Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 1138–1142, February 1991
Biochemistry

Carbohydrate ligands for endothelial–leukocyte adhesion molecule 1

(neutrophil/cell adhesion/glycolipid/inflammation)

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Communicated by Robert L. Hill, October 31, 1990

ABSTRACT **The acute inflammatory response requires that circulating leukocytes bind to and penetrate the vascular wall to access the site of injury. Several receptors have been implicated in this interaction, including a family of putative carbohydrate-binding proteins. We report here the identification of an endogenous carbohydrate ligand for one of these receptors, endothelial–leukocyte adhesion molecule 1 (ELAM-1). Radiolabeled COS cells transfected with a plasmid containing the cDNA for ELAM-1 were used as probes to screen glycolipids extracted from human leukocytes. COS cells transfected with this plasmid adhered to a subset of sialylated glycolipids resolved on TLC plates or adsorbed on polyvinyl chloride microtiter wells. Adhesion to these glycolipids required calcium but was not inhibited by heparin, chondroitin sulfate, keratan sulfate, or yeast phosphomannan. Monosaccharide composition, linkage analysis, and fast atom bombardment mass spectrometry of the glycolipids indicate that the ligands for ELAM-1 are terminally sialylated lactosylceramides with a variable number of *N*-acetyllactosamine repeats and at least one fucosylated *N*-acetylglucosamine residue.**

Adhesion of circulating leukocytes to stimulated vascular endothelium is an early event of the inflammatory response. Receptors implicated in this interaction (1) include the "LEC-CAMs" or "selectins" [gp90^{MEL}, GMP-140, and endothelial–leukocyte adhesion molecule 1 (ELAM-1); refs. 2–7], a family of putative carbohydrate-binding proteins (lectins). Although these receptors contain domains with sequence homology to calcium-dependent lectins, only gp90^{MEL} has been shown to recognize carbohydrate structures (4), and no endogenous ligands have been reported. ELAM-1 is transiently expressed on endothelial cells in response to interleukin 1 or tumor necrosis factor (6). Its role in neutrophil extravasation is suggested by the ability of a monoclonal antibody against ELAM-1 to block the adhesion of neutrophils to activated vascular endothelium *in vitro*. In addition, Bevilacqua *et al.* (8) demonstrated that human neutrophils or HL-60 cells will adhere to transfected COS cells expressing ELAM-1. We demonstrate here that COS cells transfected with a plasmid containing the cDNA for ELAM-1 can be used to directly probe for the native carbohydrate ligands. We have defined those ligands as several structurally related acidic glycolipids isolated from human leukocytes.

METHODS

Selection and Expression of ELAM-1 cDNA. A full-length cDNA encoding ELAM-1 was obtained by 35 cycles of the polymerase chain reaction (9) with 1 μg of total RNA extracted from interleukin 1β (Collaborative Research)-stimulated human umbilical vein endothelial cells and primers complementary to the untranslated flanking sequences (8) (5′-GGTGCGGCCGCGGCCAGAGACCCGAGGAGAG-3′ and 5′-GGTGTCGACCCCACCTGAGAGATCCTGTG-3′; Operon Technologies, Alameda, CA). The 2-kilobase (kb) amplified fragment generated was gel-purified, directionally cloned into the mammalian expression vector CDM8 (8), which had been modified by the insertion of a *Sal* I site into the polylinker, and propagated in *Escherichia coli* (MC1061/p3). Plasmid DNA was isolated from individual colonies and used to transfect COS cells (10). Putative ELAM-1-encoding plasmids were selected based on the ability of these transfected COS cells to support HL-60 cell adhesion 72 hr after transfection (8). The plasmid (designated pl-ELAM) used in subsequent experiments was found to contain the published sequence of ELAM-1 cDNA (11, 12).

