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Immunology

CD62 and endothelial cell–leukocyte adhesion molecule 1 (ELAM-1) recognize the same carbohydrate ligand, sialyl-Lewis x

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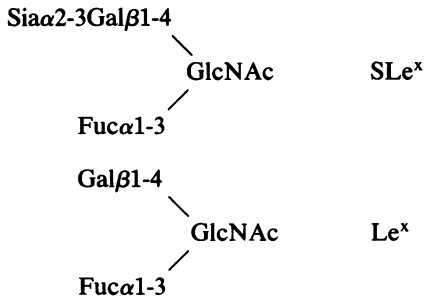
ABSTRACT **The leukocyte receptor CD62, which is expressed on activated platelets and endothelial cells, is shown to mediate cell adhesion by binding a sialylated carbohydrate structure, sialyl-Lewis x, found on neutrophils, monocytes, and tumor cells. This structure has previously been identified as the ligand for another member of the LEC-CAM family of cell adhesion molecules, endothelial cell–leukocyte adhesion molecule 1, which also binds neutrophils and monocytes. The results demonstrate that although the two LEC-CAMs differ in their biological activities by their distribution and mode of expression, they are capable of mediating cell adhesion by recognition of the same carbohydrate ligand.**

Leukocyte trafficking and recruitment to sites of inflammation are mediated by three adhesion receptor families, the integrin and immunoglobulin superfamilies and the recently described LEC-CAM, or selectin family (1–4). The known LEC-CAMs, LEC-CAM-1 (Mel-14/LAM-1), endothelial cell–leukocyte adhesion molecule 1 (ELAM-1), and CD62 [140-kDa granule membrane protein (GMP-140)/platelet activation-dependent granule external membrane protein (PADGEM)], contain an N-terminal lectin domain that mediates adhesion by binding carbohydrate ligands on opposing cells. The lectin domain is followed by an epidermal growth factor-like domain and a series of consensus repeats similar to those found in complement regulatory proteins. A flurry of recent reports have focused on the identification of the carbohydrate ligands recognized by ELAM-1 and CD62 (5–12). These two LEC-CAMs are expressed on activated endothelial cells (ELAM-1 and CD62) and platelets (CD62) and are implicated in the recruitment of neutrophils and monocytes to sites of tissue injury.

Evidence from several groups has defined the functional ligand of ELAM-1 as a terminal structure found on glycoprotein and glycolipid carbohydrate groups that contain both sialic acid (Sia) and fucose (Fuc), as typified by sialyl-Lewis x (SLe^x; refs. 5–9).



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Larsen *et al.* (10) have proposed that CD62 recognizes the nonsialylated Lewis x structure (Le^x or CD15) since neutrophil adhesion to activated platelets is inhibited both by monoclonal antibodies (mAbs) to Le^x (anti-CD15) and by oligosaccharides containing the Le^x structure. However, other groups have suggested that sialic acid is a crucial determinant of the natural ligand since treatment of neutrophils with sialidase abolishes their ability to adhere to CD62 (11, 12). Both pieces of information suggested that the ELAM-1 ligand, SLe^x, is a logical candidate as a sialylated ligand that could mediate cell adhesion by CD62.

EXPERIMENTAL PROCEDURES

Cell Isolation and Culture. Platelets were obtained from a normal human donor and washed free of plasma proteins in the presence of prostaglandin E_1 (100 nm) (13). Neutrophils were isolated from fresh human blood by centrifugation at room temperature through mono-poly resolving medium (Flow Laboratories) followed by three washes in ice-cold Hanks' balanced salt solution (GIBCO) containing 20 mM Hepes (GIBCO) and 0.2% glucose (Fisher). Human umbilical vein endothelial cells were obtained as described (6). HL-60 cells were cultured in RPMI 1640 containing 10% fetal calf serum. Chinese hamster ovary (CHO-K1) cells, glycosylation mutants LEC11 and LEC12 (14–19), and the carcinoma line (COLO-205) were cultured in the α modification of Eagle's minimal essential medium (α-MEM) containing ribonucleosides, deoxyribonucleosides, and 10% fetal calf serum.

