five serum samples, both washes and elutions were analysed for the presence of serum proteins. For ANCA-negative samples elutions were analysed for the presence of IgG by nephelometry. In addition, purified IgG was obtained from eight patients with septicaemia and 10 normal control subjects. Both washes and elutions were collected in 1-ml fractions in tubes containing neutralizing buffer (100 μl) (1·0 M Tris–HCl pH 9·0). The purity of the IgG fractions was assessed by SDS–PAGE.

Incubation of HUVEC with sera and purified IgG from patient and normal control subjects

Endothelial cells were grown to confluence overnight on 96-well

microtitre plates as previously described. Medium (50 μl, i.e. 50%) was carefully removed from the cultures and replaced with 50 μl of filter-sterilized test and control sera and purified IgG. After 24 h (maximal expression of ICAM-1 was observed between 12 h and 24 h after stimulation with cytokines) in culture the cell monolayers were washed and immediately fixed with glutaraldehyde. Endothelial cell expression of ICAM-1 was assessed employing the ELISA method described, and visualized by IIF.

Incubation of HUVEC with MoAbs to ANCA antigens

Four mouse MoAbs raised against PR-3 (1A3, 2A3 and 6A3, a generous gift from Professor Jorgen Wieslander, Staten Seruminstitut, Copenhagen, Denmark; and 7H1, a gift from Dr E. Dermott, Royal Victoria Hospital, Belfast, UK) and one MoAb reacting with MPO (AMPO; Dako) were diluted in HUVEC feeding media, patient sera and control sera to titres of 1/80, 1/320 and 1/640, filter-sterilized, and added to HUVEC cultures which had been grown to confluence overnight on 96-well microtitre plates. Following a further 24 h in culture, monolayers were washed and fixed and assayed for ICAM-1 up-regulation by ELISA.

Incubation of HUVEC with purified ANCA antigens

Purified human PR-3 (a gift from Dr T. W Johnston, Department of Biochemistry, The Queen's University, Belfast, UK) and MPO (Sigma) were diluted in serum-free feeding media and added to HUVEC monolayers at a concentration of 1 μg/ml of PR-3 and 3 μg/ml of MPO (concentrations used for coating microtitre plates in ANCA ELISAs). After a 1-h incubation period at 37°C the medium was removed, monolayers carefully washed and incubated with serum, purified IgG samples and feeding media only, respectively, for 24 h. The cells were fixed with glutaraldehyde as described and the level of ICAM-1 expression was determined by ELISA. Monolayers were assessed for bound MPO and PR-3 after 1 h and 24 h by ELISA using MoAbs to PR-3 and MPO (diluted 1:20 PBS).

Incubation of sera and purified IgG samples with purified ANCA antigens

Purified PR-3 and MPO were diluted in serum and purified IgG samples at the concentrations listed previously and incubated for 2 h at 37°C and added to HUVEC monolayers as described. Endothelial ICAM-1 expression was assessed by ELISA.

Statistical analysis

Serum samples were considered positive for ICAM-1 up-regulation when the optical density (OD) values for ICAM-1 on endothelial cells exceeded 2 s.d. above the mean value for 45 normal controls, i.e. samples which recorded an OD value of $>0{\cdot}70$. Samples treated with purified IgG were considered positive with an OD of >3 s.d. above the mean of 10 normal controls, i.e. an OD value of $>0{\cdot}52$.

RESULTS

Purification of IgG from patients' serum

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%. The ANCA/ANA-positive IgG samples and their molecular specificities are detailed in Table 1a. 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). In one sample, the staining pattern changed from cytoplasmic to perinuclear, although the molecular specificity was unchanged. 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).

Primary vasculitis (MPA, WG, PAN)

Serum samples from seven out of 11 patients with MPA, three out of seven patients with WG and one out of two patients with PAN produced an up-regulation of ICAM-1 on HUVEC (Tables 1a and 1b). These samples were positive for C-ANCA, P-ANCA or ANA. In most cases of up-regulation, the effect was reproduced with the ANCA/ANA-positive purified IgG elutions (Tables 1a and 1b). Washes and autoantibody-negative elutions did not stimulate ICAM-1 up-regulation. Up-regulation of ICAM-1 did not occur with sera which were negative for autoantibodies. Serum samples taken at different times from the same individual did not always stimulate ICAM-1 up-regulation despite having high titre ANCA or ANA. Up-regulation of ICAM-1 by sera from a patient with MPA was visualized by immunofluorescence (Fig. 1a,b).

