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Endothelial leukocyte adhesion molecule 1: Direct expression cloning and functional interactions

(cell adhesion/inflammation/endothelium/cytokine)

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ABSTRACT **A cDNA for endothelial leukocyte adhesion molecule 1 (ELAM-1) was isolated by transient expression in COS-7 cells of a subtracted cDNA library from cytokine-treated human umbilical vein endothelial cells (HUVECs), with selection of ELAM-1-expressing clones by adhesion of transfected cells to the human promyelocytic cell line HL-60. This cloning method requires neither antibody nor purified ligand. ELAM-1-expressing COS cells bind the promyelocytic cell line HL-60 by a Ca^{2+}-dependent but temperature-independent mechanism. Although ELAM-1 is homologous to mammalian lectins, its interaction with HL-60 cells is not inhibited by simple carbohydrate structures. ELAM-1-expressing COS cells also bind human neutrophils and the human colon carcinoma cell line HT-29, but not the B-cell line Ramos. However, Ramos cells adhere to cytokine-treated HUVECs but not control HUVECs, confirming the existence of other inducible adhesion molecules. In addition, the binding of HL-60 cells or neutrophils to ELAM-1-expressing COS cells is not inhibited by a monoclonal antibody (60.3) directed to an inhibitory epitope on CD18, indicating that the ELAM-1 ligand, although uncharacterized, is not a member of the CD11/CD18 family.**

Localized adhesion of neutrophils (polymorphonuclear leukocytes, PMNs) to the endothelial lining is essential for their egress from the vascular space under physiologic conditions and is a key event in the initiation and maintenance of numerous PMN-mediated pathological states (1, 2). Recent studies have delineated two fundamental mechanisms of PMN adhesion to vascular endothelium at sites of acute inflammation (reviewed in refs. 3 and 4). Certain inflammatory mediators such as leukotriene B_4 and complement fragment 5a act directly on the PMNs, which become hyperadhesive for endothelium. PMN adhesion is mediated by a group of cell surface proteins called leukocyte cell adhesion molecules (LeuCAMs), or the CD11/CD18 family (3, 5), and monoclonal antibodies (mAbs) to these cell surface proteins inhibit PMN adhesion to endothelium both *in vitro* and *in vivo* (3, 6, 7). In an alternative and complementary mechanism, inflammatory cytokines such as interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α), as well as bacterial endotoxin (lipopolysaccharide), can act directly on endothelial cells *in vitro* to substantially augment PMN adhesion (4, 8–10).

With the use of mAbs, a 116-kDa cell surface glycoprotein, endothelial leukocyte adhesion molecule 1 (ELAM-1), has been identified that contributes to the adhesion of PMNs to cytokine-treated, but not untreated, human umbilical vein endothelial cells (HUVECs) (10). It has recently been cloned (11) and characterized as a member of a new family of adhesion molecules related to mammalian lectins, epidermal growth factor, and complement regulatory proteins (11–13). Even in the continued presence of cytokines the appearance of ELAM-1 is transient and parallels the time course of extravascular PMN accumulation *in vivo* (4, 9). Moreover, immunohistologic studies show that ELAM-1 is present *in vivo* at inflammatory sites but is absent from the noninflamed vessel wall (14). Taken jointly, these results suggest that ELAM-1 is a key mediator of PMN adhesion to the inflamed vascular wall *in vivo*.

We have begun to examine the feasibility of direct expression cloning of adhesion molecules, using cell adhesion itself as the functional assay. By using the promyelocytic cell line HL-60 as the target cell, we have achieved the direct expression cloning of ELAM-1, as well as its initial functional characterization.†

MATERIALS AND METHODS

Materials. All reagents were obtained from Sigma except [^{35}S]cysteine, [^{35}S]methionine, and [*methyl*-^{3}H]thymidine (DuPont/NEN) and human serum albumin (HSA) glycoconjugates (Accurate Chemicals, Westbury, NY). Homogeneous recombinant human IL-1β (10^7 units/mg) and TNF-α (5.3×10^6 units/mg) were from Biogen stocks. mAb 60.3 was the gift of John Harlan (University of Washington, Seattle; ref. 3).

