

# IgG Antiendothelial Cell Autoantibodies from Scleroderma Patients Induce Leukocyte Adhesion to Human Vascular Endothelial Cells In Vitro

## Induction of Adhesion Molecule Expression and Involvement of Endothelium-derived Cytokines

Dulce Carvalho,\* Caroline O. S. Savage,† Carol M. Black,§ and Jeremy D. Pearson\*

\*Vascular Biology Research Centre, Biomedical Sciences Division, King's College, Kensington, London W8 7AH; †Vascular Immunobiology Group, Centre for Clinical Research in Immunology and Signalling, University Medical School, Edgbaston, Birmingham B15 2TT; and §Department of Rheumatology, Royal Free Hospital Medical School, London NW3 2QG, United Kingdom

### Abstract

IgG autoantibodies that bind human endothelial cells (AECA) were detected by ELISA in 30 of 42 samples of sera from patients with scleroderma. Pretreatment of human umbilical vein endothelial cells with AECA-positive scleroderma sera, or IgG purified from these sera, led to a dose- and time-dependent increase in the ability of the cells to bind human U937 monocytic cells. Threshold-active IgG concentrations were 1–10 µg/ml; effects were significant after 3 h and maximal after 6–12 h. IgG from AECA-negative sera or normal sera were without effect. Increased adhesion of U937 cells was accompanied by increased expression of endothelial intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin. Transfer of endothelial cell-conditioned media after pretreatment with AECA and immunodepletion of IgG demonstrated the presence of transferable activity that mimicked the effects of AECA. Treatment with neutralizing anticytokine antibodies indicated that IL-1, generated by the endothelial cells in response to AECA, was involved in the upregulation of adhesion molecules and U937 cell adhesion. We conclude that AECA can play a pathogenic role in scleroderma by activating endothelial cells, in part due to autocrine or paracrine actions of IL-1. (*J. Clin. Invest.* 1996. 97:111–119.) **Key words:** vascular damage • inflammation • immunology • connective tissue disease • mononuclear cells

### Introduction

The pathogenesis of scleroderma (systemic sclerosis; SSc)<sup>1</sup> is poorly understood. It is a connective tissue disease, character-

ized by progressive fibrosis, with a major involvement of small blood vessels, notably arterioles and capillaries, with leukocyte infiltration and damage (1). The almost invariable preexistence of Raynaud's phenomenon in SSc patients, together with the early pathological features, strongly suggests vascular dysfunction as a primary cause, as first proposed by Campbell and LeRoy in 1974 (2). In more recent years, the increasing knowledge of the roles of the endothelium in controlling vascular homeostatic functions, including vessel tone, hemostasis, and leukocyte traffic, has enhanced the search for evidence of endothelial dysfunction in the pathogenesis of SSc.

Circulating autoantibodies (anti-endothelial cell antibodies; AECA) that bind to human endothelial cells cultured in vitro have been detected in a variety of autoimmune diseases with vascular pathology, including SSc (reviewed in reference 3). AECA are distinct from the hallmark autoantibodies of these diseases, e.g., antiDNA in SLE or anti-Scl70 in SSc, and are apparently directed at heterogeneous endothelial antigens both between patients and in individual sera (4). With few exceptions, notably in Kawasaki disease, where AECA are directly cytotoxic to endothelial cells in the presence of complement and recognize cytokine-upregulated antigenic determinants, AECA have not been found to mediate complement-dependent endothelial cell damage (5–7). Thus, IgG and/or IgM AECA, which are detectable in 30–50% of SSc sera, are not directly cytotoxic to endothelial cells (4, 8–11). Although in a small fraction of cases SSc sera can induce lymphocyte-mediated endothelial cell killing (8, 10, 11), it remains unclear whether AECA play a direct role in causing endothelial dysfunction in SSc or merely represent clinical markers of disease activity or progression.

Several studies in the 1980s attempted to detect direct effects of AECA on endothelial cell functions, including release of vasoactive mediators such as prostacyclin and vWF, generally with equivocal or negative results (reviewed in reference 12). However, Tannenbaum et al. (13) and Hasselaar et al. (14) both reported that sera containing endothelial cell-binding antibodies could induce, or synergize with cytokines to induce, the production of procoagulant tissue factor by endothelial cells in vitro.

Endothelial cell activation by cytokines such as IL-1 and TNF or bacterial endotoxin (LPS) leads to the acquisition of a spectrum of changes in phenotype, notably the upregulation of leukocyte adhesion molecules intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin (15, 16). Furthermore, activated endothelial cells produce leukocyte chemoattractants and costimulatory signals for lymphocyte activation, and thus may be able to initiate or amplify inflammatory injury (17, 18).

Address correspondence to Jeremy D. Pearson, Vascular Biology Research Center, Biomedical Sciences Division, King's College, Kensington, London W8 7AH, UK. Phone: 44-171-333-4083; Fax: 44-171-333-4008.

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1. *Abbreviations used in this paper:* AECA, antiendothelial cell antibodies; ER, ELISA ratio; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; SSc, systemic sclerosis (scleroderma); VCAM-1, vascular cell adhesion molecule-1.

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Mononuclear cell infiltration across the vessel wall is prominent in SSc lesions (19–21). In vitro studies have found that PBMCs from SSc patients adhere abnormally to endothelial cells with an overall reduction in binding but with enhanced binding of a subfraction of activated cells; this is consistent with the concept that lymphocyte traffic across endothelium is chronically upregulated in SSc lesions and leads to a depletion of circulating responsive cells (22). There is now considerable recent evidence for enhanced expression of adhesion molecules on endothelium in SSc patients, both within lesions and at clinically uninvolved sites (23–26), together with increased circulating levels of soluble forms of adhesion molecules and other markers of endothelial activation or damage, including vWF, endothelin-1, and thrombomodulin (27–33).

We therefore undertook the current in vitro study to determine the effects of purified IgG AECA from SSc patients on the adhesion of leukocytes to endothelial cells.