Assays for CD62-Mediated Adhesion to Activated Platelets. Two assays were used, a fluid phase assay and a plate assay. The fluid phase assay (see Figs. 2–4) was performed essentially as described (10) for PADGEM (CD62). Platelets at 2×10^8 per ml were activated with thrombin at 0.25 units/ml for 20 min at room temperature without stirring. Twenty microliters of the activated platelet suspension was then mixed with an equal volume of a suspension of the cells (2×10^6 cells per ml) to be assessed for adhesion. Adhesion was evaluated microscopically and was scored as the percent of the test cells that had bound two or more platelets. The plate assay used was a modification of the endothelial cell–neutrophil adherence assay previously described (20). A suspension of platelets (300 μl) at 10^8 per ml was applied to each well of a 48-well plate that had previously been coated with 0.1% gelatin. The

Abbreviations: LEC-CAM, a family of cell adhesion molecules, each of which contains an N-terminal lectin domain followed by an epidermal growth factor-like domain and a series of consensus repeats similar to those found in complement regulatory proteins; ELAM-1, endothelial cell–leukocyte adhesion molecule 1; GMP-140, 140-kDa granule membrane protein; PADGEM, platelet activation-dependent granule external membrane protein; LNF, lacto-*N*-fucopentaose; mAb, monoclonal antibody; Sia, sialic acid; Fuc, fucose; SLe^x, sialyl-Lewis x; Le^x, Lewis x; Cer, ceramide.

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plate was incubated at 37°C for 15 min, centrifuged for 2 min at 90 × *g*, and then washed twice with phosphate-buffered saline (PBS) to remove nonadherent platelets. To block platelet Fc receptors, 300 μl of heat-aggregated IgG (20 μg/ml) was added to each well, and the plate was incubated at 37°C for 15 min. HL-60 cells were radiolabeled with ^{51}Cr [450 μCi (1 Ci = 37 GBq) was added to 3 × 10^6 cells in 300 μl and incubated for 60 min at 37°C], washed three times in RPMI 1640 containing 10% fetal calf serum, and resuspended to 4 × 10^6 per ml. This suspension (100 μl) was added to 1.6 ml of RPMI 1640 with or without mAb, and the mixture was incubated for 30 min at 37°C with gentle agitation. At the end of the incubation period, 300 μl of this cell suspension was added to triplicate wells of the assay plate containing bound, activated platelets. The plate was centrifuged for 2 min at 90 × *g* and then allowed to stand for 5 min at room temperature. Unbound cells were removed by three washes with PBS, and the remaining bound cells were removed from the plate with an SDS-containing buffer and then processed for radioactive counting. Adherence was scored as the percent of total HL-60 cells bound.

Assay for ELAM-1-Mediated Adhesion. ELAM-1-mediated adhesion of neutrophils to activated endothelial cells was performed essentially as described (6), with the modifications that the assay was performed at 37°C for 5.5 min and the assay wells were washed vigorously by inverting the plate and refilling four times with a repeating pipet. Adhesion was quantitated by spectrophotometric analysis of myeloperoxidase (21). The degree of ELAM-1-mediated adhesion was confirmed with the mAb for ELAM-1, P6E2, which inhibited neutrophil binding to activated endothelial cells by 80%.

Preparation of Glycolipid-Containing Liposomes. Liposomes containing glycolipids (glycolipid liposomes) were prepared as described (6). Briefly, 100 μg of glycolipid was added to 500 μg of phosphatidylcholine (egg yolk; Sigma) and 300 μg of cholesterol (Sigma) in chloroform/methanol, 2:1, and the mixture was evaporated to dryness with N_2. Before use, the pellet was dissolved in 100 μl of absolute ethanol and sonicated. PBS (1.9 ml) was added, and the mixture was sonicated for an additional 10 min. Structures of the various glycolipids examined are as follows: nLC6, Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1 ceramide (Cer); SH, Siaα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer; diLe^x, Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glcβ1-1Cer; SdiLe^x, Siaα2-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glcβ1-1Cer; SLe^x, Siaα2-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glcβ1-1Cer; SPG, Siaα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer.