Cells. HUVECs were isolated and subcultured as described (9) and used for adhesion assays or library construction at passages 3–5. HL-60, U-937, Ramos, COS-7, and HT-29 cells were obtained from the American Type Culture Collection and were propagated in RPMI-1640/10% fetal bovine serum (FBS) with 2 mM glutamine, except COS-7 cells, which were grown in Dulbecco's modified Eagle's medium (DMEM)/10% FBS with 2 mM glutamine. PMNs were isolated from anticoagulated blood by gradient centrifugation (Lymphocyte Separation Medium, Organon), 3% dextran sedimentation, and hypotonic lysis (15).

cDNA Library Construction and Subtraction. RNA was isolated from HUVECs treated with recombinant human IL-1β (10 units/ml) for 2.5 hr, and a cDNA library was constructed in the animal cell expression vector CDM8 (16, 17). To enrich for IL-1β-induced sequences the library was screened with a subtracted cDNA probe, generated by two rounds of hybridization to mRNA from uninduced HUVECs (18), resulting in a sublibrary of 864 colonies, representing about 0.09% of the original library.

Isolation of ELAM cDNA Clones by Cell–Cell Adhesion. COS cells were transfected with the sublibrary by spheroplast fusion (17) and were screened for the ability to bind

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Abbreviations: ELAM-1, endothelial leukocyte adhesion molecule 1; PMN, polymorphonuclear leukocyte (neutrophil); LeuCAM, leukocyte cell adhesion molecule; IL-1β, interleukin 1β; TNF-α, tumor necrosis factor α; FBS, fetal bovine serum; HSA, human serum albumin; HUVEC, human umbilical vein endothelial cell; mAb, monoclonal antibody.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M30640).

HL-60 cells 48 hr posttransfection. HL-60 cells were labeled with carboxyfluorescein diacetate (19) and resuspended at 10^6 per ml in RPMI-1640/1% FBS. COS cell monolayers in 100-mm tissue culture dishes were washed and then incubated with 6 ml of HL-60 cells for 15 min at room temperature, nonadherent cells were removed with three or four washes of RPMI-1640/1% FBS, and the dishes were examined for clusters of adherent HL-60 cells by fluorescence microscopy. Regions containing clusters of HL-60 cells were picked and lysed with 0.6% SDS/10 mM EDTA, pH 8.0, for plasmid rescue (17), and the DNA was used to transform into *Escherichia coli* strain MC1061/p3. Colonies were regrown for a second round of spheroplast fusion, and after the second round, individual colonies were transfected into COS cells to identify an expressing clone, designated ELAM/CDM8.

Adhesion Assays. HUVECs were grown to confluence in 48-well cluster plates and pretreated with saturating amounts of cytokine (IL-1β, 10 units/ml, or TNF-α, 10 ng/ml, unless otherwise stated) for 4 or 24 hr. Both cytokines gave similar results. COS cells (10^7 per ml in 20 mM Hepes, pH 7.05/137 mM NaCl/5 mM KCl/0.7 mM Na_2HPO_4/6 mM dextrose) were transfected by electroporation (280 V, 960 μF, Bio-Rad Gene Pulser) with 20 μg of ELAM/CDM8 DNA or CDM8 control DNA and 200 μg of sonicated salmon sperm DNA and, after 10 min, washed, resuspended in medium, and plated directly at 10^5 cells per cm^2 in 48-well plates. Cells were confluent after 48 hr and were used after 48 or 72 hr. Based on control experiments, 5–25% of the transfected cells expressed protein. Target cells (except PMNs) were labeled for 24–48 hr with [^{3}H]thymidine or [^{35}S]methionine (0.5 μCi/ml; 1 μCi = 37 kBq) and washed twice to remove excess label. All target cells were resuspended in RPMI-1640/1% FBS at 2–3 $\times$ 10^6 per ml. Adhesion assays were performed as described (10). Briefly, confluent monolayers were washed once with RPMI-1640/1% FBS, and 250 μl of target cells was added. After a 15- to 20-min incubation at room temperature, cell monolayers were washed twice, centrifuged in an inverted configuration, and washed once more. Adherent cells were assessed visually and quantified either by scintillation counting or, for adherent PMNs, by endogenous myeloperoxidase content by standard assays. For inhibition assays, target cells were preincubated for 10 min at room temperature with mAb 60.3 (50 μg/ml), cells were added to washed monolayers, and the assays were performed as above.