## Methods

**Sources of human sera.** Sera were obtained from healthy normal volunteers ( $n = 24$ ) and from patients with systemic sclerosis ( $n = 42$ ), each of whom fulfilled the diagnostic criteria of the American Rheumatism Association. Sera were collected and stored at  $-20^{\circ}\text{C}$  until use.

**Cell culture.** Human umbilical vein endothelial cells (HUVEC) were isolated as previously described (34) and cultured under standard conditions (35). The cells were used at passage two and plated onto gelatin-coated 96-well microtiter plates. Cells were cultured for 72–96 h before use. U937 cells were maintained in RPMI 1640 containing penicillin (100  $\mu\text{g}/\text{ml}$ ), streptomycin (100 U/ml), and 4 mM glutamine and supplemented with 5% FCS.

**ELISA for AECA.** The sera were screened for the presence of AECA using an ELISA that has previously been described in detail (4). Briefly, serum samples were diluted 1:400 in PBS containing 1% BSA, fraction V, and incubated for 60-min at room temperature with HUVEC which had previously been fixed with 0.1% glutaraldehyde. The bound IgG was detected by a further 60-min incubation with peroxidase-conjugated rabbit anti-human IgG (1:500; Dako, High Wycombe, UK), with subsequent quantification of peroxidase using *o*-phenylenediamine dihydrochloride and  $\text{H}_2\text{O}_2$  in citrate-phosphate buffer, pH 5. Each plate always had a blank value obtained from the optical density given by the diluting medium, a standard highly positive control sample from a patient with SSc and a negative control comprised of pooled serum from 24 normal laboratory personnel. Results were expressed as an ELISA ratio (ER) calculated as  $\text{ER} = 100 \times (S - A)/(C - A)$ ; where  $S$  is the absorbance of the sample, and  $A$  and  $C$  are the absorbances of the negative and positive controls. Values are the means of three replicate determinations.

Purified IgG were prepared by affinity chromatography using protein A columns. Fab fragments were obtained by incubating whole IgG with immobilized papain overnight at  $37^{\circ}\text{C}$  followed by rechromatography on protein A columns. The purity of both fractions (Fab and IgG) was assessed by standard SDS-PAGE, and the total protein was determined by light absorbance at 280 nm. Purified IgG and Fab fractions were concentrated and dialyzed against PBS and stored at  $4^{\circ}\text{C}$  while in use or at  $-20^{\circ}\text{C}$  when stored for long periods ( $> 3$  wk). These molecules were used in ELISAs, performed on glutaraldehyde- (0.1%) fixed HUVEC. IgG and Fab binding were detected using Fab-specific mouse anti-human IgG (1:10,000; ICN, Thame, UK).

Concomitantly, other ELISAs were performed to investigate the blocking effect of Fab fragments on whole IgG binding, in which Fab fragments were preincubated with fixed HUVEC for 60 min before addition of IgG. Binding of IgG was then assessed using an Fc-specific mouse anti-human IgG (1:5,000; ICN).

Controls in the ELISAs included anti-HLA IgG (positive) (Dako), omission of the primary IgG/Fab molecules (blank value), and mouse IgG (negative) (Dako). The results were expressed as optical densities after subtracting the blank value.

**Adhesion assay.** This assay was performed as described by Simmons and Needham (36). Briefly, U937 cells ( $2 \times 10^5$  cells/ml) were labeled with 0.075  $\mu\text{Ci}/\text{ml}$  of [ $^3\text{H}$ ]thymidine (Amersham International, Little Chalfont, UK) for 24 h. On the day of the experiment, the cells were washed three times and resuspended in prewarmed RPMI 1640 containing 20 mM Hepes and 0.2% BSA (adhesion medium). After removing the conditioned medium from the wells by aspiration, the endothelial cell monolayers were washed three times with the adhesion medium and  $2 \times 10^5$  radiolabeled U937 cells (in 0.1 ml) were added to each well and coincubated for 30 min at  $37^{\circ}\text{C}$ . The nonadherent cells were removed by a carefully standardized washing procedure, three times with PBS containing 2.5% heat-inactivated FCS. The radioactivity associated with adherent cells was quantified by  $\beta$  scintillation spectrometry after lysis with formic acid. During the assay, HUVEC integrity and U937 cell adherence were always confirmed by light microscopy. The results were expressed as percentage of added U937 cells that adhered and are presented as the means  $\pm$  SEM from not less than three replicate wells.

In some experiments, radiolabeled U937 cells were preincubated with 20 mg/ml of heat-aggregated gammaglobulin (Hyland Gamma-gard; Baxter Healthcare, Glendale, CA) for 30 min at  $37^{\circ}\text{C}$  before performing the adhesion assay, to block Fc receptors (37). In other experiments, U937 cells, cultured at an initial cell density of  $2.5 \times 10^5$  cells/ml, were pretreated for 48–72 h with 100 U/ml of recombinant human IFN- $\gamma$  (Serotec Ltd., Oxford, UK) to upregulate Fc receptor numbers (38).

Adhesion assays were performed on HUVEC monolayers which were pretreated with human sera, purified IgG, or Fab fragments diluted in medium 199 containing penicillin (100  $\mu\text{g}/\text{ml}$ ), streptomycin (100 U/ml), 4 mM glutamine, and 5% heat-inactivated FCS for 30 min over 36 h. In some experiments, cycloheximide (10  $\mu\text{g}/\text{ml}$ ) or polymixin B sulphate (100  $\mu\text{g}/\text{ml}$ ) were coincubated with purified IgG. Experiments were also performed in which HUVEC were pretreated with conditioned media that had previously been incubated with other HUVEC monolayers (see below). Adhesion experiments always included negative controls (medium alone, medium with pooled normal human sera, or IgG) and a positive control (LPS from *Escherichia coli*, 1  $\mu\text{g}/\text{ml}$ ).