Oligosaccharides. Lacto-*N*-fucopentaose I (LNF I) and Lacto-*N*-fucopentaose III (LNF III) isolated from human milk were purchased from Calbiochem and were >95% pure by HPLC as assayed by the supplier. A synthetic hexasaccharide containing the terminal SLe^x structure (22) was the generous gift of A. Hasegawa, Gifu University, Japan. Structures of the oligosaccharides are as follows: LNF I, Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc; LNF III, Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc; SLe^x-hex, NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc-O-$CH_2CH_2SiMe_3$.

Carbohydrate-Specific mAbs. mAbs to SLe^x and Le^x (6) used were specific for the carbohydrate epitope Le^x [mAb SH-1 (IgG3; anti-Le^x-1) and mAb FH-2 (IgM; anti-Le^x-2)] or SLe^x [mAb CSLEX (IgM; anti-SLe^x-1) and mAb SNH-4 (IgG3; anti-SLe^{x}2)].

Sialidase Treatment of Cells. LEC11 cells, polymorphonuclear leukocytes, and COLO-205 cells at a concentration of 3 × 10^6 in 400 μl were incubated at 37°C for 90 min with sialidase (*Clostridium perfringens*; Sigma, type X) at 1.6 units/ml.

RESULTS

Inhibition of CD62-Mediated Adhesion by mAbs to Le^x and SLe^x. In experiments in which mAbs to Le^x and SLe^x were evaluated for their ability to inhibit CD62-mediated adhesion of neutrophils to activated platelets, mAbs to Le^x inhibited slightly, whereas mAbs to SLe^x inhibited almost completely (Fig. 1). The results confirm previous reports that mAbs to Le^x give partial inhibition of CD62 adhesion (10, 12). The more complete inhibition with the mAbs to SLe^x suggested that SLe^x may be a high-affinity ligand of CD62.

CD62-Mediated Adhesion of SLe^x-Containing Cells to Activated Platelets. Examination of CHO cells and glycosylation mutants LEC11 and LEC12 (14–19) provided evidence that SLe^x could support CD62-mediated adhesion to activated platelets, whereas Le^x could not. Indeed, wild-type cells (CHO-K1), which express neither SLe^x nor Le^x, and LEC12 cells, which express only Le^x, were not bound. In contrast, LEC11 cells, which express the SLe^x determinant, were bound almost as well as the promyelocytic HL-60 cells used as a positive control (Fig. 2*A*). Sialidase treatment of LEC11 cells or neutrophils to convert SLe^x to Le^x abolished their adherence to activated platelets, further showing that Le^x alone is not sufficient for mediating adhesion by CD62 (Fig. 2*B*). A carcinoma cell line (COLO-205) that contains SLe^x (18) also bound to activated platelets and, like the other cells, its adhesion was abolished by sialidase treatment (Fig. 2*B*).

Inhibition of CD62-Mediated Adhesion by Glycolipid Liposomes. Since Le^x oligosaccharides were previously reported to inhibit CD62-mediated cell adhesion of activated platelets (10), we used a similar assay to compare liposomes containing glycolipids with the Le^x and SLe^x structures for their ability to prevent adhesion of HL-60 cells and human neutrophils. HL-60 cell adhesion (Fig. 3*A*) was partially inhibited (50%) by liposomes containing Le^x at the highest concentration tested (5 μg/ml). In contrast to partial inhibition obtained with Le^x liposomes, SLe^x liposomes gave maximal inhibition at only 1 μg/ml. Thus, SLe^x liposomes inhibited adhesion with ≥10-fold higher affinity than Le^x liposomes. Similar results were obtained with neutrophils, although inhibition of