Biosynthetic Labeling. Confluent monolayers of HUVECs, cytokine-treated or untreated, or COS cells transfected with ELAM/CDM8 or control CDM8 were washed, incubated in cysteine-free medium for 45 min, and incubated with [^{35}S]cysteine in cysteine-free RPMI-1640/10% dialyzed FBS for 2 hr at 37°C. Monolayers were washed thoroughly, and the cells were harvested nonenzymatically and extracted at 4°C with either 2% (vol/vol) Triton X-100 or 2% (vol/vol) Triton X-114. Triton X-100 extracts were fractionated by wheat germ lectin chromatography; bound proteins were eluted with 0.3 M *N*-acetylglucosamine, concentrated by acetone precipitation, and subjected to SDS/PAGE in 4–20% gradient gels. Triton X-114 extracts were phase-separated (20), and membrane proteins associated with the detergent-rich phase were acetone-precipitated and subjected to two-dimensional gel electrophoresis (21).

RESULTS AND DISCUSSION

Isolation of a cDNA Encoding Functional ELAM-1. Direct expression cloning using a combination of transient expression vectors and specific mAbs has become an established technique for the cloning of cell surface molecules (17, 22). To extend the range of direct expression cloning methods, and to obviate the need for mAb generation, we examined the feasibility of using a functional assay to clone cell surface molecules involved in cell–cell adhesion. Because ELAM-1 is absent from normal endothelium but strongly induced by cytokines (10, 11), a preliminary subtraction was performed to generate a sublibrary highly enriched for IL-1β-induced sequences. This sublibrary was transfected into COS-7 cells by spheroplast fusion, and cells expressing adhesion molecules for HL-60 cells were identified by rosette formation. In the first round of panning, 20 rosettes per plate were picked at random and plasmids were rescued into *E. coli* MC1061/p3. A second round of panning and rescue was performed in the same way. For the third round, individual clones were transfected, and from this screen a full-length cDNA expressing a functional ELAM-1 was obtained. The cDNA, designated ELAM/CDM8, was capable of supporting the adhesion of both HL-60 cells and human PMNs (see below).

In parallel experiments, miniprep DNA was prepared from 24 randomly chosen colonies of the sublibrary, and inserts were examined for size and complexity. Two contained insert DNA of 3 kilobases that hybridized to a 4-kilobase mRNA present only in IL-1β-induced HUVECs. These two inserts also hybridized to ELAM/CDM8, indicating that the ELAM mRNA represented one of the major cytokine-induced messages in HUVECs. The complete DNA sequence of the ELAM was obtained (23) from these three overlapping clones. Two of the inserts were subcloned into *Not* I-digested pNN11. The insert from ELAM/CDM8 was sequenced directly. Sequence was obtained from both DNA strands. The sequence of ELAM/CDM8 (Fig. 1) confirmed its identity with that of ELAM-1. However, our sequence includes 24 additional bases of 5′ untranslated region and also differs at 5 nucleotides. One (C $\rightarrow$ T) results in a single amino acid difference (His-477 $\rightarrow$ Tyr-477; numbering as in ref. 11). The other differences are in the 3′ untranslated region.

To date, the cloning of cell surface molecules by direct expression and functional assay has been achieved only when the cognate ligand has been defined and available as a homogeneous protein. Examples include the use of interleukin 6 to clone its receptor (24) and the use of lymphocyte function-associated antigen 1 (LFA-1) to clone intercellular adhesion molecule 2 (ICAM-2) (25). However, to our knowledge, the cloning of a cell surface protein by functional assay in the complete absence of information about its cognate ligand has not been reported.

Functional Studies of ELAM-1-Mediated Adhesion. Using HL-60 binding to either ELAM-1-transfected or control CDM8-transfected COS cells, we performed a series of functional studies (Fig. 2). HL-60 adhesion occurs at 4°C, indicating that the ELAM-1 pathway is temperature-independent. In addition, HL-60 binding is Ca^{2+}-dependent (Fig. 2). When low levels of the Ca^{2+}-selective chelating agent EGTA were present, adhesion was restored only in the presence of excess Ca^{2+}. Because ELAM-1 has an N-terminal domain related to mammalian lectins and is homologous to the murine lymphocyte homing receptor, which has established lectin-like activity, the effects of simple carbohydrates on HL-60 binding were also examined. Neither mannose 6-phosphate nor fucoidan, at concentrations known to inhibit the homing receptor-mediated binding of lymphocytes to high endothelial venules (26), inhibited the ELAM-1–HL-60 interaction (Fig. 2). In addition, no inhibition was seen with 10 mM galactose, galactose 1-phosphate, galactose 6-phosphate, fucose, mannose, or *N*-acetylglucosamine (data not shown). For some lectin-based cell–cell interactions, diffusible sugars are not inhibitory, and carbohydrates coupled to a protein carrier such as albumin are required for inhibition (27). However, HSA–fucose, HSA–mannose, or HSA–galactose (100 μM) did not inhibit adhesion (data not shown). The results suggest that if the adhesive function of ELAM-1 is dependent upon lectin-like activity, it is most likely restricted to a complex carbohydrate structure that cannot be mimicked by simple sugars.