Cell Adhesion Assays. COS cell populations transfected with the ELAM-1-encoding plasmid pl-ELAM were metabolically radiolabeled with $^{32}PO_4$ and used as probes in two assay systems to screen for recognition of glycolipids. In the first, glycolipids were adsorbed to the bottoms of polyvinyl chloride (PVC) microtiter wells, whereas in the second they were resolved on TLC plates. In both assays these immobilized glycolipids were evaluated for their ability to support adhesion of pl-ELAM-transfected COS cells, untransfected COS cells, or COS cells transfected with a control plasmid, under conditions of controlled detachment force (13, 14).

Glycolipid Extraction and Isolation. Glycolipids were extracted from chloroform/methanol-denatured leukocytes collected from patients with chronic myelogenous leukemia undergoing therapeutic leukopheresis (provided by John Klock, Glycomed). Material equivalent to about 500 ml of packed cells was extracted and partitioned by the method of Svennerholm and Fredman (15). The saponified, upper-phase glycolipids were fractionated on a DEAE-Sepharose (acetate form) column (Fast Flow, Pharmacia). The material not retained on the column and in fractions eluted by step-wise increases in salt concentration (from 5 mM to 250 mM potassium acetate in methanol) were screened for cell adhesion. The glycolipid mixture eluted from DEAE-Sepharose in 5 mM potassium acetate was subsequently fractionated on beaded silica columns (ref. 16; Iatrobead, Iatron Laboratories, Tokyo). Fractions from the first Iatrobead column, pooled by their ability to support adhesion of pl-ELAM-transfected COS cells, were separated into three active glycolipid components by using a second Iatrobead column. The more polar two of these were each resolved into two bands by preparative TLC in 1-butanol/ethanol/water, 55:35:20 (vol/vol).

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Abbreviations: ELAM-1, endothelial–leukocyte adhesion molecule 1; HPTLC, high-performance TLC; NANA, *N*-acetylneuraminic acid; PVC, poly(vinyl chloride); FAB, fast atom bombardment.

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Structural Analysis of Glycolipids. Neutral monosaccharide ratios were obtained after hydrolysis of 500 pmol of dried sample glycolipid or G_{M1} control. For hydrolysis, samples were heated in 1 M methanolic HCl at 80°C for 3 hr, dried under nitrogen, reacetylated in 1:2 pyridine/acetic anhydride (vol/vol), dried under nitrogen, and heated in 2 M CF_3COOH at 100°C for 3 hr. Samples were dried and dissolved in water prior to injection. Samples for sialic acid analysis were hydrolyzed in 0.1 M HCl at 80°C for 4 hr and then neutralized with 1 M NaOH. Chromatography was performed on Dionex Carbopac Pa-1 columns with the pulsed electrochemical detector (17). Neutral sugar chromatography used 15 mM NaOH; sialic acid chromatography used 0.6 M NaOH. Monosaccharide substituent positions were determined by methylation analysis of 2 nmol of the sample glycolipids or G_{M1} standard. The samples were methylated (18) and then hydrolyzed as above. The partially methylated monosaccharides were reduced with NaB^2H_4 and acetylated (19). The partially methylated alditol acetates were separated by gas chromatography with ion-trap mass detection. Negative-ion fast atom bombardment (FAB) mass spectra were acquired on a Finnigan (San Jose, CA) MAT 900 spectrometer with a triethanolamine matrix.

Enzyme Treatments. Nonfucosylated glycolipid material was enzymatically fucosylated by using the human milk-derived α(1–3/1–4)-fucosyltransferase (supplied by Robert L. Hill, Duke University, Durham, NC). Fifty nanomoles of the glycolipid was dried in the presence of 2 mg of sodium taurocholate and then resuspended in a final volume of 200 μl of 50 mM Mops·NaOH, pH 7.5/100 mM NaCl/5 mM $MnCl_2$ containing 20 μg of bovine serum albumin, 5 microunits of the enzyme, and 170 nmol of GDP-fucose (BioCarb, Lund, Sweden). The mixture was incubated for 6 hr at 37°C, after which fresh enzyme and GDP-fucose in the same amounts as before were added. The reaction was further incubated for 14 hr at 37°C and then stopped by the addition of 800 μl of methanol. Glycolipids were desialylated by using *Arthrobacter ureafaciens* neuraminidase (Boehringer Mannheim). Ten nanomoles of glycolipid was dried in the presence of 1 mg of sodium taurocholate and then resuspended in 100 μl of 100 mM Tris acetate, pH 6.5/5 mM $CaCl_2$ containing 10 μg of bovine serum albumin; 250 milliunits of enzyme was added, and the mixture was incubated overnight at 37°C. The reaction was stopped by the addition of 400 μl of methanol. The enzymatically modified glycolipids were recovered free of enzyme and detergent by adsorption on a C_{18} Sep-Pak column (Waters) and elution with organic solvents, and modifications were assessed by mass spectrometry (as above).