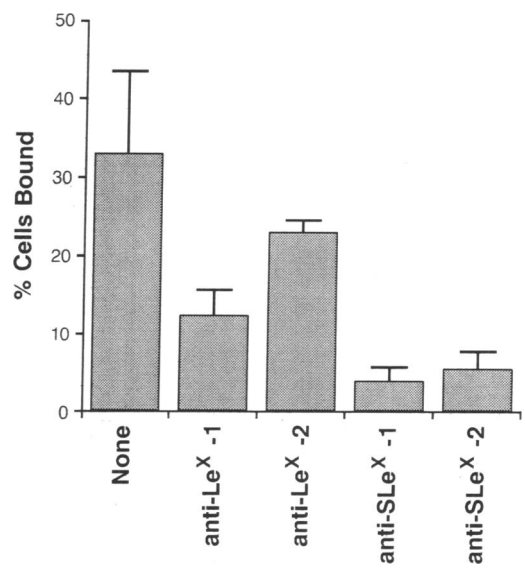


FIG. 1. Antibodies to SLe^x and Le^x inhibit CD62-mediated binding of HL-60 cells to activated platelets. mAbs to SLe^x and Le^x were examined for their ability to inhibit CD62-mediated adhesion of ^{51}Cr-labeled HL-60 cells to platelets spread on gelatin-coated plates. mAbs used (6) were specific for the carbohydrate epitope Le^x [mAb SH-1 (IgG3; anti-Le^x-1) and mAb FH-2 (IgM; anti-Le^x-2)] or SLe^x [mAb CSLEX (IgM; anti-SLe^x-1) and mAb SNH-4 (IgG3; anti-SLe^x-2)]. Error bars denote standard deviations of triplicate samples.

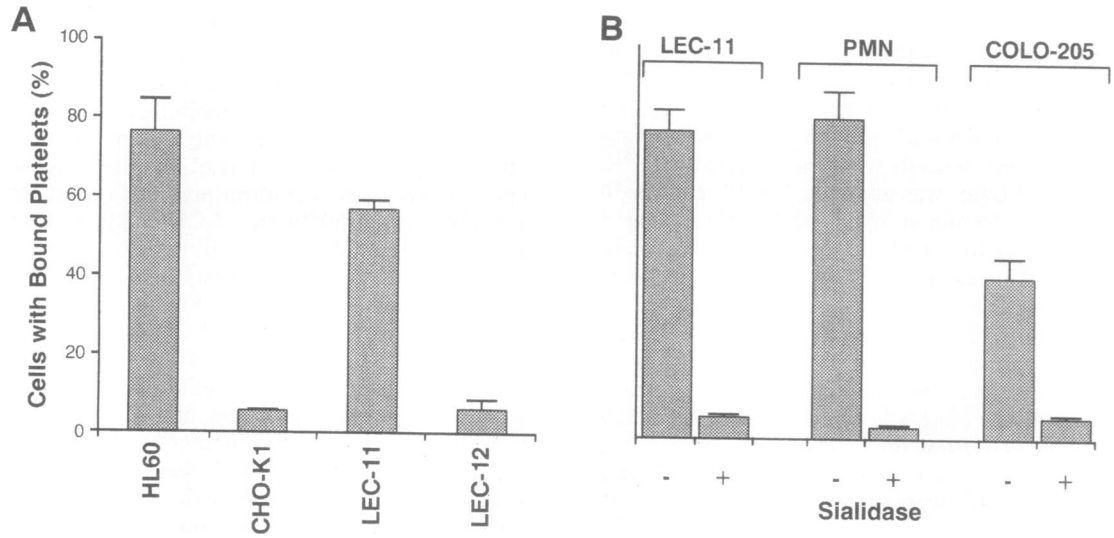


FIG. 2. SLe^x-dependent adhesion of LEC11 cells to activated platelets. (*A*) HL-60 cells, wild-type CHO-K1 cells, and glycosylation mutants LEC11 and LEC12 were evaluated for adhesion to activated platelets in a fluid-phase assay. (*B*) LEC-11 cells, neutrophils (PMN), and COLO-205 cells were evaluated for adhesion before and after treatment with *C. perfringens* sialidase.

adhesion was less sensitive and was observed only with SLe^x (Fig. 3*B*).