**ELISAs for detection of adhesion molecule expression.** HUVEC monolayers were pretreated exactly as for the adhesion experiments and then fixed with 0.1% glutaraldehyde for 10 min at  $4^{\circ}\text{C}$ . Fixed cells were preincubated with 10% nonfat dried milk diluted in PBS for 1 h at room temperature to reduce nonspecific binding. Mouse mAbs against human E-selectin, ICAM-1, or VCAM-1 (1D2, 11C8-I, 4B2; British Biotechnology, Oxford, UK) (1  $\mu\text{g}/\text{ml}$ ) were then incubated for a further hour. Bound antibodies were detected by a peroxidase-conjugated rabbit anti-mouse Ig (Dako) and quantified as in the AECA ELISA. Controls included incubation with an irrelevant isotype-matched mouse mAb and omission of the primary antibody (blanks) and mouse anti-HLA class I (positive control). The results were expressed as optical density units after subtraction of blank values.

**Depletion of IgG from conditioned media.** Human IgG were removed from conditioned media and obtained from AECA-treated HUVEC by immunoprecipitation with protein A-Sepharose CL4B which had previously been coated with rabbit anti-human IgG (I-2011; Sigma Chemical Co., Poole, UK). Immunodepletion was assessed by Western blotting. Samples were run on 12% SDS-PAGE, and proteins were transferred to nitrocellulose (BA85; Schleicher & Schuell, Dassel, Germany). The blot was blocked with 5% nonfat dried milk powder in buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20; TBST) for 1 h and incubated with rabbit anti-human IgG (0.5  $\mu\text{g}/\text{ml}$ ) (Promega Corp., Southampton, UK) for a further 1 h. After five washes in TBST the blot was incubated with alka-

line phosphatase-conjugated swine anti-rabbit Ig (1:1000, Dako). Human IgG was visualized on the blot by colorimetric detection using 0.33 mg/ml nitro blue tetrazolium (Promega Corp.) and 0.16 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (Promega Corp.) in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl<sub>2</sub>. This procedure was carried out with each batch of immunodepleted, conditioned medium, which was not used in subsequent experiments unless no immunoreactivity was observed.

**Blocking antibodies to cytokines.** In several experiments, blocking antibodies against IL-1 $\alpha$ , IL-1 $\beta$ , and/or TNF- $\alpha$  were added concomitantly with purified IgG to HUVEC or to conditioned media incubated with HUVEC. Goat anti-human IL-1 $\alpha$  was purchased from R&D Systems Inc. (Minneapolis, MN), and used at 1  $\mu$ g/ml. Sheep anti-human IL-1 $\beta$  (a gift from Dr. Steve Poole, National Institute for Biological Standards and Control, South Mimms, UK) was used at 1:2,500. Sheep anti-human TNF- $\alpha$  (a gift from Robert Forder, Zeneca, Macclesfield, UK) was used at 1:1,000. At these concentrations, these antibodies abolished the ability of each cytokine (at 100 U/ml) to induce adhesion molecule expression or increased U937 cell adhesion to HUVEC. Other antibodies used in experiments reported in this paper were EN4 (Serlab, Crawley Down, UK) and anti-CD59 (British Biotechnology).

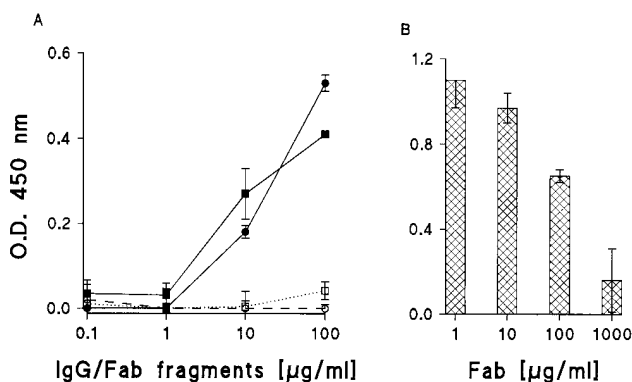
**Statistical analysis.** Statistical analysis was carried out using unpaired Student's *t* tests.

## Results

**Detection of IgG AECA.** Sera were routinely diluted 1:400 to detect AECA, since in preliminary studies it was found that this dilution gave the best discrimination between binding activity in the reference serum and in pooled normal human serum, although it was possible to detect IgG binding in positive samples with dilutions of up to 1:2,000. 25 individual normal sera were tested and gave an ER value of  $1.6 \pm 5.4$ . No normal serum sample had an ER value greater than the mean + 3 SD (ER = 18), which was used as the lower limit for detecting positive binding. With this criterion, 30 of the 42 SSc samples contained significant levels of AECA, with ER values ranging from 19 to 123%.

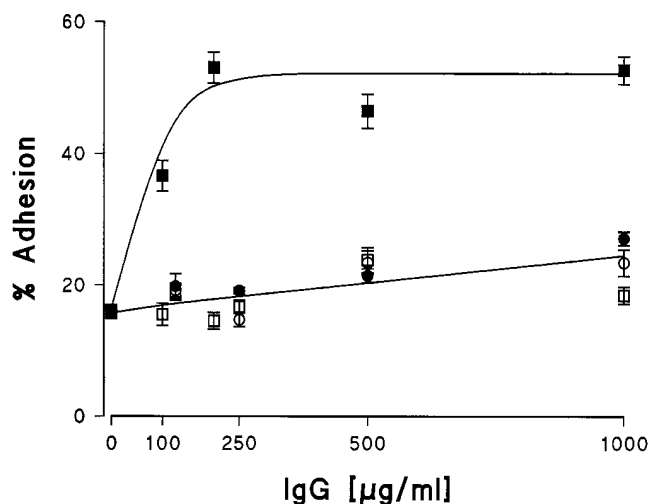
To check that, as reported previously (4), AECA binding was mediated via the Fab portion of IgG, five SSc sera, either IgG AECA positive (Nos. 9, 11, and 39, ER values 100, 103, and 92%) or negative (Nos. 3 and 20, ER values 13 and 18%) and two control sera were studied further to characterize the mechanism of IgG binding. Fig. 1 A illustrates that whole IgG or Fab fragments from AECA-positive sera bound to HUVEC in a dose-dependent manner, whereas IgG from AECA-negative sera or normal human serum did not. In addition, pretreatment of fixed HUVEC with increasing concentrations of Fab fragments (1–1,000  $\mu$ g/ml) from AECA-positive samples dose-dependently inhibited subsequent binding of whole IgG (100  $\mu$ g/ml) from the same sample (Fig. 1 B). No binding of Fab fragments (added at up to 2 mg/ml) obtained from AECA-negative SSc sera or from control sera was detected (not shown).