RESULTS

ELAM-1 Is a Calcium-Dependent Lectin That Recognizes a Subset of Myeloid-Derived Acidic Glycolipids. Glycolipids extracted from human myeloid cells were either adsorbed to the bottoms of PVC microtiter wells or resolved on TLC plates and then were probed with radiolabeled pl-ELAM-transfected COS cells under conditions of controlled detachment force. At least 30% of the COS cell populations transfected with pl-ELAM rosetted HL-60 cells, while HL-60 cells did not adhere to control-transfected COS cells. Up to 25% of the transfected COS cell population adhered to the saponified upper-phase glycolipid mixture adsorbed to PVC microtiter wells (Fig. 1). Virtually all of this ELAM-1 binding activity was eluted from a DEAE-Sepharose column with 5 mM potassium acetate (Fig. 1), suggesting that the carbohydrate ligand was monosialylated (15). Cell adhesion to these glycolipids adsorbed to PVC microtiter wells was calcium dependent and was not inhibited by a number of carbohydrates including heparin and yeast phosphomannan (data not shown). This active DEAE fraction (Fig. 1, column E) was subsequently fractionated on beaded silica columns (16). Cell adhesion to aliquots of these Iatrobead fractions adsorbed on PVC microtiter wells (Fig. 2*A*) revealed three peaks of ELAM-1 binding activity. Fractions were pooled, resolved by TLC (Fig. 2*B*), and evaluated for their ability to bind pl-ELAM-transfected COS cells (Fig. 2*C*). Autoradiographs revealed specific adhesion to glycolipids in lanes 8, 9, 10, and 11. Minor bands (arrows in Fig. 2*C*) supported adhesion of control COS on a companion plate as well, indicating nonspecific adhesion at these locations. Pools from lanes 8–11 were further fractionated into three bands supporting adhesion (Fig. 3, bands in lanes 2, 3, and 5, which we refer to as bands 2, 3, and 5) and several inactive bands (e.g., Fig. 3, bands in lanes 1 and 4, which we refer to as bands 1 and 4). The most prevalent of the active bands (band 3) yielded 0.03% of the total sialic acid found in the crude upper-phase mixture. Bands 2, 3, and 5 supported adhesion of 50–60% of the added COS cell population transfected with the ELAM-1-containing plasmid, while bands 1 and 4 supported adhesion of <5% of the same cells (data not shown). These data show that ELAM-1 is a calcium-dependent lectin that recognizes a small subset of structurally related acidic glycolipids present on cells of myelocytic lineage.

