Activation of cultured vascular endothelial cells by antiphospholipid antibodies.

R Simantov, … , J E Salmon, R L Silverstein

J Clin Invest. 1995;96(5):2211-2219. https://doi.org/10.1172/JCI118276.

Research Article

Circulating antiphospholipid antibodies (aPL) are associated with a syndrome of thrombosis, recurrent fetal loss, and thrombocytopenia. We have demonstrated the activation of cultured human umbilical vein endothelial cells (HUVEC) by IgG from patients with anticardiolipin antibodies (aCL). Incubation of HUVEC for 4 h with purified IgG (100 micrograms/ml) from patients with high-titer aCL induced a 2.3-fold increase in monocyte adhesion over that seen in HUVEC incubated with IgG's from normal subjects. The effect of aCL was not attributable to LPS contamination, Fc receptors, or immune complexes. Monocyte adhesion was not induced when the aCL were added in serum-free media but was restored by the addition of purified beta 2GP1, previously described as a necessary cofactor for aCL reactivity. Purified rabbit polyclonal IgG raised against beta 2GP1 also induced monocyte adhesion when incubated with HUVEC. Preadsorption of patient serum with cardiolipin reduced monocyte adhesion by 60%. Immunofluorescent microscopy demonstrated that endothelial cells incubated with patient IgG expressed cell adhesion molecules, including E-selectin, vascular cell adhesion molecule-1, and intracellular adhesion molecule-1. These data support the hypothesis that aPL activate vascular endothelial cells, thereby leading to a pro-thrombotic state.

Activation of Cultured Vascular Endothelial Cells by Antiphospholipid Antibodies

Ronit Simantov,* Johanna M. LaSala,* Siu K. Lo,* Azzudin E. Gharavi,‡ Lisa R. Sammaritano,‡ Jane E. Salmon,‡ and Roy L. Silverstein*

**Division of Hematology/Oncology, Department of Medicine, Cornell University Medical College, New York, 10021; and ‡Division of Rheumatology, Hospital for Special Surgery, New York, 10021*

Abstract

Circulating antiphospholipid antibodies (aPL) are associated with a syndrome of thrombosis, recurrent fetal loss, and thrombocytopenia. We have demonstrated the activation of cultured human umbilical vein endothelial cells (HUVEC) by IgG from patients with anticardiolipin antibodies (aCL). Incubation of HUVEC for 4 h with purified IgG (100 μg/ml) from patients with high-titer aCL induced a 2.3-fold increase in monocyte adhesion over that seen in HUVEC incubated with IgG's from normal subjects. The effect of aCL was not attributable to LPS contamination, Fc receptors, or immune complexes. Monocyte adhesion was not induced when the aCL were added in serum-free media but was restored by the addition of purified β_2GP1, previously described as a necessary cofactor for aCL reactivity. Purified rabbit polyclonal IgG raised against β_2GP1 also induced monocyte adhesion when incubated with HUVEC. Preadsorption of patient serum with cardiolipin reduced monocyte adhesion by 60%. Immunofluorescent microscopy demonstrated that endothelial cells incubated with patient IgG expressed cell adhesion molecules, including E-selectin, vascular cell adhesion molecule-1, and intracellular adhesion molecule-1. These data support the hypothesis that aPL activate vascular endothelial cells, thereby leading to a prothrombotic state. (*J. Clin. Invest.* 1995. 96:2211–2219.) Key words: adhesion • anticardiolipin • lupus • monocytes • thrombosis

Introduction

The presence of circulating antiphospholipid antibodies (aPL)[1] is associated with a clinical syndrome characterized by venous and arterial thrombosis, recurrent fetal loss, thrombocytopenia, and neurologic disease (1). The thrombotic disease can occur at an early age and can be associated with devastating clinical sequelae. The antiphospholipid antibody syndrome is most often seen in patients with SLE, but also occurs in patients without other evidence of collagen vascular or autoimmune disease (primary antiphospholipid syndrome [PAPS]) (2). aPL autoantibodies, including lupus anticoagulant and anticardiolipin antibody (aCL), are reactive with negatively charged phospholipids. Reactivity of aCL in vitro depends on the presence of a protein cofactor, β_2-glycoprotein 1 (β_2GP1, apolipoprotein H) (3), a 50-kD plasma protein, which may form a complex with cardiolipins to confer specificity for aCL (4).

Epidemiologic studies have demonstrated an increase in thrombosis in SLE patients who have measurable aPL, as well as an increase in thrombotic events in patients with aPL and no other underlying disease (5, 6). In addition, studies of the lupus-prone MRL mouse and animals passively immunized with aPL IgG demonstrate aPL-associated thrombosis and spontaneous abortion (7, 8). Although these represent strong evidence that aPL may be pathogenic, the molecular basis of the prothrombotic phenotype associated with these antibodies is unknown.

Numerous studies have attempted to demonstrate an effect of aPL on fluid phase coagulation enzymes or inhibitors (1). It is clear that while these antibodies often inhibit plasma thrombin generation in in vitro assays, they are not associated with a clinical anticoagulant effect. While no consistent evidence of interference with antithrombin III or with the fibrinolytic system has been found, some studies have shown that IgG fractions from patients with SLE may interfere with thrombomodulin-mediated protein C activation or with phospholipid-dependent protein C activity (9, 10). These results have not been shown to correlate with clinical thrombosis.

Most recent attention has therefore focused on the possible interaction of aPL with cellular regulators of hemostasis, especially platelets and endothelial cells. Work in our laboratory and others has failed to demonstrate platelet activation by aPL, although aPL has been shown to bind to previously activated platelets (11). Platelets opsonized by aPL may be cleared more rapidly from the circulation, and may account for the thrombocytopenia associated with aPL syndrome, although it is difficult to explain the clinical hypercoagulability on this basis.

Since the vascular endothelium is a major regulator of hemostasis, it is reasonable to speculate that the hypercoagulable state associated with aPL may be due to interference with normal endothelial cell function. In patients with SLE and aPL there is indirect evidence of endothelial cell activation, including increased circulating levels of von Willebrand factor (12). Previous studies have also shown that IgG from patients with aPL can bind to endothelial cells (13, 14). However, endothelial cell reactivity has not been shown to correlate with aCL activ-

Azzudin E. Gharavi's current address is Louisiana State University Medical Center, New Orleans, LA 70112. Address correspondence to Roy L. Silverstein, Division of Hematology and Oncology, Cornell University Medical College, 1300 York Avenue, New York, NY 10021. Phone: 212-746-2060; FAX: 212-746-8866.

Received for publication 11 April 1995 and accepted in revised form 8 August 1995.

1. *Abbreviations used in this paper:* AECA, anti-endothelial cell antibodies; aCL anticardiolipin antibody; aPL, antiphospholipid antibody; β_2GP1, β_2 glycoprotein-1; BI, binding index; GPL, gamma phospholipid unit; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; MM6, Mono Mac 6; PAPS, primary antiphospholipid antibody syndrome; VCAM-1, vascular cell adhesion molecule-1.

