ELAM-1 Mediates Cell Adhesion by Recognition of a Carbohydrate Ligand, Sialyl-Le^x

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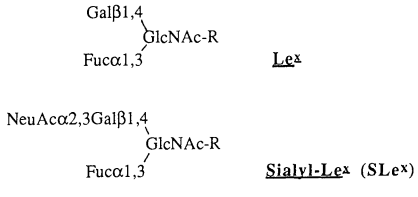
Recruitment of neutrophils to sites of inflammation is mediated in part by endothelial leukocyte adhesion molecule-1 (ELAM-1), which is expressed on activated endothelial cells of the blood vessel walls. ELAM-1 is a member of the LEC-CAM or selectin family of adhesion molecules that contain a lectin motif thought to recognize carbohydrate ligands. In this report, cell adhesion by ELAM-1 is shown to be mediated by a carbohydrate ligand, sialyl-Lewis X (SLe^x; NeuAcα2,3Galβ1,4(Fucα1,3)-GlcNAc-), a terminal structure found on cell-surface glycoprotein and glycolipid carbohydrate groups of neutrophils.

Leukocytes emigrate into tissues to destroy pathogenic microorganisms during an inflammatory response, but they can also destroy normal tissue in reperfusion injury and septic shock, and in chronic inflammatory diseases such as chronic dermatoses (for example, psoriasis) and rheumatoid arthritis (*1*). The vascular endothelium participates in the recruitment of neutrophils and other leukocytes by the expression of cell adhesion molecules in response to interleukin-1 (IL-1) and tumor necrosis factor–α (*2, 3*). ELAM-1 is produced within 2 to 4 hours of cytokine induction and mediates binding of neutrophils at endothelial foci (*3*). Subsequent extravasation of neutrophils across the endothelium into the surrounding tissue also requires the activation and interaction of the neutrophil integrin receptors LFA-1 and Mac-1 with their endothelial cell ligands, ICAM-1 and ICAM-2 (*4, 5*). The degree to which these two receptor systems may act synergistically in neutrophil recruitment and extravasation is of current interest.

ELAM-1 is a member of the selectin (LEC-CAM) family of cell adhesion molecules including Mel-14/LAM-1 and GMP-140/PADGEM, which also mediate leukocyte cell-cell interactions (*5, 6*). Because the NH_2-terminal domains of all three selectins have a lectin motif, they are postulated to recognize carbohydrate ligands (*5–8*).

We now show that ELAM-1 recognizes a carbohydrate ligand, containing the SLe^x sequence. The presence of this structure on both leukocyte and nonleukocyte cell lines is sufficient to cause ELAM-1–mediated adhesion to IL-1–activated human endothelial cells.

A survey of the glycolipids and N-linked carbohydrate groups of surface glycoproteins of neutrophils revealed uncommon terminal sequences with α(1,3)fucose residues known generically as Lewis X (Le^x) and SLe^x, as shown below, where in each case R is the remainder of the carbohydrate group (*9*).



To determine if these structures were recognized by ELAM-1, we used glycosylation mutants of Chinese hamster ovary (CHO) cells, LEC11 and LEC12, which make these structures (*10*). These glycosylation mutants differ from the wild-type CHO cells (CHO-K1) by their expression of two distinct α(1,3)fucosyltransferases that allow the synthesis of Le^x in both LEC11 and LEC12 cells, and the synthesis of SLe^x in LEC11 cells only.

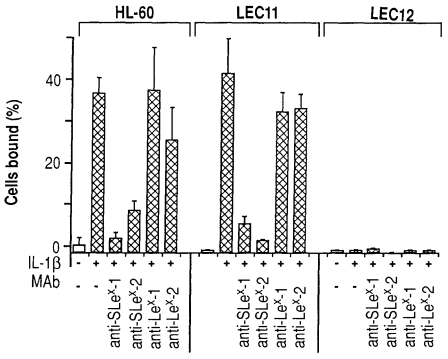


Fig. 2. Antibodies to SLe^x inhibit ELAM-1–mediated cell adhesion of HL-60 and LEC11 cells. Cell-adhesion assays were performed as in Fig. 1, except that HL-60 cells were incubated for 20 min at 37°C in the presence or absence of MAbs to carbohydrates before assay (*19*). MAbs specific for the carbohydrate epitope SLe^x were CSLEX (IgM; anti–SLe^x-1) and SNH-4 (IgG3; anti–SLe^x-2), and those specific for Le^x were SH-1 (IgM; anti–Le^x-1) and FH-2 (IgM; anti–Le^x-2).