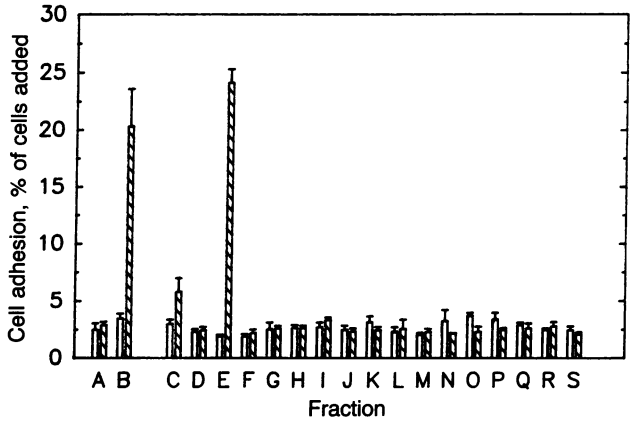

FIG. 1. ELAM-transfected COS cell adhesion to neutrophil glycolipids separated by charge. COS cells transfected with the plasmid coding for ELAM-1 were radiolabeled with $^{32}PO_4$ and harvested 72 hr after transfection (10). Neutrophil glycolipids [upper phase, saponified (15); columns B] were loaded on a 5-ml DEAE-Sepharose fast-flow column (Pharmacia) in chloroform/methanol/water, 4:8:3 (vol/vol), washed with three column volumes of methanol (columns C), and eluted with two 12-ml steps each of 5 (columns D and E), 10 (F and G), 20 (H and I), 30 (J and K), 40 (L and M), 50 (N and O), 100 (P and Q), and 250 (R) mM potassium acetate in methanol (16). Aliquots of desalted, dried fractions were resuspended in 100% ethanol containing 1 μM phosphatidylcholine and 4 μM cholesterol, and an equal volume of water was added. These solutions were added to PVC microtiter wells (50 μl per well), and the glycolipids were adsorbed for 80 min (14). Adsorbed lipids were screened for adhesion of COS cells (40,000 cells per well) transfected with an ELAM-encoding (▨) or control (□) plasmid by using a centrifugation assay (14). Columns A are the phosphatidylcholine/cholesterol control and columns S are the G_{T1b} control. Data are presented as the mean of at least three replicates ± SEM.

ELAM-1 Recognizes Sialylated, Fucosylated, *N*-Acetyllactosamine-Containing Glycolipids. Bands 1–5 of Fig. 3 were analyzed for monosaccharide composition (17), linkage assignments (18, 19), and mass spectral data. To conserve space, data are presented only for ELAM-1-recognized band 5 (Table 1 and Fig. 4), while the other structures are discussed in the text below. Data for band 5 are consistent with a lactosylceramide containing three *N*-acetyllactosamine repeats and one fucose on the middle GlcNAc residue and a terminal sialic acid residue. Glucose, fucose, and *N*-acetyl-