**Effects of SSc sera on leukocyte adhesion to HUVEC.** HUVEC monolayers were pretreated with either AECA-positive sera, AECA-negative sera, or pooled normal human serum before rinsing thoroughly and carrying out adhesion assays. AECA-positive sera, but not AECA-negative sera or normal serum, induced increased adhesion of U937 cells in a dose and time dependent manner. For example, pretreatment with SSc serum No. 32 (ER = 66) at a concentration of 25% for 12 h led to  $46.3 \pm 1.9\%$  adherent U937 cells in the 30 min



**Figure 1.** AECA-positive IgG binding to endothelial cells is Fab mediated. (A) Binding of Fab fragments or whole IgG to fixed endothelial cells was measured by ELISA, with a Fab-specific anti-IgG. Results show means  $\pm$  SEM of three observations. (●) IgG from SSc serum No. 9, ER = 100; (■) Fab fragments from the same IgG; (○) IgG from normal human serum; (□) IgG from AECA negative serum No. 30, ER = 6. (B) Dose-dependent inhibition of binding of whole IgG (100  $\mu$ g/ml; from SSc serum No. 11, ER = 103) to fixed endothelial cells after preincubation for 90 min with Fab fragments from the same IgG. Detection of whole IgG was with an Fc-specific anti-IgG. Results show means  $\pm$  SEM from three observations. Equivalent results were obtained in five other experiments and with two other AECA-positive IgG.

adhesion assay, whereas under the same conditions pretreatment with SSc serum No. 5 (ER value = 20) and with control serum led to  $18.4 \pm 0.8$  and  $23.4 \pm 2.2\%$  adhesion, respectively, not significantly different from controls ( $22.5 \pm 1.9\%$ ). The increased adhesion caused by pretreatment with AECA-positive sera was not usually significant with < 3 h pretreatment, was maximal at  $\sim$  6 h, and was still evident at 24 h (data not shown). Since increased adhesion of U937 cells was only ob-



**Figure 2.** Pretreatment of endothelial cells with IgG from AECA-positive but not from AECA-negative or normal sera, dose-dependently induces leukocyte adhesion. Endothelial cells were preincubated with IgG for 6 h. After rinsing, U937 cells were added and adhesion was measured after 30 min. Results are expressed as a percentage of added cells that adhered and show means  $\pm$  SEM from four to six observations. (■) IgG from SSc serum No. 38, ER = 120; (●) IgG from SSc serum No. 35, ER = 18; (○) IgG from SSc serum No. 3, ER = 5; (□) IgG from normal serum.

**Table 1. AECA-induced Leukocyte Adhesion to Endothelial Cells Requires Endothelial Protein Synthesis and Is Not Attributable to Endotoxin**

	IgG from normal sera	AECA positive SSc IgG	Endotoxin
	100 $\mu\text{g/ml}$	100 $\mu\text{g/ml}$	1 $\mu\text{g/ml}$
A	0.90 $\pm$ 0.10	2.37 $\pm$ 0.12*	2.93 $\pm$ 0.10*
+ polymixin B (100 $\mu\text{g/ml}$ )	0.99 $\pm$ 0.06	2.43 $\pm$ 0.16*	1.25 $\pm$ 0.11
B		1.69 $\pm$ 0.05*	1.67 $\pm$ 0.04*
+ cycloheximide (10 $\mu\text{g/ml}$ )		1.10 $\pm$ 0.03	0.94 $\pm$ 0.04

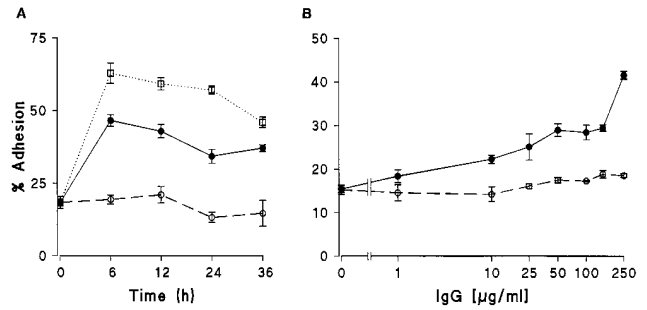
Endothelial cells were pretreated for 6 h with IgG from normal human serum, AECA-positive IgG, or endotoxin; in the absence or presence of polymixin B or cycloheximide. After rinsing, U937 cells were added and adhesion was measured after 30 min. Results are expressed relative to adhesion to untreated endothelial cells (1.00), and show means $\pm$ SEM from five (A) or four (B) observations. \*Indicates significantly higher than 1.00,  $P < 0.05$  by unpaired  $t$  test. AECA-positive IgG in (A) was from serum No. 21, ER = 43; in (B) No. 9, ER = 100. Similar results were obtained in other three experiments.

tained with AECA-positive sera, further experiments were carried out with purified IgG.

**Effect of purified IgG AECA on leukocyte adhesion.** An example of the dose-dependent increase in U937 cell adhesion after pretreatment of HUVEC for 6 h with 0–1,000  $\mu\text{g/ml}$  IgG is shown in Fig. 2. AECA-negative sera had no effect. Table I illustrates that the effects of AECA-positive IgG required endothelial protein synthesis, since they were blocked by concurrent addition of cycloheximide (10  $\mu\text{g/ml}$ ), and were not due to contamination with LPS, since polymixin B (100  $\mu\text{g/ml}$ ) did not alter the ability of IgG to enhance adhesion but blocked a similar increase in adhesion due to authentic LPS. (Purified IgG samples were checked for possible LPS contamination with the Limulus assay: none had  $> 15$  ng/ml LPS, which is below the threshold for inducing increased U937 cell adhesion.)