J. Clin. Invest.

0021-9738/95/11/2211/09 $2.00
Volume 96, November 1995, 2211–2219

ity or with the clinical manifestations of the aPL syndrome (15, 16).

In this manuscript, we report the first evidence that purified IgG from patients with aPL, even in the absence of clinical or serologic evidence of SLE, have the capacity to activate vascular endothelial cells. We show that purified IgG induce an adhesive phenotype of vascular endothelial cells, and that this effect is due to the specific antiphospholipid reactivity of the IgG.

Methods

Antibodies and proteins. Monoclonal antibody to E-selectin (F83) was a gift of Dr. M. Bevilacqua (University of California San Diego, San Diego, CA), to class I histocompatibility antigens (W6/32), a gift of Dr. Luscinskas (Brigham and Women's Hospital, Boston, MA), to intercellular adhesion molecule-1 (ICAM-1) (HU53), a gift of Dr. R. Steinman (Rockefeller University, New York, NY); monoclonal antibody to FcγRII (IV.3) was purchased from Medarex, Inc. (Annandale, NJ), and vascular cell adhesion molecule-1 (VCAM-1) from Amac, Inc. (Westbrook, ME). Human recombinant TNFα was a gift of Dr. A. Cerami (Picower Foundation, Manhasset, NY). Human serum with anti-HLA class I reactivity was kindly provided by Dr. M. Suthanthiran (Cornell Medical College, New York, NY). Mouse IgG2A was purchased from Zymed Laboratories, Inc. (South San Francisco, CA). Rabbit anti-human albumin antiserum was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). BSA was purchased from Sigma Chemical Co. (St. Louis, MO). Normal goat serum and nonimmune mouse and rabbit IgG were purchased from Pierce Chemical Co. (Rockford, IL). Purified β_2GP1 was prepared as described (17) with some modifications (18), by perchloric acid treatment of serum followed by ion-exchange chromatography on QAE-Sephadex A-50 (Pharmacia L.K.B. Biotechnology, Piscataway, NJ). The preparation was free of contaminating lipids of human origin as determined by chloroform: methanol extraction followed by high performance thin layer chromatography. Aggregated IgG was prepared as described (19), by covalent cross-linking of immune complexes with *bis*-diazotized benzidine (Sigma Chemical Co.), and sizing by gel-filtration chromatography with AcA22 (Pharmacia L.K.B. Biotechnology). Polymyxin B agarose and cardiolipin were purchased from Sigma Chemical Co.

Cell culture and isolation. Human umbilical vein endothelial cells (HUVEC) were isolated by collagenase treatment of human umbilical veins as described (20). Cultures were established in Medium 199 (BioWhittaker, Inc., Walkersville, MD) containing 20% fetal bovine serum (BioWhittaker Inc.), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (GIBCO, Grand Island, NY). HUVEC were passaged with 0.2% collagenase and 0.02% EDTA (GIBCO). HUVEC from passages 2–3 were used in these experiments. Mono Mac 6 cells (MM6), a human monocytic cell line which has been well-characterized and exhibits morphological, biochemical, and physiological phenotypes of mature monocytes, was a gift of Dr. H. Ziegler-Heitbrock (Universitat Munchen, Germany). These cells express CD 15 (the ligand for E-selectin), the β_2 leukocyte integrins (ICAM ligands), and $\alpha_4\beta_1$ (VCAM ligand) (21). MM6 cells were cultured in RPMI-1640 medium (GIBCO) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine.

Subjects. After informed consent, venous blood was obtained from 23 patients with SLE, 5 patients with PAPS and no serologic or clinical evidence of SLE, and 10 normal volunteers, from the Rheumatic Disease service at the Hospital for Special Surgery (New York, NY). All SLE patients met American Rheumatism Association Criteria for the diagnosis (22). Clinical information, including history of SLE, venous and arterial thrombosis, thrombocytopenia, or fetal loss, was recorded for each subject. The study was approved by the appropriate institutional review boards.

Purification of IgG. IgG from patients or normal subjects was purified as described (18), by protein G-Sepharose 4B affinity chromatography (Zymed Laboratories, Inc.). All IgG preparations were free of contaminating β_2GP1 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and were found to have LPS level < 0.06 ng/ml as determined by limulus lysate assay (Ameolysate; ICN Biochemical, Costa Mesa, CA). Anticardiolipin antibody titer was determined by ELISA as previously described (23), using purified IgG at a concentration of 75–100 μg/ml. The upper limit of linearity of the assay is 80 gamma phospholipid units (GPL), therefore values exceeding this level are reported as "> 80". Some patients' sera were incubated overnight at 4° with cardiolipin liposomes (5 mg/ml), which were prepared as described (24).

Rabbit polyclonal β_2 glycoprotein 1–induced antibodies. Polyclonal rabbit antiserum with both anti-β_2GP1 and anticardiolipin reactivity was produced by immunization of a New Zealand white rabbit with purified human β_2GP1 (25). As previously described, the antibody populations were non-cross-reactive, and the aCL reactivity β_2GP1-dependent.

Adhesion assay. HUVEC were seeded onto 0.1% gelatin-coated Terasaki plates (Miles Laboratories Inc., Naperville, IL) at a cell density of 1×10^6 cells/ml, allowed to grow at 37°C for 1 d, and then washed once with M199. Cells were incubated with purified human IgG (100 μg/ml) diluted in serum-containing medium, which has been shown to provide cofactor necessary for aCL binding (18), and incubated at 37°C for 4 h or other time points as specified. In some experiments, HUVEC were incubated for 15 min with purified IgG, washed three times with M199, and then incubated for various time points in serum-containing medium. HUVEC were washed in medium three times and MM6 cells (10 μl of 1×10^6 cells/ml) were added. Adhesion was allowed to proceed for 15 min at 37°C and the nonadherent cells were removed by washing three times with M199. Adherent cells on HUVEC were fixed with 1% paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA), and manually counted on an inverted phase-contrast microscope using a 10 × 10 mm grid. The values of four replicates were averaged, with variations between replicates usually < 10%. Damage to the endothelial cell monolayers was observed after prolonged incubations (> 16 h), therefore experiments were performed with incubations of up to 8 h. Control cells were treated with TNF at 200 U/ml to determine the maximal induced adhesion level.

Immunofluorescent microscopy. 8-well glass chamber slides (NUNC Inc., Naperville, FL) were treated for 15 min with 0.1 M NaOH, washed with Dulbecco's PBS (GIBCO), and coated with 0.1% gelatin, then seeded with HUVEC to near-confluence overnight. The cells were then incubated with patient IgG or medium as above and then washed twice with and fixed with 4% paraformaldehyde for 10 min at room temperature. The cells were incubated with murine monoclonal antibodies to E-selectin, ICAM-1, or VCAM-1 for 1 h at room temperature. To control for specificity, isotype-specific IgG was substituted for the primary antibody. Monoclonal antibody to class I histocompatibility antigens was used as a positive control. After washing in PBS and blocking for 15 min in 1% normal goat serum, cells were incubated with fluorescein-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersberg, MD) for 1 h at room temperature. Coverslips were mounted using Immunofluor mounting medium (ICN Biochemicals), dried overnight in the dark, and photographed with an epifluorescence microscope (Nikon Inc., Melville, NY) using slide film (Ektachrome ASA 400; Eastman Kodak Co., Rochester, NY).