LEC11 cells adhered to IL-1–activated human endothelial cells, but neither LEC12 nor wild-type CHO cells adhered (Fig. 1A), suggesting that the LEC11 unique carbohydrate structure, SLe^x, may be mediating adhesion. The binding of LEC11 cells to activated endothelium was mediated predominantly through ELAM-1, because a monoclonal antibody (MAb) to ELAM-1 blocked adhesion (Fig. 1B). Adhesion of the promyelocytic cell line HL-60 was also predominantly ELAM-1–dependent (*11*) (Fig. 1B). The reported contribution of V-CAM–mediated adhesion of HL-60 cells (*12*) was also assessed with MAbs to α_4 and was found to be a lesser component for HL-60 cells. Neither HL-60 cells nor any of the CHO cell lines tested adhered to resting endothelial cells. Thus, the results suggested that SLe^x was mediating LEC11 adhesion to ELAM-1.

To test the possibility that ELAM-1–mediated adhesion of LEC11 and HL-60 cells occurred by recognition of an SLe^x ligand, we treated both cell lines with a panel of MAbs to SLe^x and Le^x before

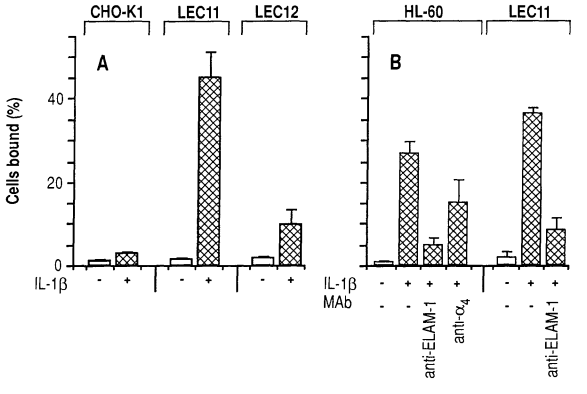


Fig. 1. ELAM-1–dependent adhesion of LEC11 and HL-60 cell lines to IL-1β–activated endothelial cells (**A**) Binding of wild-type CHO-K1 and CHO glycosylation mutants LEC11 and LEC12 to human umbilical cord vein endothelial cells (*11*) before and after activation with IL-1β. (**B**) Blocking MAbs to endothelial cell adhesion mediated by ELAM-1 (anti–ELAM-1; H18/7) and V-CAM (anti-α_4; P4C2) were used to examine the relative contribution of these molecules to adhesion of HL-60 cells and LEC11 cells.

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incubation with activated endothelial cells (Fig. 2). MAbs to SLe^x blocked adhesion of HL-60 cells and LEC11 cells by up to 90%, whereas MAbs to Le^x only inhibited adhesion slightly. Prior treatment of activated endothelial cells with MAbs to SLe^x had no effect on adhesion. In separate experiments, treatment of HL-60 cells or LEC11 cells with *Clostridium perfringens* neuraminidase to destroy SLe^x (Sigma type X; 1.6 U/ml for 90 min at 37°C) reduced their adhesion to activated endothelial cells (70 to 85%), whereas the same treatment had negligible effect on the ELAM-1–independent adhesion of Jurkat cells. Thus, ELAM-1–mediated adhesion of HL-60 and LEC11 cells is through recognition of cell-surface carbohydrate groups that contain SLe^x.