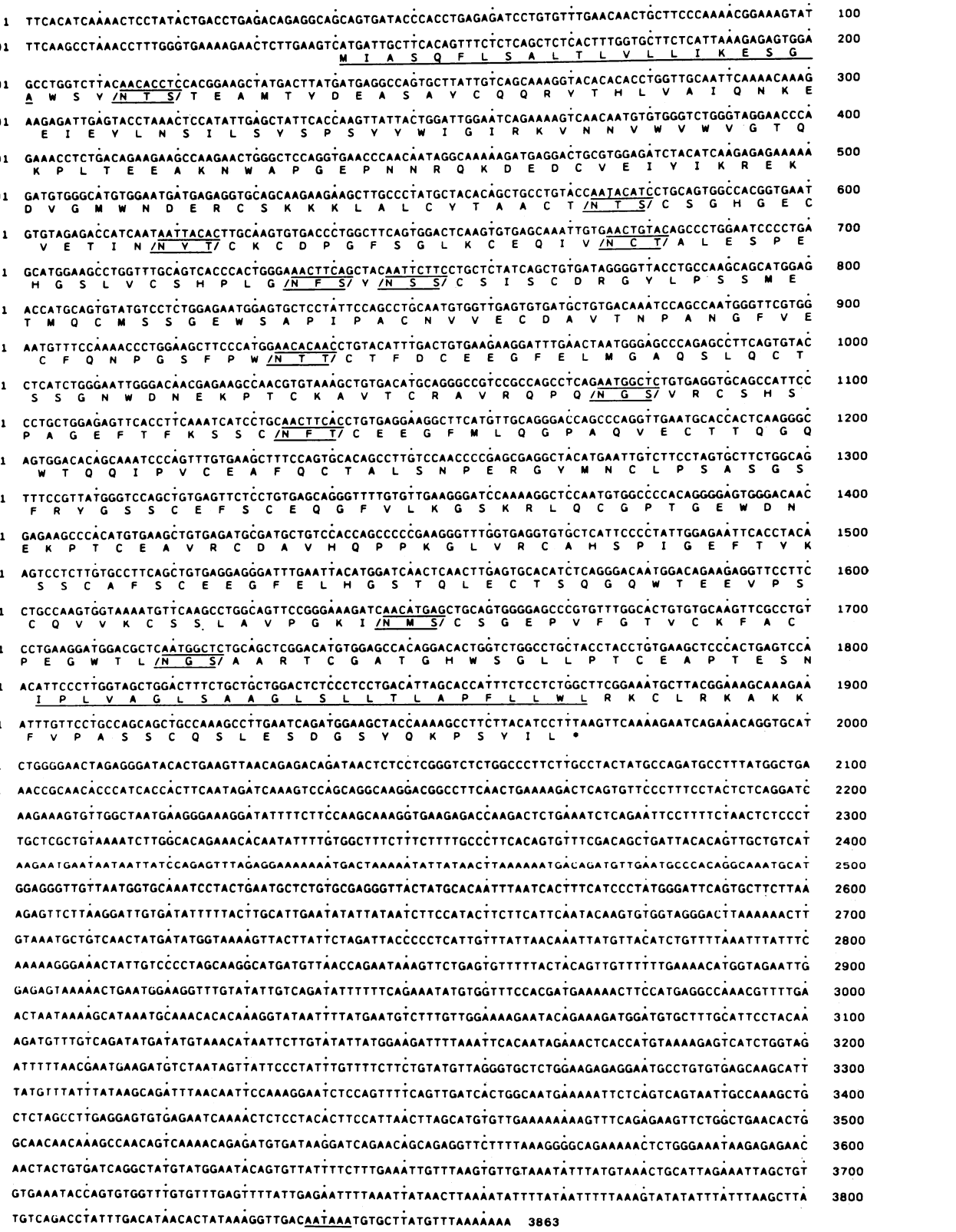

FIG. 1. Nucleotide sequence of the ELAM-1 cDNA and predicted amino acid sequence of the protein. Nucleotide numbers are given at left and at right. The putative signal sequence and transmembrane sequence are underlined, and potential N-glycosylation sites are boxed.