Antiendothelial cell antibodies (AECA). AECA were determined by ELISA as described (26). Briefly, HUVEC were seeded on 0.1% gelatin-coated 96-well tissue culture plates (NUNC Inc.), and allowed to grow to confluence over 2–3 d. The wells were washed once with Dulbecco's PBS, then fixed with 2% paraformaldehyde for 20 min. Wells were washed with a buffer of 25 mM Tris, 0.5 M NaCl, and 0.2% Tween 20 (Sigma Chemical Co.), blocked with 100 μl/well of 2% BSA in PBS for 2 h, then washed. Serial dilutions of patient IgG's (100 μl/well) were added in triplicate and incubated for 90 min. After washing, the cells were incubated with 100 μl/well 1:1,000 alkaline phosphatase–conjugated goat anti-human IgG (BioRad Laboratories, Richmond, CA) for 1 h. After washing, 50 μl of *p*-nitrophenylphosphate (Pierce Chemical Co.) at 5 mg/ml in 1 M diethanolamine/0.5 M $MgCl_2$ was then added to each well, and after 7–10 min at room temperature,

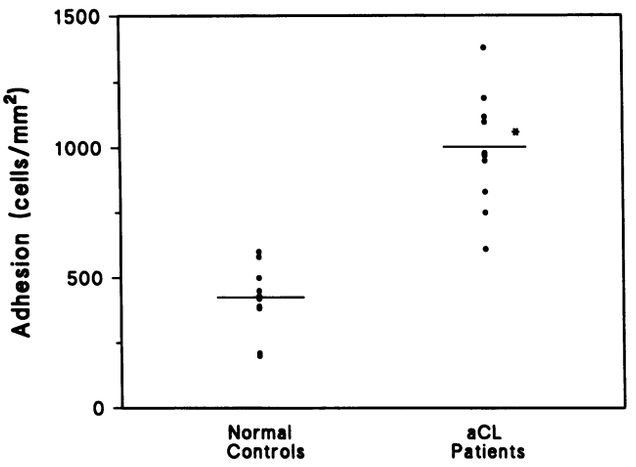


Figure 1. IgG from patients with antiphospholipid antibody syndrome promotes monocyte adhesion to vascular endothelial cells. HUVEC monolayers seeded on Terasaki plates were incubated with purified IgG (100 μg/ml) from normal controls or patients with antiphospholipid antibody syndrome, for 4 h at 37°C. After washing, HUVEC were incubated with Mono Mac 6 cells (1×10^6 cells/ml) for 15 min at 37°C; nonadherent MM6 cells were washed off, and adherent cells on HUVEC were counted on an inverted phase-contrast microscope. Each point represents the average of two to four experiments per patient sample, done in triplicate. Horizontal bars represent the mean. * $P < 0.02$

kinetic readings of absorbance (mOD/min) at 405 nm, every 9 s over 2 min, was performed using a Thermomax microplate reader (Molecular Devices Corp., Menlo Park, CA). Each experimental sample was assayed a minimum of nine times, in parallel with a positive control (purified IgG from a multiply transfused transplant patient) and a negative control (IgG from a normal subject). To control for variation in background binding to HUVEC, results are expressed as a binding index (BI) (26) by the formula BI = 100 × [(mOD/min) test sample − (mOD/min) blank]/[(mOD/min) positive control − (mOD/min) blank].

Statistical analysis. Values are expressed as means±SE. Statistical differences between the values were examined by Student's *t* tests or by one-way analysis of variance, with $P < 0.05$ considered significant.

Results

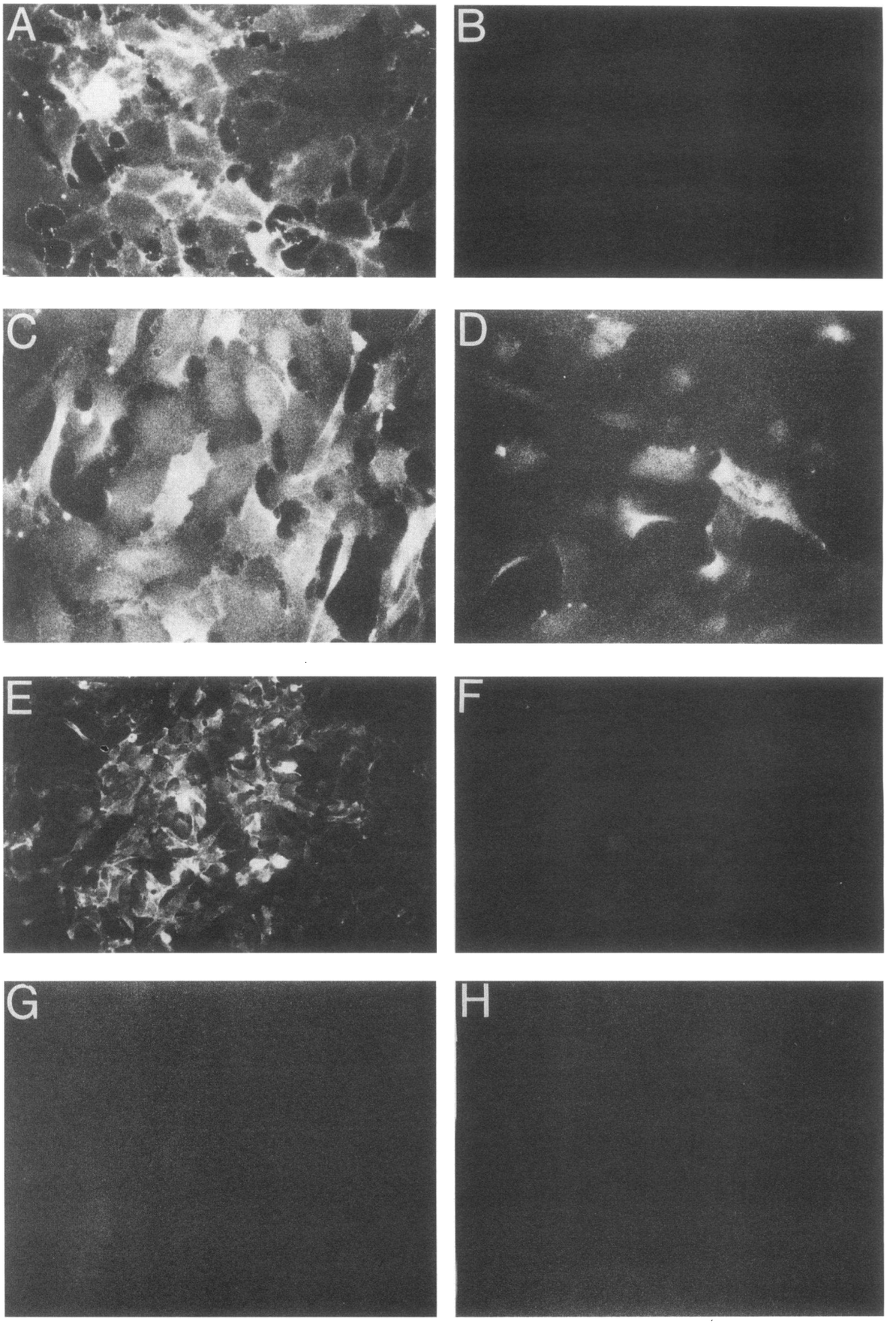
Induction of monocyte adhesion to vascular endothelial cells by IgG from patients with anticardiolipin antibodies. We studied induction of endothelial cell adhesiveness for monocytes as a biological assay to detect the activated phenotype induced by IgG isolated from patients with aPL. We used Mono Mac 6 (MM6) cells, which have been shown to express ligands for endothelial cell adhesion molecules and to behave similarly to isolated human monocytes in adhesion assays with cytokine-activated endothelium (27). In a series of preliminary studies, IgG were obtained from 10 patients with clearly identifiable clinical events and aCL titers exceeding 80 GPL, and from 10 normal subjects. As seen in Fig. 1, HUVEC incubated for 4 h with IgG from patients with aCL were significantly more adherent for monocytes than those incubated with IgG from normal controls ($P < 0.02$). The mean increase in adhesion was 2.3, with a range of up to sevenfold. Preincubation of the monocytes with patient IgG before the adhesion assay did not induce monocyte adhesion to unstimulated HUVEC, implying that IgG exerted its affect on endothelial cells, and did not directly activate monocytes.