Secondary vasculitis (MV, SLE and RA)

Sera from four patients (Sjögren's syndrome ($n=2$), ANCA-positive vasculitis ($n=1$) and vasculitis of unknown etiology ($n=1$)) out of 11 with MV produced an up-regulation of ICAM-1 on HUVEC. The serum samples were positive either for ANA or for P-ANCA. Again, not all samples from the same patient were capable of causing endothelial up-regulation of ICAM-1, despite having high autoantibody titres. None of the sera or purified IgG from patients with RA or SLE induced ICAM-1 up-regulation on HUVEC, despite having high titre ANA (Tables 1a and 1b).

Control patients

Sera and purified IgG from patients with septicaemia did not stimulate up-regulation of ICAM-1 on HUVEC, despite the presence of cytokines or endotoxin in some samples (Tables 1a and 1b).

Reproducibility and dose response

The intra-assay variation was calculated to be 8% ($n=10$), while the interassay variation was calculated to be 16% ($n=3$). Positive samples were assayed at dilutions ranging from 1:2 to 1:1280 to establish a dose–response curve. The expression levels of ICAM-1 remained high up to dilutions of 1:160 and declined thereafter (data not shown).

Anti-endothelial cell antibodies

With the exception of one patient with RA, serum samples were free of autoantibodies to cultured HUVEC.

Monoclonal antibodies

Monoclonal antibodies to the ANCA antigens, PR-3 and MPO, failed to up-regulate ICAM-1 expression on HUVEC when added to cells with feeding medium or control serum (data not shown).

Coating of HUVEC monolayers with ANCA antigens

Coating of HUVEC monolayers with purified PR-3 and MPO did not induce or enhance ICAM-1 up-regulation on endothelial cells (Table 2). To demonstrate that the antigens remained bound to the endothelium, the binding of four MoAbs to PR-3 and one monoclonal to MPO to coated and uncoated monolayers was assessed by ELISA. The results are detailed in Table 3. Anti-PR-3 MoAbs

Table 1. a. 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^+	Number IL-1β^+	Number LPS$^+$	Number P-ANCA$^+$	Number anti-MPO$^+$	Number C-ANCA$^+$	Number anti-PR-3$^+$	Number anti-(EL, LF, CG)$^+$	Number ANA$^+$
MPA	11	Sera	24	0	0	0	2	2	16	12	0	3
		IgG	17	–		–	0	0	11	4	–	–
WG	7	Sera	13	0	0	0	0	0	8	7	0	0
		IgG	11	–		–	–	–	6	7	–	–
PAN	2	Sera	3	0	0	0	3	3	0	0	1 (LF)	0
		IgG	3	–		–	–	–		–	–	–
MV	11	Sera	16	0	0	0	5	3	3	0	0	4
		IgG	6	–		–	1	1	0	0	–	4
SLE and RA	6	Sera	7	0	0	0	0	0	0	0	0	7
		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 erythematosus; LPS, endotoxin; PR-3, proteinase 3; MPO, myeloperoxidase; LF, lactoferrin; CG, cathepsin G; EL, elastase; –, not tested.

Table 1. b. The up-regulation of ICAM-1 on human umbilical vein endothelial cells (HUVEC) 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 ICAM-1 on HUVEC to >2 s.d. above the mean of 45 normal controls (OD)					
				Number P-ANCA$^+$	Number anti-MPO$^+$	Number C-ANCA$^+$	Number anti-PR-3$^+$	Number ANA$^+$	Number autoantibody-negative
MPA	11	Sera	24	1	1	9	7	1	0
		IgG	17	0	0		5	0	0
WG	7	Sera	13	0	0	4	3	0	0
		IgG	11	0	0		3	0	0
PAN	2	Sera	3	1	1	0	–	0	0
		IgG	3	1	1		–	0	0
MV	11	Sera	16	1	1	0	–	3	0
		IgG	6	1	1		–	0	0
SLE and RA	6	Sera	7	0	–	0	–	0	0
		IgG	1	0	–		–	0	0
Septicaemia	10	Sera	10	0	–	0	–	0	0
		IgG	8	0	–	0	–	0	0
Normal control	45	Sera	45	0	–	0	–	0	0
		IgG	10	0	–	0	–	0	0