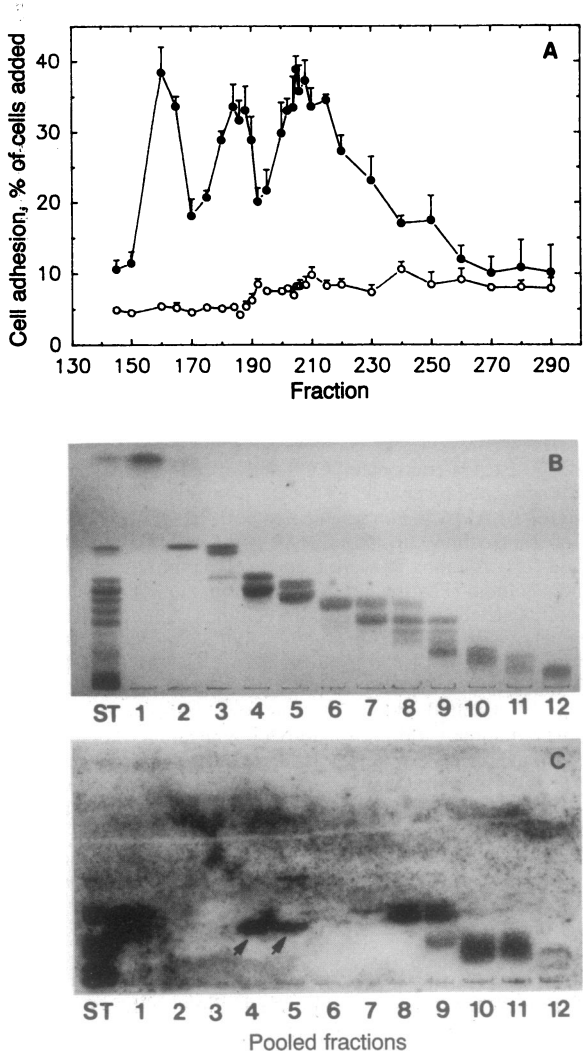

FIG. 2. Silicic acid chromatography of ELAM-recognized glycolipids. Material eluted from DEAE-Sepharose with 5 mM salt (Fig. 1, columns E) was loaded onto a 100-ml beaded silica column (Iatrobeads, 100-μm bead diameter; Iatron Laboratories) in chloroform/methanol/water, 60:35:8 (vol/vol), and eluted with the same solvent. Aliquots of fractions were screened for adhesion of pl-ELAM-transfected COS cells by the PVC microtiter well assay (*A*), expressed as the mean of at least three replicates ± SEM. Fractions were pooled based on this ELAM recognition, and the pools were chromatographed on high-performance-silica TLC (HPTLC) plates in chloroform/methanol/0.25% aqueous KCl, 60:35:10 (vol/vol), and stained with resorcinol (*B*). Companion plates (unstained) were coated with poly(isobutyl methacrylate) (0.005% in hexane) and probed with radiolabeled pl-ELAM-transfected (*C*) or control (not shown) COS cells (13). Lanes: ST, total 5 mM fraction; 1–12, pooled fractions 18–23, 25–30, 31–44, 45–69, 70–85, 135–147, 148–159, 160–170, 171–192, 193–220, 221–245, and 246–300, respectively. Minor bands (arrows in *C*) supported adhesion of control COS on a companion plate as well, indicating nonspecific adhesion at these locations.

neuraminic acid (NANA) were found in equimolar ratios, with 3 mol of GlcNAc and 4 mol of galactose per mol of glucose. Mass spectrometry (Fig. 4) revealed a molecular ion at 2393, with signals at 2102, 1940, 1737, 1575, 1225, 1063, 860, 698, and 536, suggesting a structure composed of a deoxyhexose, an NANA, three repeats of hexose/*N*-acetylhexosamine, two hexoses, and a ceramide with a C16:0 fatty acid. The signal at 2247 may include a molecular ion of nonfucosylated material as well as the loss of fucose from 2393. The fragments at 1737, 1575, and 1225 suggest that the most probable location of the deoxyhexose (fucose) is the

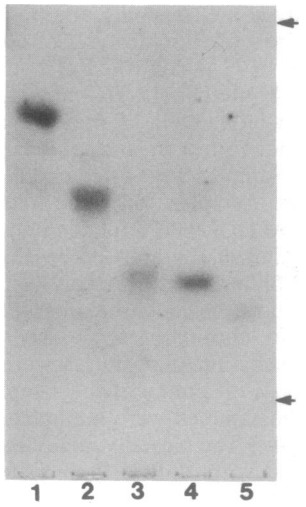

FIG. 3. TLC of purified neutrophil glycolipids. Glycolipid pools supporting specific pl-ELAM-transfected COS cell adhesion (pools 8–11 in Fig. 2) were further purified by a second Iatrobead column. The more polar material (slower mobility) in lanes 3 and 4 was further separated by preparative HPTLC in 1-butanol/ethanol/water, 55:35:20 (vol/vol). These materials were resolved on HPTLC plates in chloroform/methanol/0.25% aqueous KCl, 60:35:10 (vol/vol), and stained with resorcinol. (Lanes: 1, band 1; 2, band 2; 3, band 3; 4, band 4; 5, band 5). Arrows indicate relative positions of G_{M1} (upper arrow) and G_{T1b} (lower arrow) standards.

middle *N*-acetylhexosamine. Presence of an internal fucose was also indicated by the susceptibility of the material (after removal of sialic acid) to exo-β-galactosidase and exo-β-hexosaminidase (data not shown). The 3,4-disubstituted GlcNAc (Table 1) was converted to 4-monosubstituted GlcNAc after removal of the fucose, indicating that the fucose was (1-3)-linked. A small amount of 6-substituted galactose was detectable, suggesting that a small percentage of the NANA may be (2-6)-linked.