A time course analysis revealed that incubation of HUVEC with aCL IgG for 15 min, 1 h, or 2 h was not sufficient to induce adhesion above background levels (107±66 adherent cells/mm², 154±33 cells/mm², 176±59 cells/mm², respectively), while increased adhesion was maximal after 4 h (991±214 cells/mm²) and remained apparent after 8 h (780±486 cells/mm²). A similar time course of increased adhesion was seen when HUVEC were incubated with five different aCL patient IgG's for 15 min, then washed and incubated in medium alone before the adhesion of monocytes. Mean adhesion was 543±111 cells/mm² at 4 h after washing out the aCL IgGs, vs 666±217 cells/mm² after a parallel 4-h incubation with the same patient IgG.

The 4-h time lag required for the development of the adhesive phenotype suggests the synthesis and expression of new adhesion proteins by HUVEC. We therefore performed immunofluorescence microscopy on HUVEC which had been incubated for 4 h with patient IgG, using antibodies to known cell surface adhesion molecules. We found, as shown in Fig. 2, that endothelial cells incubated with aCL IgG expressed surface E-selectin, VCAM-1, and ICAM-1, while endothelial cells incubated with normal patient IgG expressed only low levels of ICAM-1. We then performed the adhesion assay in the presence of blocking monoclonal antibodies to cell adhesion molecules. Antibody to E-selectin blocked 80% of monocyte adhesion; the combination of all three antibodies blocked all monocyte adhesion to the endothelial cells ($P < 0.05$) (Fig. 3). Although the antibody to VCAM-1 is known to have blocking activity (28), it did not block monocyte adhesion to endothelial cells in our system (Fig. 3). Nonimmune mouse IgG was used as a negative control, and had no effect. These data show that the adhesive phenotype produced by endothelial cell exposure to aCL was due to new expression of specific adhesion receptors.

Specificity of aCL effect on HUVEC. LPS contamination did not account for the effects of aCL on the endothelial cells. All of IgG preparations had undetectable levels of LPS (< 0.06 ng/ml) by limulus lysate assay. Furthermore, incubation of three aCL-positive patient IgGs with polymyxin B–coated agarose to remove any potentially contaminating LPS did not abrogate the adhesion induced by these samples (Fig. 4).

To exclude the possibility that Fcγ receptor interactions accounted for the proadhesive activity of IgG from patients with aCL, we performed adhesion studies in the presence of blocking antibodies. Immunofluorescence and flow cytometric analysis revealed that MM6 expressed FcγRII. Although they did not react with a panel of FcγRI antibodies, the MM6 exhibited low levels of binding to murine IgG2A, suggesting FcγR1 activity (Lo, S.K., and J.E. Salmon, unpublished observations). Saturating concentrations of anti-FcγRII monoclonal antibody (IV.3) or IgG2A (50 μg/ml) that block the ligand binding sites of FcγRII and FcγRI, respectively, had no effect on adhesion mediated by IgG from three PAPS patients and two SLE patients with aCL (Fig. 4). Incubation of HUVEC with aggregated IgG (100 μg/ml) did not lead to monocyte adhesion (331±276 cells/mm²), nor did aggregated IgG affect aCL-induced adhesion (985±171 vs 891±92 cells/mm²). Monocyte adhesion was also not caused by incubation of HUVEC with a combination of rabbit anti–human albumin (100 μg/ml) with 50–200 μg/ml human serum albumin (90±84 cells/mm²), arguing against an immune complex–mediated effect.


A
B
C
D
E
F
G
H

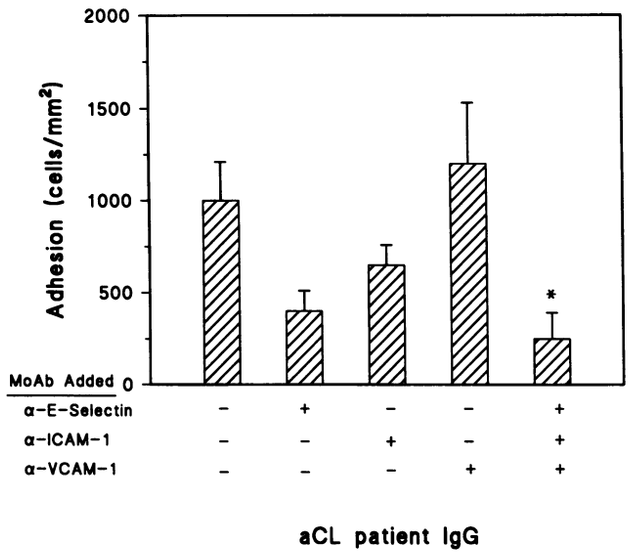


Figure 3. Inhibition of monocyte adhesion to aCL-activated endothelial cells by monoclonal antiadhesion receptor antibodies. HUVEC were incubated with aCL IgG as in Fig. 1 and monocytes were added in the presence of murine monoclonal antibodies to E-selectin (F-83), ICAM-1 (HU53), or VCAM-1, or a combination of the three, at concentrations of 10 μg/ml. Adhesion was measured as in Fig. 1. Each bar represents the mean of duplicate experiments, with four replicates per experiment. * $P < 0.02$