SLe^x structures are found to terminate the carbohydrate groups of both glycoproteins and glycolipids (*9*). To investigate the potential for an SLe^x ligand to competitively block ELAM-1–mediated cellular adhesion, we incorporated selected glycolipids with terminal sequences containing SLe^x, Le^x, and closely related structures (Table 1) into liposomes and exposed them to activated endothelial cells at 4°C (Fig. 3). Subsequent challenge with HL-60 cells revealed that liposomes containing glycolipids with SLe^x (S-diLe^x) inhibited adhesion up to 90%, whereas liposomes that contained glycolipids with Le^x (di-Le^x) or other related carbohydrate structures were minimally inhibitory. Comparable results were obtained with LEC11 cell adhesion. When the same experiment was done at 37°C, HL-60 cell adhesion was reduced by liposomes containing glycolipids with the SLe^x structure (S-diLe^x, 70%), and also to a lesser extent by liposomes containing Le^x (diLe^x, 40%), suggesting that Le^x may also interact with ELAM-1, but with lower affinity.

In contrast to adhesion with HL-60 and LEC11 cells, Jurkat cells bind to IL-1–activated endothelial cells predominantly through the V-CAM (endothelial cell)–VLA-4 (Jurkat cell) adhesion pair (*13*). Jurkat cell adhesion was not inhibited by liposomes containing SLe^x, but was completely inhibited by a MAb to the α subunit of the integrin molecule VLA-4. Thus, SLe^x liposome inhibition of HL-60 cells and LEC11 cells is not a steric effect attributed to binding of liposomes to endothelial cells. Instead, SLe^x liposomes inhibit adhesion of HL-60 cells through a direct competition with the ligand binding site of ELAM-1.

Identification of a carbohydrate ligand for ELAM-1 is an example of protein-carbohydrate recognition mediating cell-cell interactions. SLe^x is expressed on blood cells of the myeloid lineage, but not erythrocytes or most lymphocytes, consistent with the selective adhesion of myeloid cells to ELAM-1 (*9*). As documented for the LEC11 glycosylation mutants, expression of SLe^x is most likely regulated by the expression of a specific α(1,3)fucosyltransferase (*10, 14*). SLe^x is also expressed on a subset of human NK cells (*15*) and a variety of lung and colon carcinomas (*16*). Thus, ELAM-1 might also have a role in the adhesion of other cell types that carry the SLe^x ligand.

Because ELAM-1 is believed to be involved in the recruitment of leukocytes to sites of inflammation in both acute and chronic pathological conditions in vivo (*1, 17*) and may establish metastasis of certain carcinomas (*18*), blocking ELAM-1 function may be of therapeutic value. As shown by the inhibition of ELAM-1–mediated cell adhesion by liposomes containing SLe^x (Fig. 3), a strategy for inhibiting leukocyte adhesion aimed at the carbohydrate ligand could be a viable alternative to current approaches with MAbs to cell adhesion molecules.

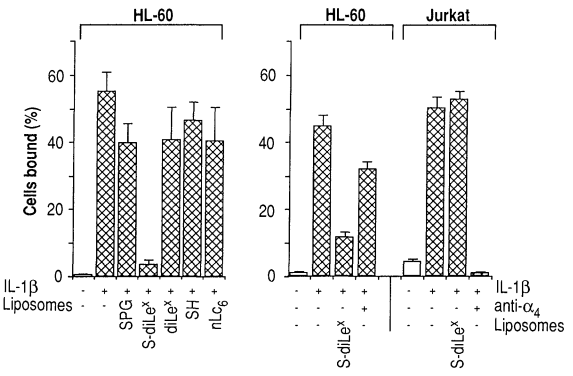

Fig. 3. Liposomes containing glycolipids with the SLe^x ligand block binding of HL-60 cells but not Jurkat cells to IL-1β–activated endothelial cells. Before the binding assays (*11*), activated endothelial cells were incubated with liposomes (*20*) containing a glycolipid (5 μg/ml) with terminal structures representing SLe^x (S-diLe^x), Le^x (diLe^x), or other related structures as listed in Table 1. Jurkat cells were used as a control to assess the ability of liposomes containing SLe^x glycolipids to influence V-CAM mediated adhesion. V-CAM adhesion was inhibited with a MAb to α_4 (*11*).

Table 1. Glycoplipids tested for liposome inhibition of ELAM-1–mediated cell adhesion in Fig. 3.