Similar data obtained from the ELAM-recognized band 2 (data not shown) are consistent with a lactosylceramide (C16:0 fatty acid) with two *N*-acetyllactosamine repeating units, a terminal NANA, and a fucose on one of the GlcNAc residues. Data for another active band (band 3) suggest that it too is structurally related to band 5, varying only in the ceramide. Bands 1 and 4, material unable to support adhesion of pl-ELAM-transfected COS cells, appear to contain the

Table 1. Monosaccharide ratios and substituent positions from band 5 glycolipid

	Position of substitution	Sugar ratio
Glucose	4	0.9
Galactose	3 or 6	4
GlcNAc	4 or 3, 4	2.7
Fucose	ND	0.9
NANA	ND	1.1

Neutral monosaccharide ratios were obtained after hydrolysis of 500 pmol of dried sample glycolipid or G_{M1} control as described in *Methods*. Chromatography was performed on Dionex Carbopac Pa-1 columns with the pulsed electrochemical detector. Monosaccharide substituent positions were determined by methylation analysis on 2 nmol of the sample glycolipids or G_{M1} standard as previously described: 2,3,6-OMe-glucitol acetate, 4-substituted glucose; 2,4,6-OMe-galactitol acetate, 3-substituted galactose; 2,3,4-OMe-galactitol acetate, 6-substituted galactose; 3,6-OMe-*N*-Me-2-acetamido-2-deoxyglucitol acetate, 4-substituted GlcNAc; 6-OMe-*N*-Me-2-acetamido-2-deoxyglucitol acetate, 3,4-disubstituted GlcNAc. Trace amounts of 2,6-OMe-galactitol acetate were also detected. ND, not determined.