ELAM-1 Biosynthesis. In the absence of an antibody, we chose to use lectin chromatography to demonstrate ELAM-1 biosynthesis. ELAM-1 is very cysteine-rich and was readily labeled relative to other proteins with [^{35}S]cysteine. Moreover, fractionation of labeled, Triton X-100-extracted cells by wheat germ lectin chromatography readily demonstrated the presence of proteins of apparent M_r 96,000 and 110,000 in ELAM-1-transfected COS cells, and IL-1β-treated (4 hr) HUVECs, respectively, but not in appropriate controls (data not shown), consistent with previously reported data (11). Labeled transfected COS cells and cytokine-treated HUVECs were also extracted with Triton X-114 and subjected to

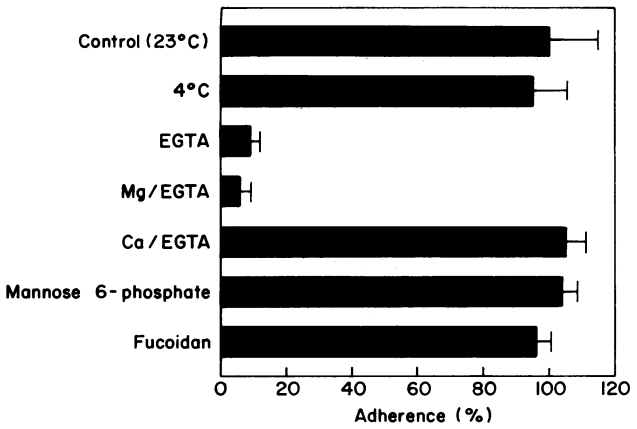

FIG. 2. Functional studies. The percentage of HL-60 cells bound to ELAM-1-expressing COS cells (mean + SEM) is indicated. For divalent cation studies, both cell types were washed three times with Hanks' balanced salt solution without Ca^{2+} and Mg^{2+}. HL-60 cells were preincubated for 15 min at 23°C with additives (2 mM Ca^{2+} or Mg^{2+} and 0.5 mM EGTA) prior to assay. Other assays were performed in RPMI-1640/10% FBS. For carbohydrate studies, both cell types were preincubated for 15 min at 23°C with 10 mM mannose 6-phosphate or 25 μg of fucoidan per ml. For temperature-dependence studies, both cell types were preincubated for 60 min at 4°C.

two-dimensional gel electrophoresis. ELAM-1 was extracted into Triton X-114, as expected for a typical membrane protein, and was readily detected upon two-dimensional electrophoresis (Fig. 3). ELAM-1 can be resolved into several closely related species, with acidic isoelectric points of about 5.0. The observed charge and size heterogeneity probably reflects ongoing carbohydrate processing (11).

The Ligand for ELAM-1 Is Not a LeuCAM. LeuCAMs are heterodimers with a common β subunit, CD18, and distinct α subunits, CD11a, CD11b, and CD11c, for LFA-1, Mac-1, and gp150,95, respectively. mAbs to inhibitory epitopes either on the individual α chains or on the common β-chain effectively inhibit adhesion of activated PMNs to HUVECs