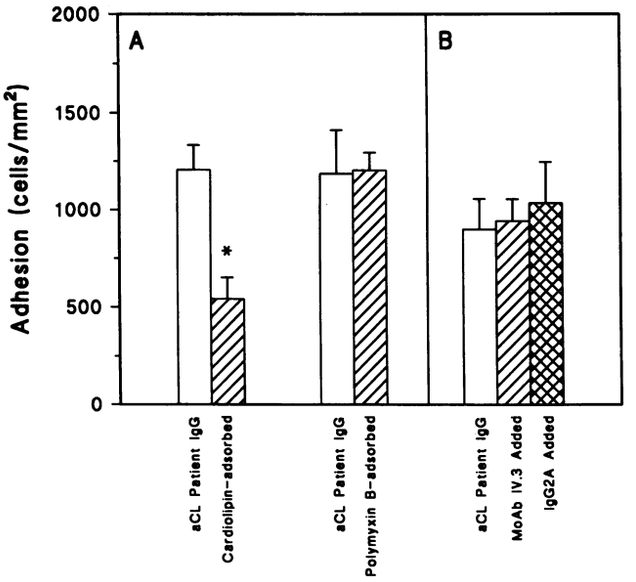


Figure 4. Specificity of aCL effect on HUVEC activation. *(A)* Serum from patients with aCL was preadsorbed with cardiolipin liposomes for 16 h at 4°C, or with Polymyxin B agarose for 1 h at 4°C before incubation with HUVEC as in Fig. 1. Monocyte adhesion was then measured. *(B)* IgG from patients with aCL was incubated with HUVEC as in Fig. 1. MM6 cells were then added in the presence of blocking antibody to FcγRII *(mAb IV.3)* or excess murine IgG2A (50 μg/ml) to block FcγRI; adhesion was then measured as above. Each bar represents the average±standard error of five experiments using purified IgG from two patients with high-titer aCL. * $P < 0.01$

Endothelial cell reactivity of patients IgGs. We found that all of the IgG preparations from patients with SLE and PAPS bound to endothelial cells, as determined by enzyme-linked immunoassay. We quantitated these AECA for 21 of our patients, and showed that binding of the IgG to HUVEC was not sufficient to induce the expression of an activated phenotype. As shown in Table I, many patient IgG's with high levels of antiendothelial cell reactivity did not induce increased adhesion. Furthermore, purified IgG from a multiply transfused patient with known anti-HLA class I reactivity, which bound to endothelial cells and was used as a positive control in the AECA assay (BI = 100), did not lead to monocyte adhesion when incubated with HUVEC, nor was adhesion induced by a monoclonal antibody to class I histocompatibility antigens expressed by resting endothelial cells. The mean BI of five normal IgG's tested was 16±8; none of the normal IgGs induced increased monocyte adhesion. Finally, AECA reactivity did not correlate with aCL titer, level of monocyte adhesion, or with clinical manifestations. These data suggest that the endothelial cell activation by aPL was distinct from the previously described AECA found in patients with autoimmune disease (26).

aCL IgG activation of endothelial cells requires β_2 glycoprotein-1. Binding of aCL to anionic phospholipids is dependent on the presence of a normal plasma protein, β_2GP1 (4). Bovine serum, which contains β_2GP1, has been shown to have cofactor activity for human aCL as well (18). We therefore sought to determine if the endothelial activation effect mediated by the aCL IgG was similarly dependent upon the presence of β_2GP1. As shown in Fig. 5, aCL IgG's did not induce monocyte adhesion to HUVEC when the IgG was added in serum-free medium, which lacks bovine β_2GP1. When exogenous purified human β_2GP1 (20 μg/ml) was added to the serum-free system, however, the adhesive phenotype was restored, suggesting that the effect of aCL IgG was β_2GP1 dependent.

To assess further the anticardiolipin specificity of our IgG fractions in promotion of monocyte adhesion to endothelial cells, we incubated serum from two aCL patients with cardiolipin liposomes. Preadsorption with cardiolipin decreased the level of anticardiolipin antibody in the remaining preparation as assayed by ELISA (> 80 vs 12 GPL; > 80 vs 16 GPL), but had no effect on other SLE-related autoantibodies, including ANA and anti-DNA (data not shown). As seen in Fig. 4, when endothelial cells were incubated with these preadsorbed patient sera, we observed a significant reduction in the level of monocyte adhesion ($P < 0.01$). This is further evidence for the

Figure 2. Incubation of HUVEC with aCL IgG induces the expression of cell adhesion molecules. HUVEC seeded on chamber slides were incubated with aCL IgG (*A, C, E,* and *G*), control IgG (*B, D,* and *F*), or IgG from a patient with SLE without aCL *(H)* at a concentration of 100 μg/ml for 4 h at 37°C. After washing and fixing with 4% paraformaldehyde, cells were incubated with murine monoclonal antibodies to E-selectin (F83) (*A, B,* and *H*), ICAM-1 (HU53) (*C* and *D*), VCAM-1 (*E* and *F*), or nonimmune mouse IgG *(G)*. Bound antibody was determined with fluorescein-labeled goat anti-mouse IgG. ×400 (*A–D, G,* and *H*) ×100 (*E* and *F*).

Table I. Patient Characteristics and Antibody Titers

Patient	Sex	Diagnosis	Complication	aCL titer	AECA	Adhesion
					BI±SE	*cells/mm²*
1	F	SLE	Fetal loss Venous thrombosis	> 80	N.D.	1,090
2	F	SLE	Fetal loss Venous thrombosis	> 80	N.D.	1,100
3	F	SLE	Fetal loss Venous thrombosis	> 80	N.D.	970
4	F	SLE	Fetal loss	> 80	N.D.	1,200
5	F	SLE	Fetal loss	> 80	N.D.	985
6	F	SLE	Fetal loss	> 80	N.D.	990
7	F	SLE	Fetal loss	> 80	N.D.	800
8	F	SLE	Fetal loss	> 80	N.D.	1,400
9	F	SLE	Fetal loss	> 80	N.D.	760
10	F	SLE	Fetal loss	> 80	N.D.	620
11	F	SLE	None	5	40±11	570
12	F	SLE	None	2	34±3	290
13	M	PAPS	Venous thrombosis	78	47±5	790
14	F	SLE	None	12	36±4	720
15	F	SLE	Venous thrombosis	4	53±12	580
16	F	SLE	Cardiac disease	> 80	80±8	900
17	F	PAPS	Cerebrovascular disease	51	35±9	670
18	F	SLE	Arterial thrombosis cerebrovascular disease thrombocytopenia	72	76±19	610
19	F	PAPS	Fetal loss	80	64±15	1,040
20	F	SLE	None	3	24±15	520
21	F	SLE	Fetal loss cerebrovascular disease	46	28±8	710
22	F	SLE	Cerebrovascular disease thrombocytopenia	> 80	52±4	880
23	F	SLE	Thrombocytopenia	43	72±13	860
24	M	PAPS	Cerebrovascular disease thrombocytopenia cardiac valvular disease	54	19±4	1,070
25	F	PAPS	Fetal loss cerebrovascular disease	> 80	38±6	940
26	F	SLE	None	8	79±7	610
27	F	SLE	Cerebrovascular disease	6*	55±16	560
28	F	SLE	None	4	61±14	740

Purified IgG from patients with SLE and PAPS was assayed for aCL, expressed as GPL and AECA, expressed as a binding index (*BI*)±standard error, as described in the Methods. Values above 80 GPL, the upper limit of linearity of the assay, are reported as "> 80." Patient IgG were incubated with HUVEC and adhesion of MM6 cells, expressed as cells/mm², was determined as described in the Methods. Adhesion values are the mean of two to three experiments, each done in triplicate. N.D., not done. * Patient had positive lupus anticoagulant.

specific role of aCL, rather than other SLE-related autoantibodies, in activation of endothelial cells.