Generic	IUPAC	Structure
nLc_6	nLc_6	Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer
diLe^x	III^{3}V^{3}Fuc$_2$nLc$_6$	Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer 3 3 ↑ ↑ Fucα1 Fucα1
SPG	IV^{3}NeuAcnLc$_4$	NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer
SH	VI^{3}NeuAcnLc$_6$	NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer
S-diLe^x	III^{3}V^{3}Fuc$_2$VI^{3}NeuAcnLc$_6$	NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer 3 3 ↑ ↑ Fucα1 Fucα1

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9. Our approach was based on the assumption that ELAM-1 recognizes a carbohydrate ligand. Accordingly, published reports of carbohydrate structures of neutrophils and the promyelocytic cell line HL-60 [S. J. Collins, R. C. Gallo, R. E. Gallagher, *Nature* **270**, 347 (1977)] were surveyed for ligand candidates [A. Mizoguchi, S. Takasaki, S. Maeda, A.

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11. ELAM-1–mediated binding was measured as previously described [A. Dobrina *et al.*, *Immunology* **67**, 502 (1989)]. MAbs were added to appropriate wells as follows: antibody to ELAM-1 (anti–ELAM-1) [H18/7, immunoglobulin G2a (IgG2a)] (*3*) was used at 2 μg/ml; antibody to α4 (anti-α4) (P4C2, IgG3) (*13*) was used as a 1:100 dilution of ascites fluid. Cell adhesion was performed at 4°C to reduce nonspecific adhesion of LEC11 cells. Each experiment was repeated with at least two independently derived endothelial cell cultures. Data is expressed as the mean percent of input cells bound for triplicate samples and the SEM is given in each case.
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19. ELAM-1 cell adhesion was measured as in Fig. 1, except that washed, ^{51}Cr-labeled HL-60, LEC11, or LEC12 cells were incubated for 20 min at 37°C and chilled for 20 min at 4°C in the presence of MAb containing hybridoma culture supernatants [anti–SLe^x-2; SNH-4, IgG3 (A. K. Singhal, E. Nudelman, S. Hakomori, unpublished results); anti–Le^x-1; SH-1, IgG3 and anti–Le^x-2; FH-2, IgM (A. Singhal *et al.*, *Cancer Res.* **50**, 1375 (1990)], or purified MAb diluted to 10 μg/ml in DMEM containing 5% fetal calf serum (FCS) anti–SLe^x-1; CSLEX-1, IgM) [K. Fukushima *et al.*, *ibid.* **44**, 5279 (1984)] and anti–ELAM-1 (*3*), before binding of the cells to IL-1β activated human umbilical vein endothelial cells (HUVEC).
20. HUVEC, which had been activated for 4 hours with IL-1β, were washed twice with RPMI 1640 containing bovine serum albumin (BSA) at 5 mg/ml and then incubated in the same buffer with liposomes containing various glycosphingolipids at a final concentration of 5 μg/ml (300 μl per well) for 30 min at 37°C and the plates were held an additional 15 min at 4°C. Approximately 10^5 ^{51}Cr-labeled HL-60 or Jurkat cells (*13*) were added to the incubation mixture in 50 μl and cell adhesion was measured as described for Fig. 1. Liposomes were formed as follows: 100 μg glycolipid was added to 500 μg phosphatidylcholine (Sigma, egg yolk) and 300 μg cholesterol (Sigma) in chloroform-methanol (2:1) and the whole solution evaporated to dryness by N_2 in 15-ml screw-cap tubes. Before use, the pellet was dissolved in 100 μl of absolute ethanol and sonicated for 2 min. Phosphate-buffered saline (PBS) (1.9 ml) was added while sonicating for an additional 10 min. The liposome suspension was diluted 1:10 in RPMI 1640 containing BSA (5 mg/ml) before addition to the HUVEC. Glycolipids listed in Table 1 were either purified or biosynthetically produced at the Biomembrane Institute and were characterized by nuclear magnetic resonance (NMR) and mass spectrometry and found to be identical to previously reported structures [S. Hakomori, E. Nudelman, S. B. Levery, R. Kannagi, *J. Biol. Chem.* **259**, 46729 (1984); Y. Fukushi, E. Nudelman, S. B. Levery, S. Hakomori, H. Rauvala, *ibid.*, p. 10511]. S-diLe^x was synthesized enzymatically by adding fucosyl residues with a cell line as enzyme source and SH (Table 1) as substrate. diLe^x was similarly synthesized with nLc6 as substrate [E. H. Holmes, G. K. Ostrander, S. Hakomori, *J. Biol. Chem.* **261**, 3737 (1986)]. SPG and SH (Table 1) were purified from bovine red blood cells, and nLc6 was produced by chemical removal of terminal sialyl residue from SH.
21. We thank N. Kovack for technical advice; J. Harlan and H. Grey for advice and insightful discussions during the course of this work; M. Bevilacqua for his continued interest during this work and for the H18/7 MAb to ELAM-1; P. Terasaki for anti-SLe^x MAb (CSLEX); E. A. Wayner for the P4C2 MAb and the Jurkat cell line; P. Stanley for the LEC11, LEC12, and CHO-K1 cell lines; and M. A. Weiss and the obstetrical nurses of Sharp Memorial Hospital, San Diego, for helping to obtain umbilical cords.