Inhibition of Binding of Neutrophils to Activated Platelets by Free Sugars Containing SLe^x and Le^x. It has been reported (10) that CD62 binding of neutrophils to activated platelets was inhibited by a soluble human milk oligosaccharide that contains the Le^x structure (LNF III). Maximal inhibition required a concentration of 200 μg/ml. In the present study, LNF III (Le^x) and a related synthetic hexasaccharide containing the terminal SLe^x structure were compared for their ability to inhibit binding of neutrophils to activated platelets. As shown in Fig. 4, the SLe^x sugar was approximately a 30-fold more potent inhibitor than the nonsialylated Le^x sugar, which required 2 μg/ml and 54 μg/ml to achieve 50% inhibition of neutrophil adhesion, respectively.

Inhibition of CD62-Mediated and ELAM-1-Mediated Adhesion by Glycolipid Liposomes. Previous reports have demonstrated that another LEC-CAM, ELAM-1, mediates adhesion of neutrophils using SLe^x as a ligand (6, 7). To directly compare the specificity of the two LEC-CAMs, CD62 and ELAM-1, glycolipid liposomes were tested for their ability to inhibit adhesion of neutrophils to activated platelets and

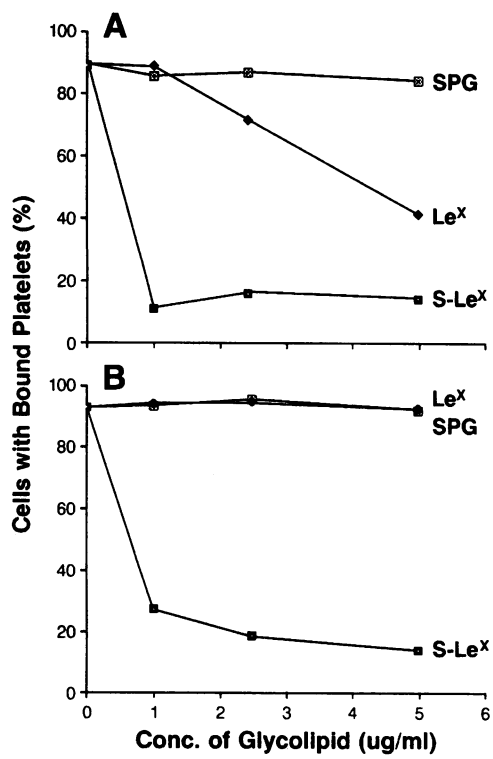


FIG. 3. Liposomes containing glycolipids with the SLe^x ligand block binding of HL-60 cells (*A*) and neutrophils (*B*) to thrombin-activated platelets. Thrombin-activated platelets were incubated at room temperature for 20 min with the liposome preparations containing glycolipids or with buffer as a control at the indicated concentrations expressed as the final concentration of glycolipid. HL-60 cells (*A*) or neutrophils (*B*) were then added and adherence was assessed. The glycolipids used contained the terminal carbohydrate structures representing SLe^x (SdiLe^x), Le^x (diLe^x), and Siaα2-3Galβ1-4GlcNAc- (SPG).