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

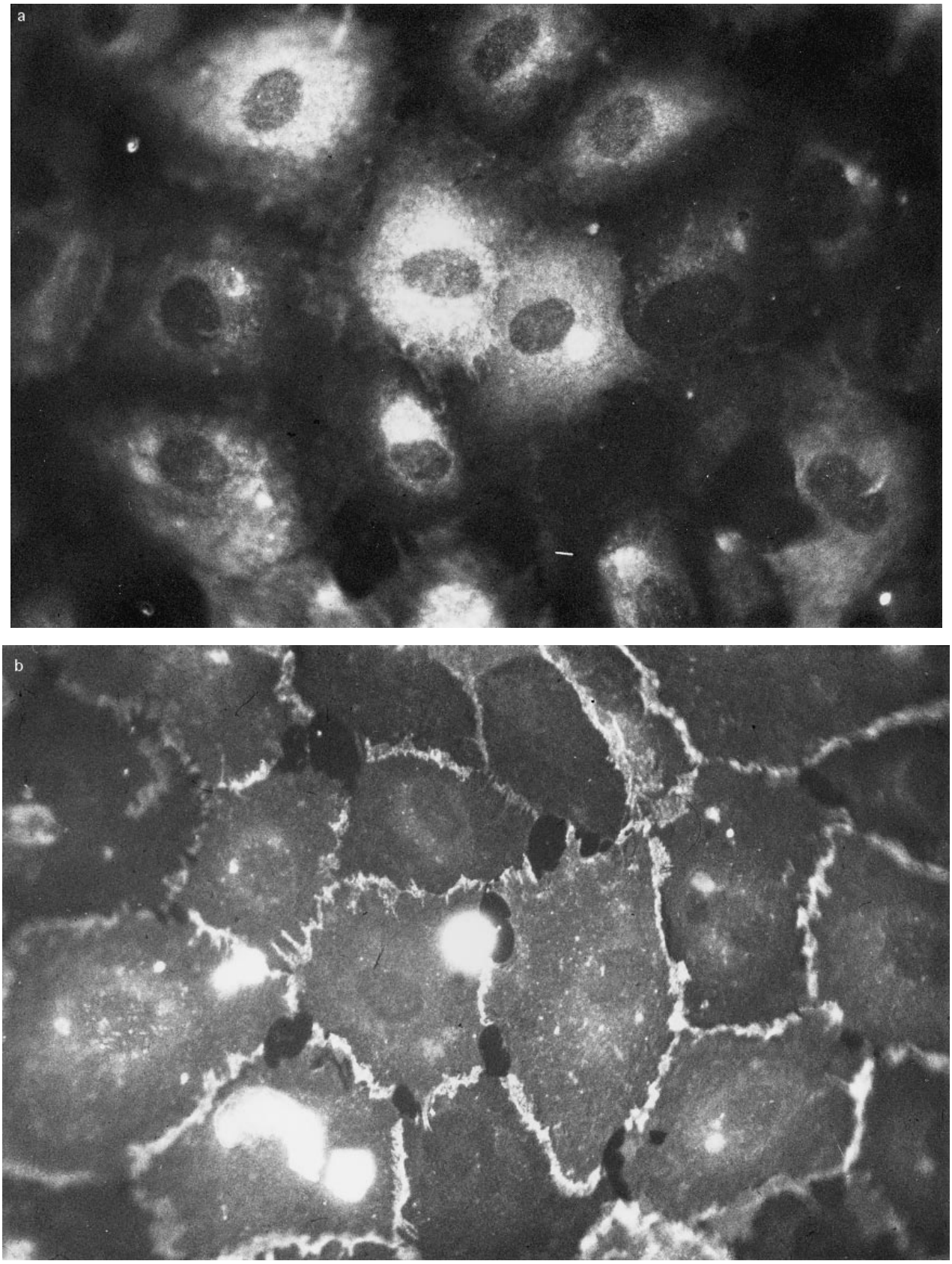

Fig. 1. ICAM-1 expression on (a) resting human umbilical vein endothelial cells (HUVEC) and (b) HUVEC incubated with sera containing C-ANCA from a patient with microscopic polyarteritis demonstrating adhesion molecule up-regulation.

bound to HUVEC which were coated with PR-3, but did not react with untreated cells, or with cells which had been coated with MPO. The level of binding diminished with time. However, the MoAbs still bound to coated cells after 24 h incubation. Anti-MPO MoAbs demonstrated only slight anti-HUVEC activity after coating with MPO. They did not react with untreated cells or cells coated with PR-3 (Tables 2 and 3).