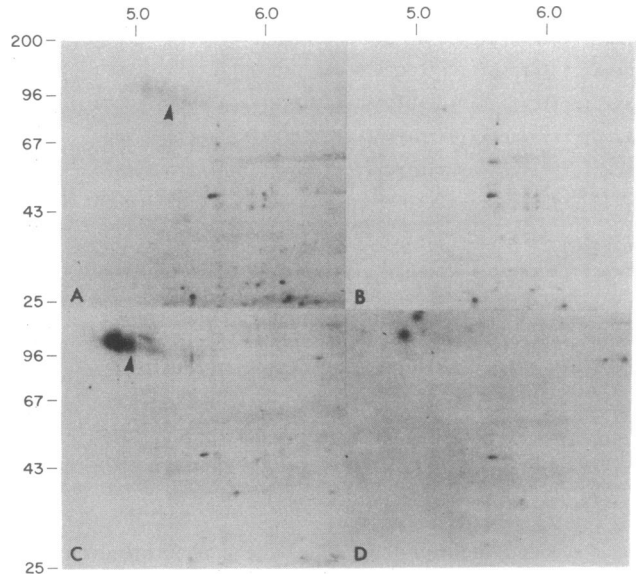

FIG. 3. Two-dimensional gel electrophoresis of Triton X-114 extracts of [^{35}S]cysteine-labeled COS-7 cells (*A* and *B*), either transfected with ELAM/CDM8 (*A*) or with CDM8 control (*B*), or HUVECs (*C* and *D*) either treated for 4 hr with IL-1β (10 units/ml) (*C*), or untreated (*D*). ELAM-1 (arrowheads) was present only in transfected COS cells or cytokine-treated HUVECs. Molecular weights ($\times 10^{-3}$) are indicated at left, and approximate isoelectric points are indicated at top.

in vitro (3, 5, 28). They also inhibit significantly the adhesion of unactivated PMNs to cytokine-treated HUVECs (3, 5, 28, 29), indicating that LeuCAMs are also involved in PMN adhesion under conditions where the ELAM-1 pathway is operative. To examine the contribution of LeuCAMs to ELAM-1-dependent adhesion, we performed adhesion assays in the presence or absence of mAb 60.3, which binds a common inhibitory epitope on all three LeuCAMs. In our assays mAb 60.3 inhibited the binding of PMNs to cytokine-treated (4 hr) HUVECs by about 70% but had no effect on the binding of PMNs to COS cells transfected with ELAM-1 (Fig. 4*A*). mAb 60.3 had no effect on the binding of HL-60 cells to either cytokine-treated HUVECs or ELAM-1-transfected COS cells (Fig. 4*B*), although the mAb bound to HL-60 cells as assessed by flow cytofluorometric analysis. Our results provide direct evidence that LeuCAMs are not obligate ligands for ELAM-1 and that ELAM-1 is recognized by an as yet undefined structure on the leukocyte surface.

ELAM-1-Mediated Adhesion of Other Cell Types. ELAM-1-expressing COS cells bind human peripheral blood PMNs (Fig. 4) and the promyelocytic cell lines HL-60 (Fig. 4) and U-937 (data not shown). Lymphocytes and lymphocytic cell lines show increased binding to cytokine-treated HUVECs (30, 31). Fig. 5*A* compares the binding of HL-60 and the B-cell line Ramos to cytokine-treated HUVECs. HL-60 cells bind well to HUVECs 4 hr after cytokine treatment but show substantially reduced binding at 24 hr, in agreement with the time course of ELAM-1 induction, and with previously published data (10, 11). In contrast, the increased binding of Ramos 4 hr after cytokine treatment does not diminish but is increased at 24 hr. Consistent with these observations, ELAM-1-expressing COS cells bind HL-60 cells but not Ramos cells (Fig. 5*B*). Since Ramos binding is not inhibited by mAb 60.3 (data not shown), thus eliminating a role for the intercellular adhesion molecule/lymphocyte function-associated antigen 1 (ICAM/LFA-1) pathway in this inter-