U937 cells possess low levels of Fc receptors. To check that these were not significantly contributing to the ability of U937 cells to bind to IgG-treated HUVEC, adhesion was measured after preincubation of U937 cells with heat-aggregated IgG (to block Fc receptors) or IFN- $\gamma$  (to upregulate Fc receptors) as described in Methods. Adhesion was unaltered by these treatments (80 $\pm$ 4.3 and 69 $\pm$ 3.8% adhesion, respectively; adhesion of untreated U937 cells 67 $\pm$ 4.5%).

The effect of IgG AECA pretreatment of HUVEC on U937 cell adhesion was dose and time dependent (Fig. 3). The minimally effective concentration of IgG was between 1 and



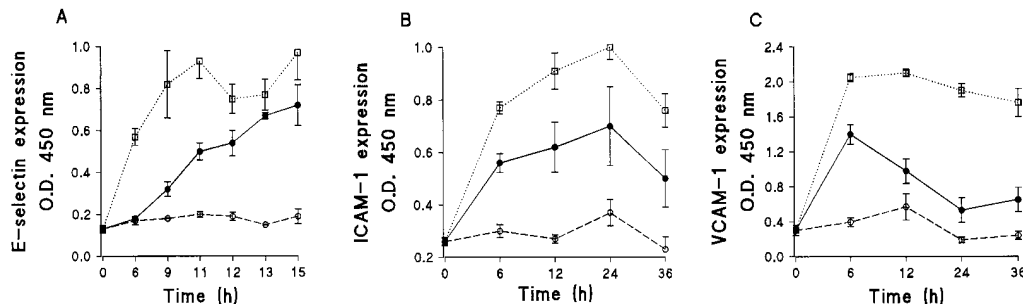
**Figure 3.** Time and dose dependence of induction of leukocyte adhesion by pretreatment of endothelial cells with IgG from AECA-positive serum. (A) Endothelial cells were pretreated for up to 36 h with IgG (100  $\mu\text{g/ml}$ ) from either SSc serum No. 9 (●; ER = 100) or control serum (○), or with endotoxin (□; 1  $\mu\text{g/ml}$ ). After rinsing, U937 cells were added and adhesion was measured after 30 min. (B) Endothelial cells were pretreated for 12 h with IgG from SSc serum No. 9 (●; ER = 100) or IgG from normal human serum (○), before U937 cell adhesion was measured as in A. Results show means $\pm$ SEM of three (A) or four (B) observations. These results are representative of those found in more than six similar experiments and with five different sera.

10  $\mu\text{g/ml}$ , and maximum adhesion was obtained after 6–12 h pretreatment, as with AECA-positive whole sera.

To determine whether the effect of IgG AECA could be replicated by pretreatment with antibodies known to bind to HUVEC surface antigens, HUVEC were pretreated for 6 h with IgG antibodies to HLA class I, ICAM-1, EN4, or CD59 (all at 1–500  $\mu\text{g/ml}$ ). None enhanced U937 cell adhesion (data not shown).

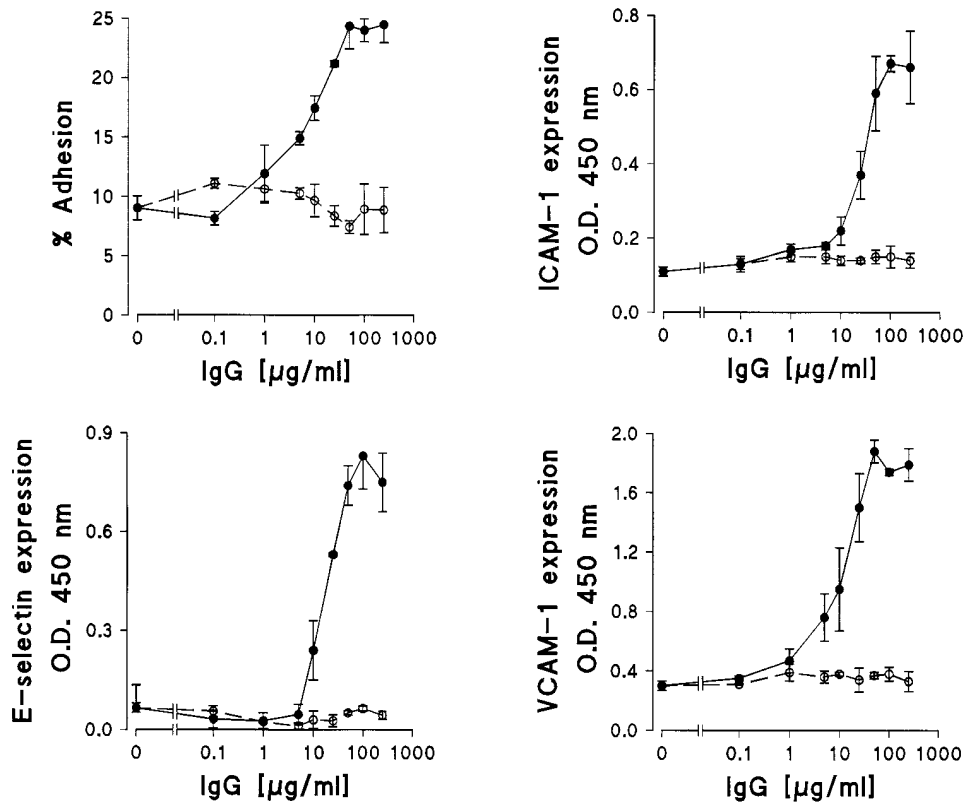
**Effects of IgG AECA on endothelial cell adhesion molecule expression.** Fig. 4 shows that the enhanced adhesion of U937 cells to endothelial cells after pretreatment with AECA-positive IgG from SSc sera was accompanied by increased expression of E-selectin, ICAM-1, and VCAM-1. Three experiments are shown in Fig. 4, each illustrating a different adhesion molecule, performed on different days, with a different batch of HUVEC, and two different sera (No. 9 in A and C, and No. 38 in B). Expression of each of the three endothelial cell adhesion molecules followed a similar time course, in any given experiment. One feature that is clear (Fig. 4 A) and was often seen was that the rate or time to reach maximal expression could be slower than when adhesion molecule expression was enhanced by LPS.