Inhibition studies with purified ANCA antigens

Purified ANCA antigens PR-3 and MPO did not interfere with the up-regulation of ICAM-1 on HUVEC by sera or purified IgG from patients with ANCA. The purified PR-3 had previously been shown to inhibit the binding of anti-PR-3 antibodies in sera from patients with vasculitis to PR-3-coated microtitre plates in an ELISA (data not shown).

Table 3. The binding of anti-myeloperoxidase (MPO) and anti-proteinase 3 (PR3) monoclonals to MPO- and PR-3-coated human umbilical vein endothelial cells (HUVEC) (optical density read at 405 nm)

	HUVEC+ MPO		HUVEC+ PR-3		Untreated HUVEC	
Antibody	1 h	24 h	1 h	24 h	1 h	24 h
AMPO	0·435	0·243	0·098	0·186	0·192	0·086
1A3	0·085	0·017	0·631	0·488	0·007	0·000
2A3	0·015	0·063	0·831	0·522	0·076	0·052
6A3	0·002	0·018	0·925	0·327	0·010	0·034
7HI	0·041	0·009	0·926	0·611	0·025	0·036

DISCUSSION

This study demonstrates that ANCA-positive sera and purified IgG from patients with vasculitis up-regulate ICAM-1 on the surface of HUVEC. The effect was seen with both classes of antibody, C-ANCA and P-ANCA, and with ANCA-negative/ANA-positive samples. Sera and purified IgG samples from patients with secondary vasculitis (RA and SLE) did not up-regulate ICAM-1 on HUVEC despite the presence of high-titre ANA.

The mechanism of endothelial activation by ANCA and ANA is not clear. It has recently been demonstrated that anti-endothelial cell antibodies (AECA) from patients with WG can stimulate adhesion molecule up-regulation on endothelial cells [22]. In contrast to some previous reports [23–30], we found little evidence for AECA in patients with vasculitis, and activation of the HUVEC is unlikely to be due to strong anti-endothelial cell activity. In these earlier reports of AECA in patients with vasculitis, an AECA ELISA employing cells cultured on gelatin-coated surfaces was utilized [23–30]. Cross-reactivity between AECA and gelatin-coated surfaces leading to high background values has recently been described [31]. In our initial efforts to establish an AECA ELISA a similar problem of sera cross-reactivity with gelatin was observed. To eliminate high background values a rigorous AECA ELISA was developed employing endothelial cells grown on fibronectin-coated surfaces. This ELISA may not have identified weak anti-endothelial cell activity and may partly explain the apparent disparity between the low level of AECA in our study *versus* the earlier reports. Recently, Chan and co-workers demonstrated that anti-DNA (ANA) antibodies may act as AECA and that binding to the endothelium is enhanced during active disease compared with identical concentrations of ANA-positive serum samples obtained from patients during remission [32]. This suggests that ANA may interact with endothelial cells and that weak interactions may not always be identified by AECA assays. They propose that enhancement of cellular binding by ANA during disease exacerbation is due to increased avidity of binding to the same set of antigenic determinants. This may explain why in this study not all autoantibody-positive samples taken from the same patient retained the capacity to induce ICAM-1 up-regulation on endothelial cells.

Table 2. The effect of sera and IgG on ICAM-1 expression after incubation of human umbilical vein endothelial cells (HUVEC) (optical density read at 405 nm)

Sample number		Untreated HUVEC	HUVEC +MPO	HUVEC +PR-3
MPA 5	Sera	1·421	1·261	1·006
	IgG	1·231	1·007	1·153
MPA 6c	Sera	1·362	1·115	1·059
	IgG	0·942	1·016	0·817
PAN 3c	Sera	0·983	0·825	0·996
	IgG	0·759	0·711	0·821
MPA 6a	Sera	0·432	0·392	0·315
	IgG	0·236	0·184	0·217
WG 5d	Sera	0·436	0·380	0·336
	IgG	0·125	0·205	0·163
AMPO		0·210	0·258	NT
1A3		0·182	NT	0·256
2A3		0·203	NT	0·199
6A3		0·2	NT	0·253
7HI		0·295	NT	0·301

NT, Not tested.