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Recognition by ELAM-1 of the Sialyl-Le^x Determinant on Myeloid and Tumor Cells

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Endothelial leukocyte adhesion molecule-1 (ELAM-1) is an endothelial cell adhesion molecule that allows myeloid cells to attach to the walls of blood vessels adjacent to sites of inflammation. ELAM-1 recognizes the sialyl-Lewis X (sialyl-Le^x) determinant, NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc-, a granulocyte carbohydrate also found on the surface of some tumor cell lines. Binding of myeloid cells to soluble ELAM-1 is inhibited by a monoclonal antibody recognizing sialyl-Le^x or by proteins bearing sialyl-Le^x, some of which may participate in humoral regulation of myeloid cell adhesion. Stimulated granulocytes also release an inhibitor of ELAM-1 binding that can be selectively adsorbed by monoclonal antibody to sialyl-Le^x.

INTEGRAL MEMBRANE ADHESION PROteins of a nascent family share a common extracellular domain organization consisting of an NH_2-terminal lectin-like segment, an epidermal growth factor (EGF)–related element, and multiple complement receptor motifs (*1, 2*). One member of this family, ELAM-1 (*3*), mediates the adhesion of myeloid cells to endothelial cells activated by interleukin-1 (IL-1), tumor necrosis factor, or substance P (*4*).

To identify the ELAM-1 domains necessary for cell adhesion, we first localized the binding sites for two monoclonal antibodies (MAb) to ELAM-1 (anti–ELAM-1): H18/7, which blocks leukocyte adhesion to activated endothelium, and H4/18, which does not. Progressive deletion from the COOH-terminus showed that the lectin plus EGF-repeat elements were required for expression of H18/7 binding, whereas H4/18 reactivity required the first three complement-related repeats as well (Fig. 1). A restriction fragment exchange between ELAM-1 cDNA and the related Leu-8 (LECAM-1) cDNA (*2*) showed that H18/7 bound to a determinant encoded in the first 75% of the lectin domain, and that the equivalent determinants for MAbs to Leu-8 (anti–Leu-8) and TQ1 were similarly encoded in the first 75% of the LECAM-1 lectin domain (Fig. 1).

To study the possible lectin-carbohydrate interactions suggested by epitope mapping, we prepared a soluble ELAM-1 protein chimera (ELAM-Rg) consisting of the ELAM-1 extracellular domain joined to the hinge and CH2 and CH3 domains of human immunoglobulin G1 (IgG1) (*5–7*) as a disulfide-linked dimer from supernatants of transfected COS-7–m6 cells (Fig. 1).

When plastic dishes coated with goat antibodies to human IgG were incubated with supernatants containing ELAM-Rg, the treated plastic acquired the ability to specifically bind granulocytes and the myeloid cell lines HL-60 and THP-1. Other myeloid cells and some carcinoma cells bound to the ELAM-1–coated plastic, whereas dishes coated with CD8 fusion protein (*5*) showed negligible affinity for all cells tested (*8*). Adhesion to ELAM-1 correlated with the presence of the CD15 [Le^x or lacto-*N*-fucopentaose III (*9, 10*)] determinant, but not with the determinants associated with CD17 [lactosyl ceramide (*11*)], CD65 [VI^{3}NeuAcIII^{3}FucnorLcnOse$_6$Cer (*12*)], or sulfatides (*13*). However, the correlation with CD15 was imperfect, and digestion of

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