Fig. 5 shows an example of an experiment where the dose-dependent increases in U937 cell adhesion and adhesion mole-



**Figure 4.** Time course of induction of endothelial adhesion molecule expression in response to AECA-positive IgG. Endothelial cells were incubated with IgG (150  $\mu\text{g/ml}$ ) from SSc serum No. 9 (●; ER = 100) (A and C), from SSc serum No. 39 (●; ER = 92) (B), from normal serum (○), or with endotoxin (□; 1

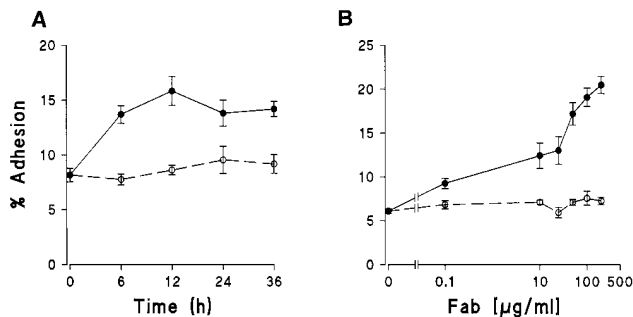
$\mu\text{g/ml}$ ) before fixation and measurement of E-selectin, ICAM-1, and VCAM-1 expression. Results show means $\pm$ SEM of three observations. These results are representative of those found in  $> 10$  experiments and with nine different sera.



**Figure 5.** Dose dependence of AECA-positive IgG-induced leukocyte adhesion to endothelium and adhesion molecule expression. Endothelial cells were pretreated for 8 h with IgG from SSc serum No. 21 (●; ER = 43) or control IgG (○). After rinsing, U937 cells were added and adhesion was measured after 30 min. In parallel wells, endothelial cells were fixed and adhesion molecule expression was measured by ELISAs. Results show means  $\pm$  SEM of three observations. Similar results were obtained in more than five experiments.

cule expression were measured with the same batch of HUVEC after pretreatment with IgG. Maximal cell adhesion was obtained with 100  $\mu$ g/ml of IgG AECA, which also induced maximal expression of adhesion molecules on the endothelial cell surface. The threshold active concentration for a detectable increase in expression of each adhesion molecule was 10  $\mu$ g/ml, whereas this concentration induced about one-third of the maximum U937 cell adhesion.

Fig. 6 illustrates that time- and dose-dependent increases in U937 cell adhesion could also be achieved by pretreating HUVEC with Fab fragments from AECA-positive IgG. As for whole IgG, Fab fragments induced adhesion molecule expression on HUVEC (data not shown).

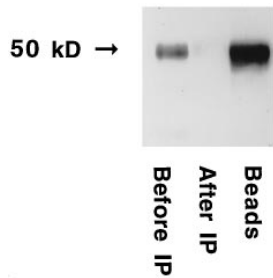


**Figure 6.** Time and dose dependence of leukocyte adhesion induced by pretreatment of endothelial cells with Fab fragments from AECA-positive IgG. Endothelial cells were pretreated with Fab fragments (100  $\mu$ g/ml for time course; 8 h for dose response) from SSc sera No. 9 (●; ER = 100) and No. 20 (○; ER = 18) (A) or pooled normal human sera (○) (B). Results show means  $\pm$  SEM of three (time course) or five (dose response) observations. Similar results were also found in two other experiments.

*Effects of conditioned media.* As noted above, the time course of AECA-induced adhesion molecule expression varied between experiments, although in any one experiment the pattern was always broadly similar for the three adhesion molecules. Enhanced adhesion and expression commonly peaked at around 12 h and had disappeared by 24 h, but in some experiments AECA-induced adhesion molecule expression lasted for 36 h or longer. The more rapid induction of responses by LPS suggested that AECA could act on HUVEC to induce the synthesis of a secondary mediator, which then acts in a paracrine fashion to activate the cells. We therefore examined whether HUVEC pretreated with AECA-positive IgG produced a transferable factor that could activate other HUVEC in the absence of IgG.

In preliminary experiments we found that direct transfer of conditioned medium from AECA-positive IgG-treated HUVEC to new cells could induce enhanced adhesion molecule expression and U937 cell adhesion, again after a time lag and peaking after several hours, while conditioned medium from HUVEC pretreated with IgG from normal human serum or AECA-negative SSc sera did not (data not shown). This effect could be due either to a soluble mediator released by AECA-activated endothelium or to IgG AECA still present in the conditioned medium. Although the latter seemed unlikely, we immunodepleted samples of conditioned media (as described in Methods) to remove human IgG. As shown in the example in Fig. 7, human IgG was present in the conditioned medium and on the protein A beads but not detectable in the immunodepleted conditioned medium.

Table II shows that induction of U937 cell adhesion and of expression of adhesion molecules was not distinguishably different when HUVEC were treated for 6 h with AECA-positive IgG, with conditioned medium from HUVEC pretreated

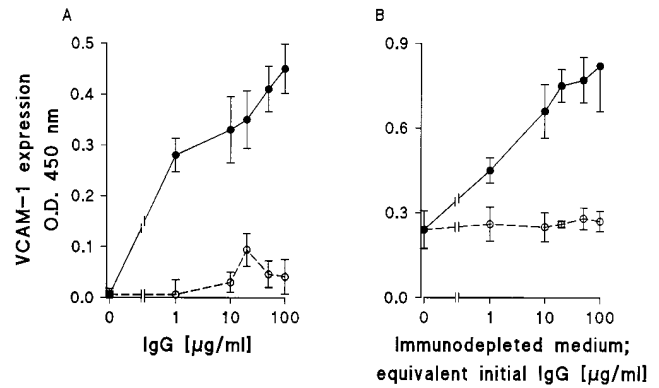


**Figure 7.** Western analysis to confirm depletion of human IgG from conditioned media by immunoprecipitation (IP). Conditioned media from HUVEC pretreated with 100  $\mu\text{g/ml}$  of IgG, were collected, pooled, and immunoprecipitated using protein A–Sepharose CL4B pre-coated with rabbit anti-human IgG. 10- $\mu\text{l}$  fractions of conditioned media, immunodepleted conditioned media, and extracts from beads were run in 12% SDS-PAGE under reducing conditions, transferred to nitrocellulose, and blotted with anti-IgG.

in the same way, or with immunodepleted conditioned medium. Fig. 8 demonstrates that when immunodepleted conditioned medium is used in concentrations that are equivalent to those of the initial IgG-containing medium the dose-response curve (in this example for VCAM-1 expression) is also equivalent.