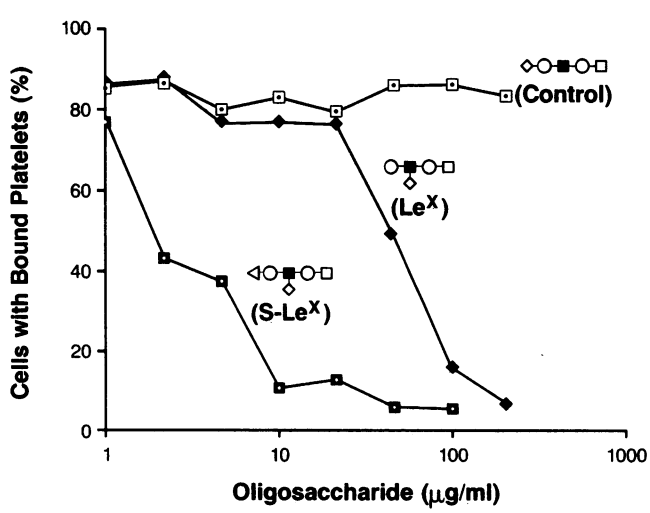


FIG. 4. Soluble sugars containing the SLe^x and Le^x structures inhibit CD62-mediated binding of neutrophils to thrombin-activated platelets. Thrombin-activated platelets were incubated at room temperature for 20 min with a control oligosaccharide (LNF I) or oligosaccharides containing the Le^x (LNF III) or SLe^x (SLe^x-hex) structures. The indicated concentration is the final concentration. Neutrophils were then added and adherence was assessed as described in *Experimental Procedures* for the fluid-phase assay. Structures are indicated in symbol form as given in Fig. 5.

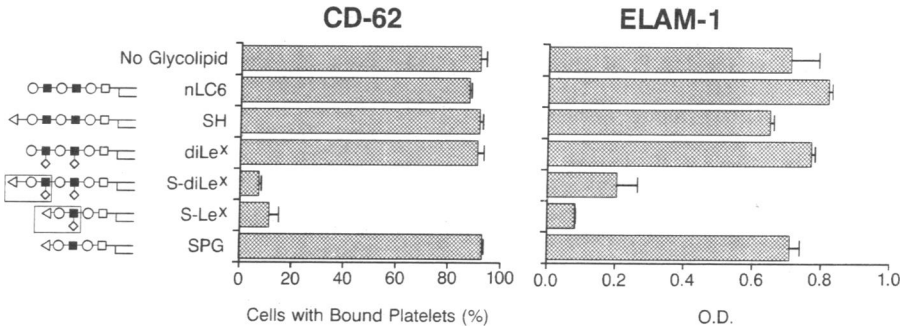


FIG. 5. CD62 and ELAM-1 exhibit similar specificity for SLe^x as a high-affinity ligand. Liposomes containing various glycolipids of related structure were evaluated for their ability to inhibit neutrophil adhesion to activated platelets (CD62) and interleukin 1β-activated endothelial cells. Structures are indicated at the left in symbol form, and the SLe^x determinant found in two of the glycolipids is highlighted in the rectangular box. ◁, Sia; ◇, Fuc; ○, Gal; ■, GlcNAc. CD62-dependent adhesion of neutrophils to thrombin-activated platelets was performed by the fluid-phase assay. Liposome-induced inhibition of ELAM-1-mediated cell adhesion to activated endothelial cells was performed as described in *Experimental Procedures*.

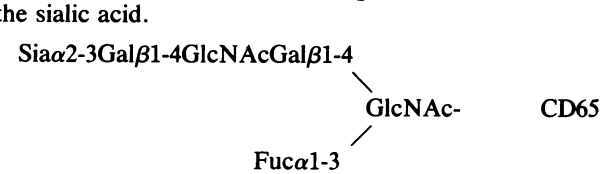
ELAM-1-mediated adhesion of neutrophils to interleukin 1β-activated human vascular endothelial cells (Fig. 5). For each assay the concentration of liposomes used was fixed empirically as the concentration that gave maximal inhibition when SLe^x glycolipid was used—namely, 2 μg/ml for CD62 and 14 μg/ml for ELAM-1. Under conditions of the assays used in the present study, the specificity of the two LEC-CAMs was virtually identical for the glycolipid structures tested, being inhibited only by those containing the SLe^x structure.