Furthermore, IgG isolated from a rabbit immunized with purified β_2GP1, that was previously shown to react with cardiolipin in a β_2GP1-dependent manner (25), also activated HUVEC. As seen in Fig. 6, HUVEC incubated with this IgG acquired the capacity to bind monocytes in a concentration-dependent fashion. In addition, this effect was diminished when the IgG preparation was preadsorbed with β_2GP1 (1,230±237 vs 583±299 cells/mm²). Normal rabbit IgG did not increase adhesion above control (Fig. 6), indicating a specific effect of the β_2GP1 antibody.

Relationship of endothelial cell activation by aCL to SLE. To determine if the presence of SLE in patients with aCL contributed to these findings, we studied IgG from five patients with primary antiphospholipid syndrome, five patients with antiphospholipid antibody syndrome and concomitant SLE, and eight patients with SLE but no detectable aCL. As shown in Fig. 7 and Table I, IgG derived from patients with either PAPS or from patients with SLE and aCL induced significantly more adhesion as compared to IgG from normal subjects. The highest mean level of adhesion was induced by IgG from patients with PAPS, although there was no statistically significant difference between this and adhesion induced by IgG from patients with antiphospholipid antibody syndrome and concomitant SLE. IgG from patients with SLE but without detectable aCL induced a low level of monocyte adhesion, which was not statistically significant compared with control. Because some patients have antiphospholipid antibodies that do not react with cardiolipin (1), we performed activated partial thromboplastin times on all patients with negative anticardiolipin antibody titers to detect the presence of lupus anticoagulant. We found that only one of

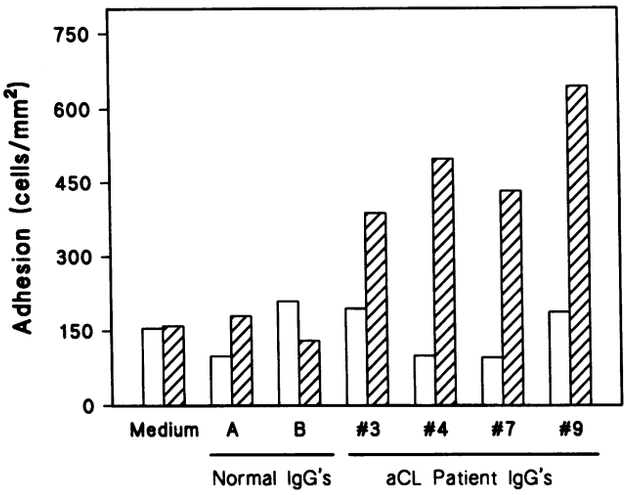


Figure 5. Induction of monocyte adhesion by aCL IgG is dependent on the presence of β_2-glycoprotein 1. HUVEC were incubated with aCL or control IgG in serum-free medium for 4 h at 37°C in the presence *(hatched bar)* or absence *(open bar)* of purified β_2-glycoprotein. Cells were then washed and monocyte adhesion was measured as in Fig. 1.

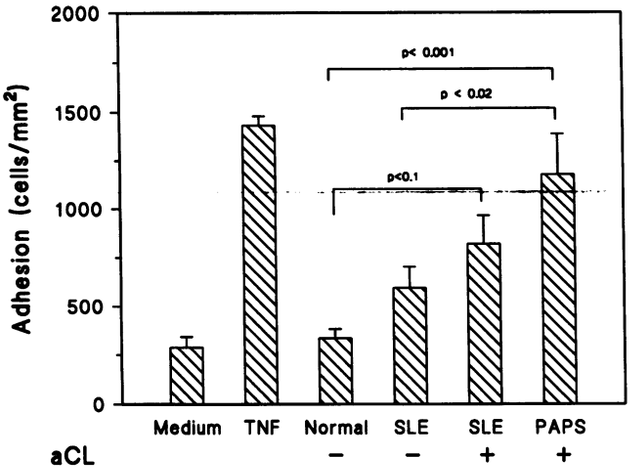


Figure 7. Relationship of endothelial cell activation by anticardiolipin IgG to diagnosis of SLE. HUVEC were incubated with IgG (as in Fig. 1), from normal subjects (n = 5), patients with SLE and no evidence of aCL (n = 8), SLE with high titer aCL (n = 5), and patients with PAPS (n = 5). The first two bars show the negative control of medium alone, and the positive control of TNF (200 U/ml). Each bar represents the average±standard error of five experiments, with each sample done in four replicates per experiment.

the eight SLE patients without detectable aCL had a positive lupus anticoagulant (patient 27, Table I). This did not change the statistical significance of our findings.

Discussion

The vascular endothelium is the major site of preservation of the nonthrombogenic state. This state is maintained by constitutive expression of natural anticoagulant systems, including thrombomodulin, heparan sulfate proteoglycans, plasminogen activators, and specific binding sites for fibrinolytic enzymes. Conversion of the normal antithrombotic endothelial phenotype to a prothrombotic state may be the primary pathophysiological event in many acquired hypercoagulable states. For example, inflammatory cytokines such as TNFα and interleukin-1, and bacterial products such as LPS have been shown to induce an activated endothelial phenotype characterized by expression of tissue factor procoagulant, plasminogen activator inhibitor, and leukocyte adhesion molecules, with concomitant loss of thrombomodulin expression (29, 30). In this manuscript, we show that the antiphospholipid antibody syndrome, a hypercoagulable state associated with high circulating titers of autoantibodies against anionic phospholipids, may be the result of such endothelial activation by a unique antibody-dependent mechanism.

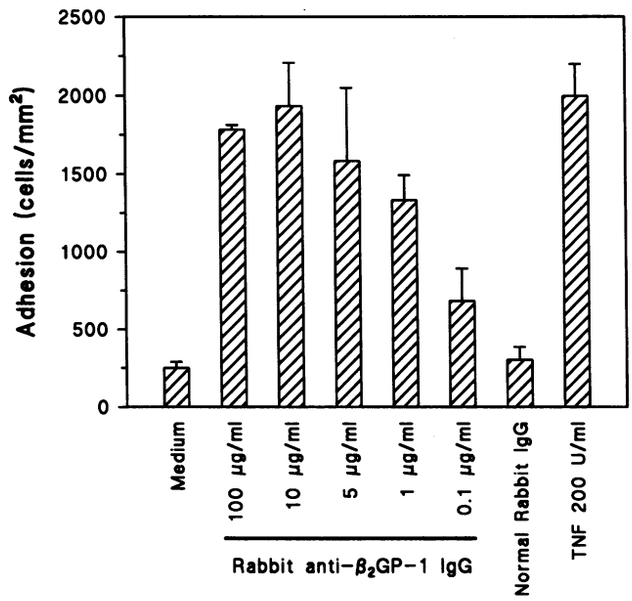


Figure 6. Rabbit anti-β_2-glycoprotein 1 IgG induces monocyte adhesion to HUVEC. HUVEC were incubated with rabbit anti-β_2-glycoprotein 1 antibody at varying concentrations, and monocyte adhesion was measured as in Fig. 1. Each bar represents the mean±standard error of five experiments, with four replicates in each experiment.