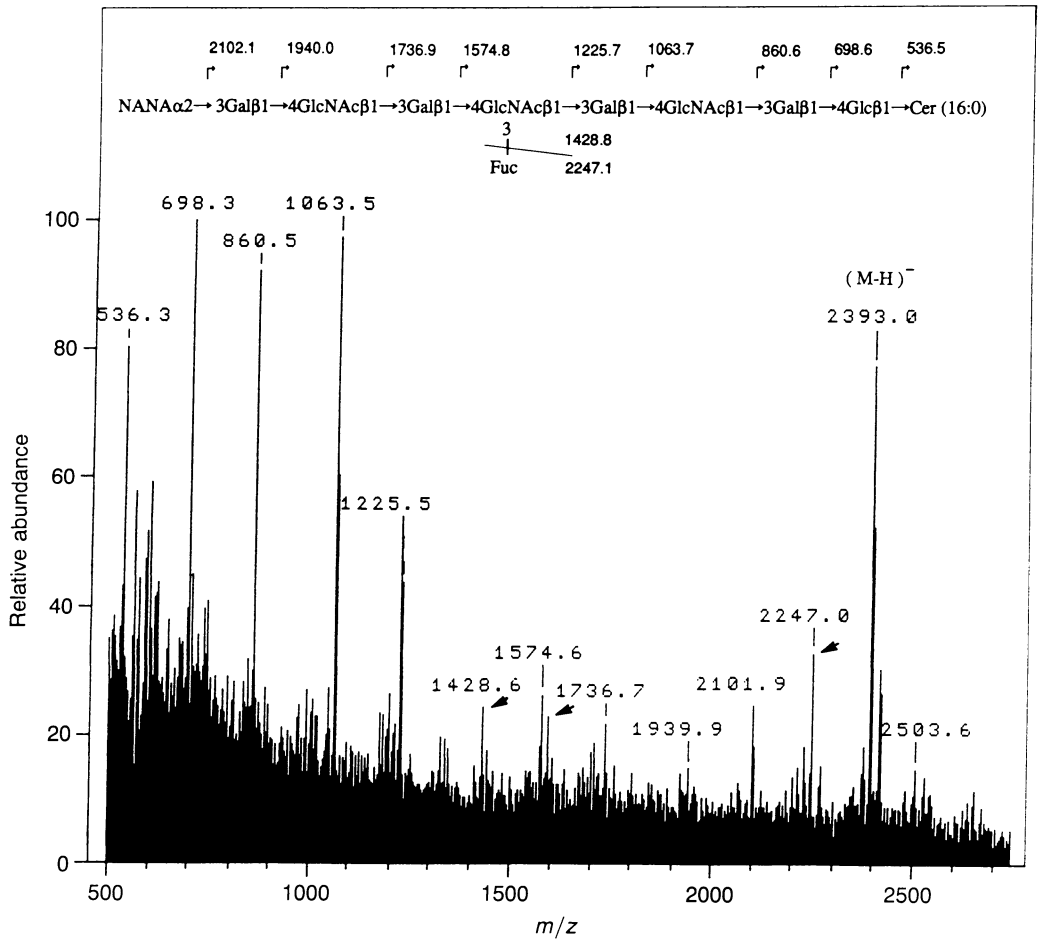

FIG. 4. Mass spectrum of band 5, with a proposed structure based on the mass spectrum, composition, and linkage analysis. Liquid secondary ion mass spectra were acquired on a Finnigan MAT 900 mass spectrometer with a triethanolamine matrix. The proposed structure was inferred from data presented in this paper as well as known structures reported from similar sources (20, 21). The signal at 2247 may include a molecular ion of nonfucosylated material, which may fragment to contribute to signals seen at 1591.6 and 1428.6 (arrows).

same oligosaccharides as bands 2 and 5, respectively, but lack the fucose residue. Therefore, it appears that the minimum structure isolated from myeloid cells possessing binding activity contains two *N*-acetyllactosamine units, a terminal sialic acid, and one fucose residue.

Removal of NANA and fucose from the mixed glycolipids by mild acid hydrolysis (22) eliminated ELAM-COS binding, suggesting the requirement for at least one of these sugars. To confirm this hypothesis, band 1, an unrecognized lactosylceramide with two *N*-acetyllactosamine units and a terminal sialic acid residue, was enzymatically fucosylated. This fucosylation converted the nonbinding band 1 into a mixture of fucosylated glycolipids capable of supporting adhesion of pl-ELAM-transfected COS cells (Fig. 5*A*). This fucosylated band 1, when enzymatically desialylated, no longer supported adhesion (Fig. 5*B*). These data confirm that ELAM-1 requires both sialic acid and fucose for recognition.

DISCUSSION

In this study we have utilized cell adhesion assays that detect the direct interaction of cell surface-expressed ELAM-1 with glycolipid ligands. The data presented here indicate that ELAM-1 recognizes terminally sialylated lactosylceramides with a variable number of *N*-acetyllactosamine repeats and at least one fucose linked (α1-3) to an internal GlcNAc residue. Enzymatic fucosylation and desialylation experiments demonstrate the absolute requirement of both fucose and sialic acid for ELAM-1 binding (Fig. 5).

The mass spectrum of the ELAM-1-recognized band 5 (Fig. 4) as well as enzyme digestion studies on other ELAM-1 ligands indicate an internal location of the fucose. The ELAM-1-recognized structure proposed in Fig. 4 is inferred from the data presented here as well as known structures reported from similar sources (20, 21). While oligosaccharides with fucose only on the external-most GlcNAc were not found, we cannot rule out the possibility that such a structure (sialyl-Le^x) might be recognized by ELAM-1 as well. Macher *et al.* (21) report that oligosaccharides with the structural features we have described here for the endogenous ELAM-1 ligands are primarily restricted to human myeloid cells. They indicate that this structure is present in higher quantities on neutrophils and more highly differentiated leukemia cells (23).