Mayet and co-workers demonstrated that purified anti-PR-3 IgG from patients with WG induced an up-regulation of the adhesion molecules E-selectin and vascular cell adhesion molecule-1 (VCAM-1) on the surface of HUVEC [33,34]. They suggest that activation occurs by binding of anti-PR-3 antibodies to PR-3 on the surface of the endothelial cells. However, others have been unable to demonstrate the expression of PR-3 by endothelial cells [35]. In addition, the production of PR-3 by endothelial cells has been observed only in cytokine-activated cells [36], yet the cells in this study were not primed with cytokines and were in the resting state prior to incubation with sera and purified IgG samples. Clearly, some cellular activation would need to occur initially, irrespective of PR-3 production by the HUVEC. Furthermore, this theory does not account for the activation of the HUVEC monolayers by P-ANCA (anti-MPO) sera and IgG samples. As yet there is no evidence for the production of MPO or other ANCA antigens by endothelial cells.

Recent studies have demonstrated that both MPO and PR-3 can, by virtue of their positive charge, bind to the surface of the endothelium, thus presenting molecular targets for ANCA [16,17]. In order to investigate further the nature of this cellular activation, the endothelium was coated with the antigens MPO and PR-3. Up-regulation by the sera or IgG components was neither induced nor enhanced, suggesting that activation may not merely be due

to ANCA reacting with endothelial-bound antigens. Preincubation of the sera and IgG fractions with the purified antigens did not interfere with the ability of the samples to up-regulate endothelial ICAM-1, suggesting that the molecular specificity of the autoantibody may not be involved in the activation process. Alternatively, disassociation of the antibody–antigen complex during incubation may have occurred. However, the purified PR-3 was previously shown to inhibit binding of anti-PR-3 antibodies in patients' serum in anti-PR-3 ELISAs (Dr T Johnston, Department of Biochemistry, The Queen's University of Belfast, personal communication). MoAbs to the ANCA antigens failed to induce endothelial ICAM-1 up-regulation. This would again suggest that the molecular specificity of the antibody is not the only factor in cellular activation. To investigate this further we made an intensive effort to cleave the intact IgG molecules into Fc and $F(ab)_2$ fragments, but we experienced technical difficulties in recovering sufficient mass of IgG fragments following pepsin cleavage and affinity chromatography. Unfortunately, we were unable to demonstrate whether the $F(ab)_2$ or Fc portion of the autoantibody molecule is primarily responsible for inducing ICAM-1 up-regulation.

Keogan and co-workers suggested that ANCA may activate neutrophils by simply cross-reacting with a receptor involved in neutrophil activation [37]. It may be possible that similar receptors exist on endothelial cells. Furthermore, it is possible that patients develop autoantibodies with epitopes other than those involved in antigen binding which interact with endothelial receptors, causing cellular activation. However, for this to be the case the interaction must be a weak one, otherwise these ANCA would act as AECA.

An alteration in the predominant subclass of the antibody may account for variation in the ability of ANCA/ANA to up-regulate ICAM-1 expression. A recent study has suggested that the subclass of the antibody is important in cellular activation, and describes a link between stimulating capacity and ANCA of subclass IgG3 [38]. A variation in affinity of ANCA may explain the altered ability of ANCA from certain individuals to up-regulate ICAM-1 expression, if activation was associated with the molecular specificity of the antibody. A prospective study of patients presenting with vasculitis would permit an assessment of temporal changes in the ability of ANCA from an individual patient to stimulate adhesion molecule up-regulation. It will be of interest to correlate the effects of ANCA on adhesion molecule expression *in vitro* with the perceived clinical activity of the disease and the amount and duration of immunosuppressive therapy.

This study also raises interesting questions as to the exact mechanism of ICAM-1 up-regulation. Autoantibodies may have a direct ability to increase endothelial ICAM-1 expression independent of cytokines. In our investigations, HUVEC were not primed with cytokines and endothelial ICAM-1 up-regulation was observed within hours of incubation with autoantibody-containing sera. Alternatively, the effects of ANCA may be indirect and mediated by ANCA-induced cytokine release from endothelial cells. Such endothelial cell-derived cytokines could then operate in an autocrine fashion to stimulate ICAM-1 expression.

As with all *in vitro* studies, one cannot assume that the findings demonstrated here reflect the pathophysiological process occurring *in vivo*. Binding or interaction of these autoantibodies with HUVEC in culture may not be synonymous with binding to arterioles or capillaries *in vivo*. In addition, the use of endothelial cells which have been isolated from large vessels and serially passaged in culture may not reflect the *in vivo* situation, as the phenotype of the cells may change with time in culture. Nevertheless, the *in vitro* model of ANCA sera interactions with endothelial cells has generated interesting data directly implicating these autoantibodies in adhesion molecule up-regulation.

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