We used induction of endothelial cell adhesiveness for MM6 cells as a biologic assay to detect the activated phenotype induced by IgG purified from patients with aCL. The adhesive phenotype was shown to be the result of induced expression of specific adhesion molecules, including E-selectin, VCAM-1, and ICAM-1. Adhesion was not attributable to contaminating endotoxin, immune complexes, or to Fc receptor interactions, but rather was mediated by the specific anticardiolipin reactivity of the IgG. The endothelial-activating capacity of the IgG's was blocked by adsorption with cardiolipin liposomes, and was dependent on the presence of β_2GP1, a protein cofactor previously shown to be necessary for aCL reactivity. Furthermore, rabbit IgG with anticardiolipin reactivity that was raised by immunization with purified β_2GP1 also produced a similar activation of cultured endothelial cells.

We showed that IgG's from patients with aCL bind to HUVEC as determined by enzyme-linked immunoassay, however activation of endothelial cells was not dependent upon the level of endothelial cell reactivity of our IgG preparations. In addition, binding by control antibodies with endothelial cell reactivity was not sufficient to induce activation. As others have pre-

viously shown, we found that aPL antibodies were distinct from the AECA described in patients with autoimmune disease (13–16). We demonstrated that the effect of aPL in activating endothelial cells was independent of the level of endothelial cell reactivity. AECA did not correlate with clinical events, aCL titer, or with the level of endothelial cell activation as measured by adhesion. We conclude, therefore, that endothelial cell activation by IgGs from patients with aPL is dependent on specific antiphospholipid reactivity, and not on other antiendothelial cell reactivities.

The molecular mechanism by which aPL activate vascular endothelial cells is not known. Our data suggest that exogenous β_2GP1, a circulating glycoprotein, is necessary, and previous work has suggested that aCL recognize a complex antigen that includes β_2GP1 and anionic phospholipid (4). It is likely that the interaction of circulating β_2GP1 with endothelial cell anionic phospholipid induces formation of a neoepitope that confers recognition specificity for aCL. Although β_2GP1 has been shown to be necessary for the anticardiolipin-mediated pathophysiological effect, we cannot exclude the possibility that a protein other than β_2GP1 can serve as a cofactor for aCL binding to endothelial cells. We doubt that an endothelial cell membrane protein acts as a cofactor, however, because the aCL did not activate HUVEC in a serum-free system. Furthermore, although β_2GP1 has been shown to bind to HUVEC (31), we found no evidence by Northern analysis or Western blot that HUVEC express endogenous β_2GP1 mRNA or antigen (data not shown).

While we studied leukocyte adhesion as a convenient marker for endothelial activation, it is also highly likely that monocyte adhesion to the vascular endothelium may contribute to a hypercoagulable state. Several mechanisms could account for this. We have shown recently that upon adhesion of monocytes to E-selectin expressed on activated endothelium, the monocytes undergo a phenotypic transformation that includes surface expression of tissue factor procoagulant (27). Furthermore, activated endothelial cells also secrete inflammatory mediators, such as interleukin-1, that may in turn activate monocytes and induce expression of tissue factor (30). aPL may also bind to adherent monocytes and activate them via an Fc receptor–mediated mechanism. Some experimental evidence suggests that aCL may directly activate monocytes. Kornberg et al. have shown that a monoclonal anticardiolipin antibody induced procoagulant activity (32).

Thus far, there has been no reliable predictor of thrombotic events in patients with aCL. Recent studies have reported that aPL may interfere with the protein C anticoagulant pathway, inhibiting phospholipid-dependent activated protein C activity (10). However, not all patients with aPL develop thrombotic complications, and the clinical manifestations of aCL in SLE and non-SLE patients are heterogeneous (1, 6). It is likely, therefore, that a constellation of activities of antiphospholipid antibodies, including effects on coagulation enzymes and endothelial cells, is necessary to tip the balance toward the clinical phenotype. It is possible that these patients have ongoing endothelial cell activation, and clinically relevant events may occur when the patients are subjected to an additional factor, which then leads to vascular thrombosis or fetal loss in certain settings. It remains to be seen whether the level of endothelial cell activation in vitro correlates with clinical thrombotic events. In our study, the highest level of endothelial cell activation was induced by aCL from patients with PAPS, however further work needs to be done to more accurately predict the risk of thrombosis in patients with aPL.

Acknowledgments

The authors would like to thank Dr. Eric Jaffe of Cornell University Medical College for the generous donation of human umbilical vein endothelial cells. We thank Qinghu Zheng, George Lam, and Ying Weng for their technical assistance, and Ms. A. René Crombie for her helpful advice.

This work was supported by National Institutes of Health (NIH) grants P50 AR42588, RO1 HL-42540, and PO1 HL46403 (R.L. Silverstein). S.K. Lo was supported by NIH grant HL-49883 and an American Heart Association Grant-in-Aid. A.E. Gharavi was supported by NIH grant AR32929. R. Simantov and J. LaSala were supported by fellowships from the Louis and Rose Klosk Fund.

References

1. Sammaritano, L. R., A. E. Gharavi, and M. D. Lockshin. 1990. Antiphospholipid antibody syndrome: immunologic and clinical aspects. *Semin. Arthritis Rheum.* 20:81–96.

2. Harris, E. N., A. E. Gharavi, and G. R. V. Hughes. 1985. Antiphospholipid antibodies. *Clin. Rheum. Dis.* 11:591–609.

3. Lozier, J., N. Takahashi, and F. W. Putnam. 1984. Complete amino acid sequence of human plasma Beta-2-glycoprotein I. *Proc. Natl. Acad. Sci. USA.* 81:3640–3644.

4. McNeil, H. P., R. J. Simpson, C. N. Chesterman, and S. A. Krilis. 1990. Antiphospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation: beta-2-glycoprotein I (apolipoprotein H). *Proc. Natl. Acad. Sci. USA.* 87:4120–4124.

5. Love, P. E., and S. A. Santoro. 1990. Antiphospholipid antibodies: anticardiolipin and the lupus anticoagulant in systemic lupus erythematosus (SLE) and in non-SLE disorders: Prevalence and clinical significance. *Ann. Int. Med.* 112:682–698.

6. Ginsburg, K. S., M. H. Liang, L. Newcomer, S. Z. Goldhaber, P. H. Schur, C. H. Hennekens, and M. J. Stampfer. 1992. Anticardiolipin antibodies and the risk for ischemic stroke and venous thrombosis. *Ann. Int. Med.* 117:997–1002.

7. Gharavi, A. E., R. C. Mellors, and K. B. Elkon. 1989. IgG anti-cardiolipin antibodies in murine lupus. *Clin. Exp. Immunol.* 78:233–238.