DISCUSSION

The potential significance of CD62 and ELAM-1 mediating cell adhesion through the same carbohydrate ligand can be inferred from emerging information on the biological roles of these receptors. These LEC-CAMs mediate adhesion of neutrophils and monocytes to vascular endothelial cells and platelets, but in different biological contexts (23–26). CD62 is stored in intracellular secretory granules of endothelial cells and platelets and is rapidly expressed on the cell surface following degranulation, within minutes of exposure to activating agents such as thrombin, histamine, or phorbol esters (23, 24). In contrast, ELAM-1 is expressed only on endothelial cells and requires *de novo* synthesis (1–4 hr) following induction by inflammatory cytokines such as interleukin 1β and tumor necrosis factor (25). Since CD62 is expressed on both platelets and endothelial cells, it has been proposed that this adhesion receptor may provide a link between the hemostatic and inflammatory responses (24, 26). Indeed, since CD62 and ELAM-1 are differentially expressed in response to various complex stimuli, it is possible that the same carbohydrate ligand is used to coordinate both rapid (CD62) and more prolonged (ELAM-1) recruitment of neutrophils and monocytes to sites of tissue injury.

Other carbohydrate structures have been proposed as ligands for CD62 and ELAM-1 in addition to SLe^x. Larsen *et al.* (10) demonstrated that an oligosaccharide containing the nonsialylated analog, Le^x (LNF III), was capable of inhibiting CD62-mediated adhesion. This observation is completely consistent with results presented in this report that suggest that Le^x is a low-affinity ligand of CD62 (Fig. 4), but will not by itself support cell adhesion (refs. 11 and 12; Fig. 2).

Tiemeyer *et al.* (8) have proposed that the natural ligand for ELAM-1 is a variation of SLe^x referred to as CD65, or VIM-2, where the fucose is two sugars further removed from the sialic acid.



The glycolipid SdiLe^x (27), previously shown to be an inhibitor of ELAM-1 (6), incorporates both the SLe^x and CD65 structures (see Fig. 5). However, the shorter chain SLe^x glycolipid examined in this report does not incorporate the CD65 structure, yet it inhibits ELAM-1-mediated adhesion and CD62-mediated adhesion (Fig. 5). Thus, the SLe^x structure appears to be sufficient to mediate high-affinity interactions with both LEC-CAMs.

Although both CD62 and ELAM-1 exhibit similar specificities toward the carbohydrate structures examined in this report, it is premature to conclude that they have identical specificities for cell surface receptor molecules. Although the SLe^x structure is found to terminate both glycoprotein and glycolipid carbohydrate groups of human neutrophils, it has not been established if either or both serve as biologically relevant neutrophil ligands. It is possible, however, that recognition of protein epitopes by either LEC-CAM could modify their ligand specificity enabling them to recognize specific glycoproteins. In this regard, however, it is of interest to note that LEC11 cells and COLO-205, which have no myeloid-specific proteins, are capable of participating in LEC-CAM-mediated cell adhesion. Further, it has been recently demonstrated that binding of neutrophils to soluble GMP-140 is not inhibited by synthetic SLe^x–bovine serum albumin conjugates (12). The underlying basis for this observation is not clear and may reflect the fine specificity of GMP-140 for SLe^x-containing carbohydrate groups on glycoproteins and glycolipids. Finally, recent reports have demonstrated that skin-homing T cells bind ELAM-1 even though T cells do not appear to contain the SLe^x ligand (28–31). These data suggest that ELAM-1 may additionally recognize a carbohydrate ligand other than SLe^x. Further information on the fine specificities of ELAM-1 and CD62 will be required to determine if these two LEC-CAMs have identical or merely overlapping specificities for their carbohydrate ligands.

Note Added in Proof. Since SdiLe^x- and SLe^x-containing glycolipids were prepared from precursors derived from bovine erythrocytes, the sialic acids were 85% *N*-glycollylneuraminic acid and 15% *N*-acetylneuraminic acid. In separate experiments, SLe^x glycolipids containing only *N*-acetylneuraminic acid were found to be equipotent to the SLe^x glycolipids used in this report.

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