Glycolipids with structures similar to those described here have been identified in fetal tissue and in a variety of human cancers (20, 24). Glycoproteins have also been described that contain the ELAM-1-recognized oligosaccharide structures (25). Although protein–protein interactions in cell recognition have been recognized for some time, only recently has the role of carbohydrates in physiologically relevant recognition been widely considered (26, 27). Oligosaccharides are well-positioned to act as recognition molecules because of their cell surface location and structural diversity. Many oligosaccharide structures can be created through the differential activities of a small number of specific glycosyltransferases, suggesting a plausible mechanism for establishing the information necessary to direct a wide range of cell–cell interac-

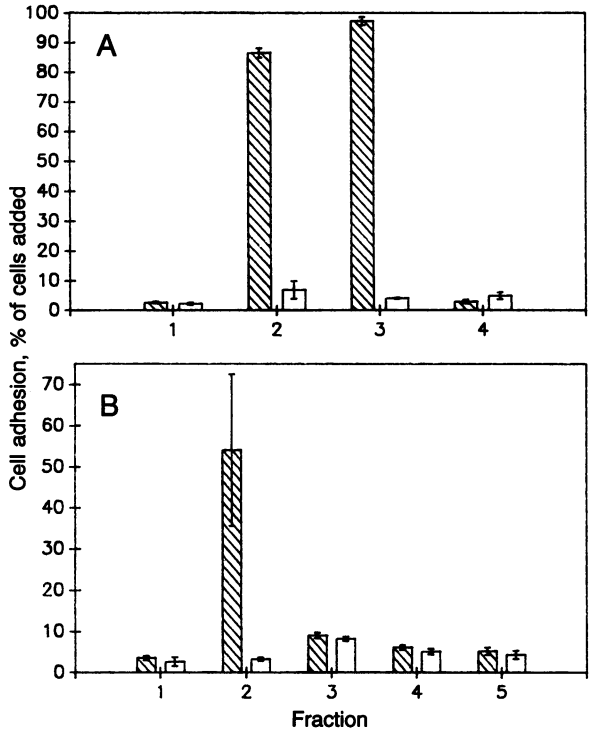

FIG. 5. Contribution of fucose and sialic acid to ELAM-1 binding. (*A*) Band 1, a lactosylceramide with two *N*-acetyllactosamine units and a terminal sialic acid residue that is not recognized by ELAM-1, was enzymatically fucosylated. After purification of the glycolipid by adsorption on a C_{18} Sep-Pak and elution with chloroform/methanol, 100 pmol of the glycolipids were adsorbed to microtiter wells and probed with pl-ELAM-transfected (▨) or control-transfected (□) COS cells as described. Columns: 1, band 1; 2, enzymatically fucosylated band 1; 3, glycolipid pool from lane 10 of Fig. 2*B* as the positive control; 4, phosphatidylcholine/cholesterol as the negative control. (*B*) This fucosylated band 1 was then enzymatically desialylated, purified, and probed in an identical manner. Columns: 1, band 1; 2, fucosylated band 1; 3, desialylated band 1; 4, desialylated fucosylated band 1; 5, phosphatidylcholine/cholesterol. Data are presented as the mean of triplicate measurements ± SEM.

tions. Examples of differential expression of cell surface carbohydrates and putative lectins on interacting cells have been described (28–30). Our data indicate that some acidic glycolipids on human leukocytes recognized by ELAM-1 on activated vascular endothelium may function in one such oligosaccharide–lectin pair. Since many protein–protein interactions have been implicated in neutrophil-endothelial transmigration (for review, see ref. 1), this lectin–carbohydrate interaction may be an early step in a series that results in neutrophil extravasation.

Note Added in Proof. Since submission of this manuscript, four papers on the carbohydrates recognized by ELAM-1 have been published (31–34). For a review of these papers in relation to the data presented here, refer to ref. 35.

We thank Dr. John Klock for providing the human cells used in this study, Dr. Robert Hill for providing the fucosyltransferase, Dr. Brian Seed for the gift of the CDM8 plasmid, and Drs. Robert Stack, Roger Laine, and Ron Schnaar for helpful discussions.

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