8. Branch, D. W., D. J. Dudley, M. D. Mitchell, K. A. Creighton, T. M. Abbott, E. H. Hammond, and R. A. Daynes. 1990. Immunoglobulin G fractions from patients with aPL antibodies cause fetal death in BALB/c mice: a model for autoimmune fetal loss. *Am. J. Obstet. Gynecol.* 163:210–216.

9. Cariou, R., G. Toblem, S. Bellucci, J. Soria, C. Soria, J. Maclouf, and J. Caen. 1988. Effect of lupus anticoagulant on antithrombogenic properties of endothelial cells-inhibition of thrombomodulin-dependent protein C activation. *Thromb. Haemostasis.* 60:54–58.

10. Smirnov, M. D., D. T. Triplett, P. C. Comp, N. L. Esmon, and C. T. Esmon. 1995. On the role of phosphatidylethanolamine in the inhibition of activated protein C activity by antiphospholipid antibodies. *J. Clin. Invest.* 95:309–316.

11. Shih, W., B. H. Chong, and L. N. Chesterman. 1993. Beta-2-glycoprotein 1 is a requirement for anticardiolipin antibody to activated platelets: differences with lupus anticoagulants. *Blood.* 81:1255–1262.

12. Lindsey, N. J., R. A. Dawson, F. I. Henderson, M. Greaves, and P. Hughes. Stimulation of von Willebrand factor antigen release by immunoglobulin from thrombosis prone patients with systemic lupus erythematosus and the anti-phospholipid syndrome. 1993. *Br. J. Rheum.* 32:123–126.

13. Cines, D. B., A. P. Lyss, M. Reeber, M. Bina, and R. J. DeHoratius. 1984. Presence of complement-fixing anti-endothelial cell antibodies in systemic lupus erythematosus. *J. Clin. Invest.* 73:611–625.

14. Del Papa, N., P. L. Meroni, A. Tincani, E. N. Harris, S. S. Pierangeli, W. Barcellini, M. O. Borghi, G. Balestrieri, and C. Zanussi. 1992. Relationship between anti-phospholipid and anti-endothelial cell antibodies: further characterization of the reactivity on resting and cytokine-activated endothelial cells. *Clin. Exp. Rheum.* 10:37–42.

15. McCrae, K. R., A. DeMichele, P. Samuels, D. Roth, A. Kuo, Q.-H. Meng, J. Rauch, and D. Cines D. 1991. Detection of endothelial cell-reactive immunoglobulin in patients with anti-phospholipid antibodies. *Br. J. Haematol.* 79:595–605.

16. Hess, D. C., J. C. Shepard and R. J. Adams. 1993. Increased immunoglobulin binding to cerebral endothelium in patients with antiphospholipid antibodies. *Stroke.* 24:994–999.

17. Polz, E., H. Wurm, and G. M. Kostner. 1980. Investigation on β_2-glycopro-

tein I in the rat: isolation from serum and demonstration in lipoprotein density fractions. *Int. J. Biochem.* 11:265–270.

18. Sammaritano, L. R., M. A. Lockshin, and A. E. Gharavi. 1992. Antiphospholipid antibodies differ in aPL cofactor requirement. *Lupus.* 1:83–90.

19. Ishizaka, K., and T. Ishizaka. 1960. Biological activity of aggregated gamma-globulin II. A study of various methods for aggregation and species differences. *J. Immunol.* 85:163–166.

20. Jaffe, E. A., R. L. Nachman, C. G. Becker, and C. R. Minick. 1973. Culture of human endothelial cells derived from umbilical cord veins. Identification by morphologic and immunologic criteria. *J. Clin. Invest.* 52:2745–2756.

21. Ziegler-Heitbrock, H. W., E. Thiel, A. Futterer, V. Herzog, A. Wirtz, and G. Riethmuller. 1988. Establishment of a human cell line (Mono Mac 6) with characteristics of mature monocytes. *Int. J. Cancer.* 41:456–461.

22. Tan, E. M., A. S. Cohen, J. F. Fries, A. T. Masi, D. J. McShane, N. F. Rothfield, J. C. Schaller, N. Talal, and P. J. Winchester. 1982. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis & Rheum.* 25:1271–1277.

23. Gharavi, A. E., E. N. Harris, R. A. Asherson, and G. R. V. Hughes. 1987. Anticardiolipin antibodies: isotype distribution and phospholipid specificity. *Am. Rheum. Dis.* 46:1–6.

24. Sammaritano, L. R., A. E. Gharavi, C. Soberano, R. A. Levy, and M. D. Lockshin. 1992. Phospholipid binding of antiphospholipid antibodies and placental anticoagulant protein. *J. Clin. Immun.* 12:27–35.

25. Gharavi, A. E., L. R. Sammaritano, J. Wen, and K. B. Elkon. 1992. Induction of antiphospholipid antibodies by immunization with β_2-glycoprotein I (apolipoprotein H). *J. Clin. Invest.* 90:1105–1109.

26. Rosenbaum, J., B. E. Pottinger, P. Woo, C. M. Black, S. Loizou, M. A. Byron, and J. D. Pearson. 1988. Measurement and characterization of circulating anti-endothelial cell IgG in connective tissue diseases. *Clin. Exp. Immunol.* 72:450–456.

27. Lo, S. K., A. Cheung, Q. Zheng, and R. L. Silverstein. 1995. Induction of tissue factor on monocytes by adhesion to endothelial cells. *J. Immunol.* 154:4768–4777.

28. Thornhill, M., S. M. Wellicome, D. L. Mahiouz, J. S. S. Lanchbury, U. Kyang-Aung and D. O. Haskard. 1991. TNF combines with IL-4 or IFN-gamma to selectively enhance endothelial cell adhesiveness for T cells: the contribution of vascular cell adhesion molecule-1-dependent and independent mechanisms. *J. Immunol.* 146:592–598.

29. Nachman, R. L., and R. L. Silverstein. 1993. Hypercoagulable states. *Ann. Intern. Med.* 119:819–827.

30. Bevilacqua, M. P., J. S. Pober, M. E. Wheeler, R. S. Cotran, and M. A. Gimbrone, Jr. 1985. Interleukin-1 acts on cultured human vascular endothelium to increase the adhesion of polymorphonuclear leukocytes, monocytes, and related leukocyte cell lines. *J. Clin. Invest.* 76:2003–2011.

31. Del Papa, N., G. Conforti, D. Gambini, W. Barcellini, M. O. Borghi, C. Fain, A. Tincani, G. Balestrieri, F. Tedesco, and P. L. Meroni. 1993. Characterization of anti-endothelial cell antibodies in antiphospholipid syndrome. *In* Molecular Basis of Human Diseases. E. E. Polli, editor. Elsevier Science Publishers, Amsterdam. 67–73.

32. Kornberg, A., M. Blank, S. Kaufman, and Y. Schoenfeld. 1994. Induction of tissue factor-like activity in monocytes by anti-cardiolipin antibodies. *J. Immunol.* 153:1328–1332.