*Effects of blocking antibodies to cytokines on effects of conditioned media.* The potential contribution of endothelial cell-generated cytokines to the transferable effects of conditioned media was examined with blocking antibodies. HUVEC were treated for 6–12 h with conditioned media in the absence or presence of these antibodies, and then adhesion molecule expression and U937 cell adhesion were measured. Table III shows the results from an experiment of this type, in which antibody to IL-1 $\alpha$ , or a combination of antibodies to IL-1 $\alpha$  and IL-1 $\beta$ , but not antibody to TNF, substantially inhibited or blocked enhanced U937 cell adhesion and VCAM-1 expression. This pattern of inhibition was reproduced in three further experiments, whereas in one other antibodies had no significant effect. Attempts to measure IL-1 $\alpha$  and TNF in conditioned media revealed just detectable levels (10–25 U/ml) of IL-1 $\alpha$  in three of nine samples but no detectable TNF (data not shown).

*Effects of blocking antibodies to cytokines on direct effects of IgG AECA.* Table IV demonstrates that a mixture of blocking antibodies to IL-1 $\alpha$ , IL-1 $\beta$ , and TNF was also capable of significantly reducing, though not abolishing, the direct effect of AECA-positive IgG on adhesion molecule expression and subsequent U937 cell adhesion, at concentrations which



**Figure 8.** Dose-dependent induction of VCAM-1 expression by immunodepleted conditioned medium. (A) Endothelial cells were incubated for 8 h with IgG from SSc serum No. 1 (●; ER = 83) or from No. 5 (○; ER = 20). The media were removed, the cells were fixed, and adhesion molecule expression was then measured by ELISA. (B) The media from A were collected and depleted of human IgG. The depleted media, at concentrations equivalent to that containing 1–100  $\mu\text{g/ml}$  IgG in original media, were then incubated with a fresh batch of endothelial cells for 8 h before VCAM-1 expression was determined as in A. Results show means  $\pm$  SEM of five observations. Equivalent results were obtained for ICAM-1 and E-selectin and were also found in two other experiments.

blocked a similar level of enhanced adhesion induced by individual cytokines. Control antibodies (goat or sheep serum) had no effect. As for the effects on conditioned media, neutralizing antibodies usually, but not in every experiment, reduced U937 cell adhesion: as an example in an experiment with four different IgG, inhibition was No. 9 = 0%, No. 38 = 95%, No. 39 = 53%, and No. 21 = 66%.

## Discussion

We believe that this is the first study reporting functional effects of AECA on endothelial cell interactions with leukocytes. Our results demonstrate that pretreatment of human endothelial cells in vitro with SSc sera containing IgG AECA leads to a consequent increased adhesion of monocytic U937 cells. This effect is not found with SSc sera that contain no detectable AECA and can be replicated by incubating the endothelial cells with purified IgG AECA. Increased leukocyte ad-

**Table II.** Treatment of Endothelial Cells with AECA-positive IgG or Conditioned Medium from IgG Pretreated Endothelial Cells Induces Comparable Leukocyte Adhesion and Adhesion Molecule Expression

	U937 cell adhesion	E-selectin	ICAM-1	VCAM-1
	percent added cells	OD	OD	OD
Control	13.4 $\pm$ 0.6	0.03 $\pm$ 0.03	0.17 $\pm$ 0.05	0.11 $\pm$ 0.02
AEC positive IgG	37.7 $\pm$ 2.2	0.51 $\pm$ 0.10	0.41 $\pm$ 0.06	1.10 $\pm$ 0.30
Conditioned medium	31.7 $\pm$ 2.0	0.45 $\pm$ 0.06	0.47 $\pm$ 0.05	0.86 $\pm$ 0.08
IgG-depleted conditioned medium	33.7 $\pm$ 5.9	0.46 $\pm$ 0.07	0.52 $\pm$ 0.12	0.86 $\pm$ 0.09

Endothelial cells were treated for 12 h with AECA-positive IgG (100  $\mu\text{g/ml}$ ; from serum No. 39, ER = 92) or with conditioned medium from wells of endothelial cells pretreated in the same way, either directly transferred or depleted of human IgG before transfer. After rinsing, U937 cells were added and adhesion was measured at 30 min. In parallel wells, adhesion molecule expression was measured by ELISAs. Results show means  $\pm$  SEM from four observations. Equivalent results were obtained in four other independent experiments with different AECA-positive IgG.

**Table III. Blocking Antibodies to IL-1 Can Reduce the Effects of IgG-depleted Conditioned Medium on Leukocyte Adhesion to Endothelial Cells and Endothelial Adhesion Molecule Expression**

	U937 cell adhesion		VCAM-1	
	percent added cells		OD	
Basal	9.3±1.6		0.28±0.06	
IgG-depleted conditioned medium	20.1±0.6* (43%)		2.10±0.07*	
IgG-depleted conditioned medium + anti-IL-1α	12.3±1.1 (72%)		0.85±0.24 (69%)	
+ anti-IL-1β	15.5±0.8* (43%)		1.80±0.18* (16%)	
+ anti-IL-1α/anti-IL-1β	10.5±1.3 (89%)		0.25±0.05 (100%)	
+ anti-TNF	19.6±0.4* (5%)		1.88±0.07* (12%)	
+ anti-IL-1α/anti-IL-1β/anti-TNF	12.1±0.3 (82%)		0.40±0.05 (93%)	

Endothelial cells were treated for 6–36 h with individual AECA-positive IgG (100 µg/ml). The conditioned medium from these cells were pooled, depleted of human IgG, and transferred to new endothelial cells for 12 h, in the absence or presence of blocking antibodies to cytokines (IL-1α, IL-1β, TNF-α). After rinsing, U937 cells were added and adhesion was measured after 30 min. In parallel wells, adhesion molecule expression was measured by ELISA. Results show means±SEM from three (U937 cell adhesion) or four (VCAM-1) observations. The data in parentheses show the percent inhibition. \**P* < 0.05 by comparison with basal. Similar results were obtained in more than five experiments and for ICAM-1 and E-selectin expression (see text for details).

hesion required endothelial cell protein synthesis, was dose dependent with a threshold of 1–10 µg/ml IgG, was detectable within ~ 3 h of exposure to IgG, and was maximal at 6–12 h.