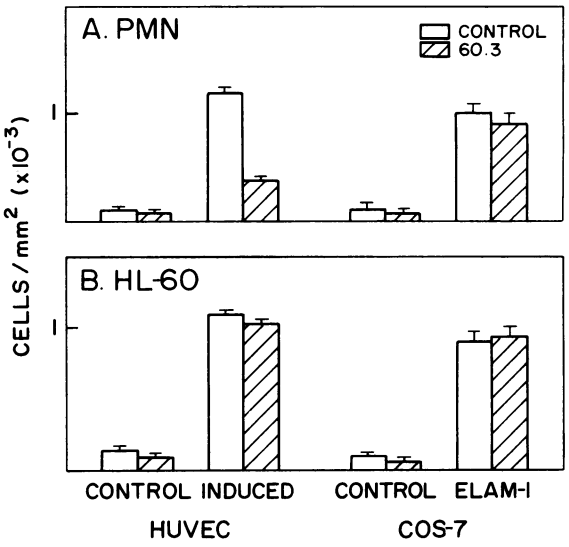

FIG. 4. Effects of mAb 60.3 on PMN and HL-60 binding. (*A*) PMN adhesion. In the absence of antibody (open bars), PMNs showed low, basal binding to control HUVECs or COS-7 cells but bound well to cytokine-treated (TNF-α-induced) HUVECs or ELAM-1-transfected COS cells. mAb (hatched bars) did not affect basal binding of PMNs to either cell type but significantly inhibited PMN adhesion to induced HUVECs. In contrast, 60.3 had no effect upon PMN adhesion to COS cells expressing ELAM-1. (*B*) HL-60 adhesion. In the absence of mAb 60.3 (open bars), HL-60 cells also showed low, basal binding to control HUVECs or COS cells and bound well to induced HUVECs or ELAM-1-transfected COS cells. mAb 60.3 (hatched bars) had no effect upon HL-60 adhesion to either cell type. Results are expressed as mean + SEM.

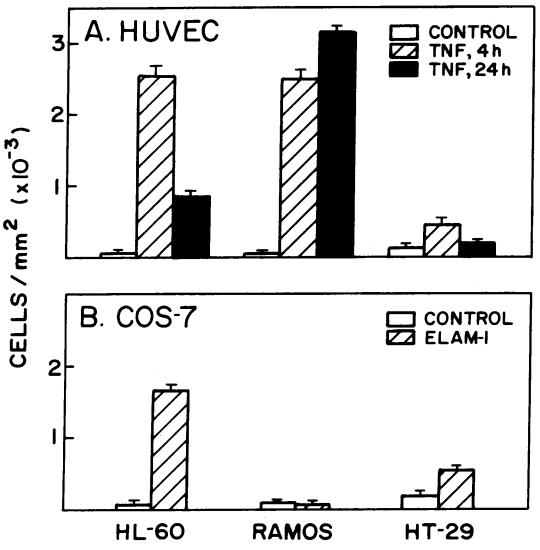

FIG. 5. Adhesion of HL-60, Ramos, and HT-29 cells to HUVECs (*A*) and COS-7 cells (*B*). (*Left*) HL-60 cells bound well to HUVECs treated with TNF-α for 4 hr (hatched bar) but showed diminished binding to cells treated for 24 hr (black bar). HL-60 cells bound well to ELAM-1-transfected COS cells (hatched bar). (*Center*) Ramos cells bound well to HUVECs treated with TNF-α for either 4 or 24 hr but did not bind to ELAM-1-transfected COS cells. (*Right*) HT-29 colon carcinoma cells showed a small but significant increase in adhesion to 4-hr-treated HUVECs but little adhesion to 24-hr-treated HUVECs. HT-29 cells bound to ELAM-1-transfected COS cells (lower right). Results are expressed as mean + SEM.

action, the results provide direct evidence not only for the existence of other adhesion molecules for lymphocytic cell lines but also for their interaction with non-LeuCAM ligands.

Two recent studies indicate that certain established cell lines derived from solid tumors show increased binding to cytokine-treated endothelium (32, 33). In one of these reports a series of colon carcinoma lines, including HT-29, was found to adhere maximally at 4–6 hr after cytokine treatment, with reduced adhesion at 24 hr, a time course consistent with ELAM-1-mediated binding (32). We therefore examined the binding of HT-29 cells to cytokine-treated HUVECs and to ELAM-1-transfected COS cells. Consistent with the published results, HT-29 cells showed a small but consistent increase in adhesion to cytokine-treated HUVECs at 4 hr, with diminished binding at 24 hr (Fig. 5*A*). HT-29 cells showed increased adhesion to ELAM-1-transfected COS cells (Fig. 5*B*), indicating that the adhesion of HT-29 cells to cytokine-treated HUVECs is mediated, at least in part, by ELAM-1. These data are consistent with the suggestion (32, 33) that circulating tumor cells derived from solid tumors may subvert the host inflammatory response and perhaps enhance their metastatic spread, by using induced leukocyte adhesion molecules such as ELAM-1 at sites of inflammation.

In summary, we have demonstrated that cell adhesion molecules such as ELAM-1 can be cloned by direct expression and functional assay, without requiring the generation of specific mAbs or even knowledge of the cognate ligand. The availability of the gene for ELAM-1 should allow us to rapidly dissect its role in PMN adhesion *in vitro* and to begin to understand its role in extravascular PMN accumulation at sites of acute inflammation under both physiologic and pathologic conditions.

Note Added in Proof. We have also cloned by direct expression a previously unreported endothelial cell adhesion molecule for lymphocytes, vascular cell adhesion molecule 1 (VCAM-1; ref. 34).

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