We used U937 cells for the adhesion experiments: these cells possess several characteristics of circulating immature monocytes (39), but of most importance for our experiments, they express the counterligands for ICAM-1, VCAM-1, and E-selectin and thus provide a reproducible and valid model for studies of leukocyte adhesion to endothelium (36). ELISAs

demonstrated that increased adhesion of U937 cells was concomitantly associated with enhanced expression of each of the three adhesion molecules on endothelial cells. Comparison of the time course of adhesion molecule expression in response to IgG AECA showed that, by comparison with LPS, the effect of AECA was often slower and more prolonged, perhaps indicating an indirect action of AECA due to production by the endothelial cells of a secondary mediator.

Endothelial cells secrete a wide variety of inflammatory and immunomodulatory mediators in response to stimuli. Potentially, the most interesting in the context of this study is IL-1α, since this cytokine induces endothelial cell adhesion molecule expression, and both IL-1α and IL-1β are synthesized by endothelial cells in response to IL-1α itself or other inflammatory cytokines, although only a small proportion of IL-1α is secreted (40, 41). We initially obtained evidence that a stable, transferable, factor was present in the conditioned medium from IgG AECA-treated endothelial cells, which could reproduce the effects of IgG on fresh endothelial cell cultures. Subsequently, by using blocking antibodies and attempting to measure IL-1α levels in conditioned medium, we were able to show that the action of AECA could, at least in part, be attributed to endothelial production of IL-1. A contribution from secreted IL-1α was, however, not detectable in every experiment, suggesting that other as yet undefined products may also play a role.

The finding that endothelial cell activation by AECA from SSc sera can include synthesis of IL-1 is also of potential relevance to the fibrotic process in SSc, since IL-1 and IL-6 (another secreted product from cytokine-stimulated endothelium; [42]) stimulate fibroblast proliferation and collagen synthesis (43).

An outstanding problem in understanding the mechanisms underlying the stimulatory effect of AECA on endothelium is the lack of definition of their target antigens. It is clear from our data that the effects are Fab mediated, and, interestingly, can be obtained with Fab fragments from IgG AECA, indicating that cross-linking of target antigens is not necessary to induce the endothelial response. Since binding of antibodies to known endothelial surface antigens (MHC class I, CD59, etc.) did not mimic the effect of AECA, we presume that specific

**Table IV. Blocking Antibodies to Cytokines Can Reduce AECA-positive IgG-induced Leukocyte Adhesion to Endothelial Cells and Endothelial Adhesion Molecule Expression**

	U937 cell adhesion		Adhesion molecule expression		
	–blocking Ab	+blocking Ab	ICAM-1	E-selectin	VCAM-1
	percent U937 cells added		OD	OD	OD
Basal	15.2±1.3		0.14±0.01	0.04±0.01	0.11±0.06
+IL-1α	37.1±3.9	11.8±1.3 (113%)			
+IL-1β	35.1±4.8	15.2±0.6 (100%)			
+TNF	44.0±4.3	17.0±0.7 (94%)			
+AECA positive IgG	41.1±4.3	25.0±0.2 (63%)	0.44±0.02	0.68±0.03	1.41±0.07
+AECA positive IgG + blocking Ab			0.28±0.01 (87%)	0.14±0.03 (84%)	0.61±0.08 (61%)

Endothelial cells were treated for 12 h with medium alone, IL-1α (100 U/ml), IL-1β (100 U/ml), TNF-α (100 U/ml), or AECA-positive IgG (100 µg/ml; from serum No. 21, ER = 43), in the absence or presence of blocking antibodies to individual cytokines (for the cytokine-treated cells) or a mixture of all three antibodies (for the IgG-treated cells). The cells were then rinsed. U937 cells were added and adhesion was measured after 30 min. In parallel wells, adhesion molecule expression was measured by ELISAs. Results show means±SEM for three (U937 cell adhesion) or four (adhesion molecule) observations. The data in parentheses show the percent inhibition in the presence of blocking antibodies.

targets must be involved. Other groups have tried to identify antigenic targets for AECA in various autoimmune diseases (44, 45). In unpublished studies we, like them, have been able to find a small number of consistent bands on Western blots of endothelial proteins probed with AECA but have yet to identify any at the molecular level, and further work is needed.

Elevated levels of anticytokine autoantibodies have been demonstrated in several autoimmune diseases (46), including in SSc anti-IL-8 and IL-6/anti-IL-6 complexes retaining IL-6 activity (47, 48), though their significance is obscure and it is difficult to imagine that they are related to AECA. Whether autoantibodies to cytokine receptors could constitute part of the AECA repertoire remains to be determined, but it is worth noting that AECA present in autoimmune vasculitic diseases such as SSc do not bind significantly to leukocytes (4).

In conclusion, the pathogenesis of SSc is complex and involves at least three cell types: lymphocytes, endothelial cells, and fibroblasts. Nonetheless, there is increasing evidence that endothelial cell activation is an early feature of the disease. Activation of endothelial cells will facilitate leukocyte traffic, inflammatory injury, and profibrotic reactions, thus initiating, amplifying, or perpetuating the disease process. Raised levels of cytokines in SSc (49) could cause endothelial activation, but our present results define another mechanism and for the first time assign a pathogenic role for AECA in SSc. We have recently obtained similar results (unpublished) with AECA purified from SLE sera, suggesting that these autoantibodies contribute to the vascular pathology associated with other auto-immune diseases in which AECA are found.

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