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ELAM-1-Dependent Cell Adhesion to Vascular Endothelium Determined by a Transfected Human Fucosyltransferase cDNA

John B. Lowe,*† Lloyd M. Stoolman,† Rajan P. Nair,* Robert D. Larsen,* Terry L. Berhend,† and Rory M. Marks‡
* Howard Hughes Medical Institute
† Department of Pathology
‡ Department of Internal Medicine
The University of Michigan Medical School
Ann Arbor, Michigan 48109-0650

Summary

Adhesion of circulating leukocytes to the vascular endothelium during inflammation is mediated in part by their interaction with the endothelial-leukocyte adhesion molecule ELAM-1. ELAM-1, a member of the LEC-CAM family of cell adhesion molecules, expresses an N-terminal carbohydrate recognition domain (CRD) homologous to various calcium-dependent mammalian lectins. However, the contribution of the CRD to cell adhesion and its carbohydrate binding specificity have not been elucidated. This study demonstrates that transfection of a human fucosyltransferase cDNA into nonmyeloid cell lines confers ELAM-1-dependent endothelial adhesion. Binding activity correlates with de novo cell surface expression of the sialylated Lewis x tetrasaccharide, whose biosynthesis is determined by the transfected fucosyltransferase cDNA. We propose that specific α(1,3)fucosyltransferases regulate cell adhesion to ELAM-1 by modulating cell surface expression of one or more α(2,3)sialylated, α(1,3)fucosylated lactosaminoglycans represented by the sialyl Lewis x carbohydrate determinant.

Introduction

The LEC-CAM/SELECTIN family of cell adhesion molecules mediates adhesive interactions between circulating leukocytes and the vascular endothelium (Stoolman, 1989; Geng et al., 1990). These molecules participate in the recruitment of neutrophils and monocytes to inflammatory lesions (Carlos and Harlan, 1990; Jutila et al., 1989), in the adhesion of activated platelets to leukocytes (Larsen et al., 1989a), in the recirculation of normal lymphocytes through the lymphoid system (Yednock and Rosen, 1989), and in the hematogenous dissemination of lymphoid malignancies (Bartgatze et al., 1987). Three structurally related receptors have thus far been identified: ELAM-1 (Bevilacqua et al., 1989), GMP-140/PADGEM (Bonfanti et al., 1989; Johnston et al., 1989), and LEC-CAM1 (consensus term referring to the antigen expressing the Mel14 epitope in the mouse [Bowen et al., 1989; Lasky et al., 1989] and its human homologs, Leu8/TQ1 [Camerini et al., 1989], LAM [Tedder et al., 1989], and DREG [Kishimoto et al., 1990]). The N-terminal domains of these molecules are homologous to one another and to a variety of calcium-dependent carbohydrate recognition domains (CRDs) containing a structural motif originally described by Drickamer and colleagues (Drickamer, 1988). The CRD of LEC-CAM1 mediates adhesion to high endothelial venules in vitro (Geoffroy and Rosen, 1989; Yednock and Rosen, 1989) and appears to initiate lymphocyte recirculation through binding to one or more sialylated ligands on the high endothelial venules in vivo (Rosen et al., 1985, 1989).

The presence of a CRD in ELAM-1 therefore suggests that specific oligosaccharide determinants may constitute part or all of its endogenous ligand. The current study provides experimental support for this hypothesis by linking expression of a family of α(2,3)sialylated, α(1,3)fucosylated lactosaminoglycans at the cell surface to ELAM-1-dependent endothelial adhesion and by demonstrating that transfection of a specific α(1,3)fucosyltransferase cDNA into nonmyeloid cell lines results in the de novo expression of functional ligands for ELAM-1-mediated cell adhesion.

Results

ELAM-1-Dependent Cell Adhesion Correlates with Sialyl Lewis x Expression in Variants of the HL-60 Cell Line

The ELAM-1 receptor mediates the adhesion of neutrophils, monocytes, and the related cell lines HL-60 and U937 to cytokine-stimulated human umbilical vein endothelial cells (HUVECs) (Bevilacqua et al., 1987, 1989). The surfaces of these leukocytic cells are unusually rich in fucosylated derivatives of neutral and α(2,3)sialylated polylactosamine ($[Gal\beta1,4GlcNAc]_n$) moieties (Fukuda et al., 1984, 1985; Spooncer et al., 1984). The structurally and biosynthetically related members of this group include the sialyl Lewis x tetrasaccharide (NeuAcα2→3-Galβ1→4(Fucα1→3)GlcNAc; sLex; Fukushima et al., 1984; Figure 1A), its nonsialylated trisaccharide analog Lewis x (Galβ1→4(Fucα1→3)GlcNAc; Lex or SSEA-1; Gooi et al., 1981; Figure 1A), and related neutral and α(2,3)sialic acid-substituted structures containing single (VIM-2 [Macher et al., 1988]) or multiple (FH4, FH5 [Fukushi et al., 1984], and FH6 [Fukushi et al., 1985]) internal α(1,3)-linked fucose residues. By contrast, these oligosaccharide determinants are generally not detected on circulating cells that lack the ligand(s) for ELAM-1 (Fox et al., 1983; Fukushima et al., 1984; Fukushi et al., 1985). These observations prompted us to measure expression of sLex and Lex epitopes on two variants of the HL-60 line showing markedly different levels of ELAM-1-dependent adhesion to TNFα-treated HUVECs.

Figure 2 depicts a series of experiments with two spontaneously arising variants of the HL-60 line. Both cell lines expressed the myeloid differentiation antigens CD11b, CD19, and CD33 (data not shown). Using an assay that maximizes carbohydrate-dependent cell adhesion (Stoolman et al., 1987; Experimental Procedures), HL-60 line B cells bound avidly to TNFα-treated HUVECs, with 55% of

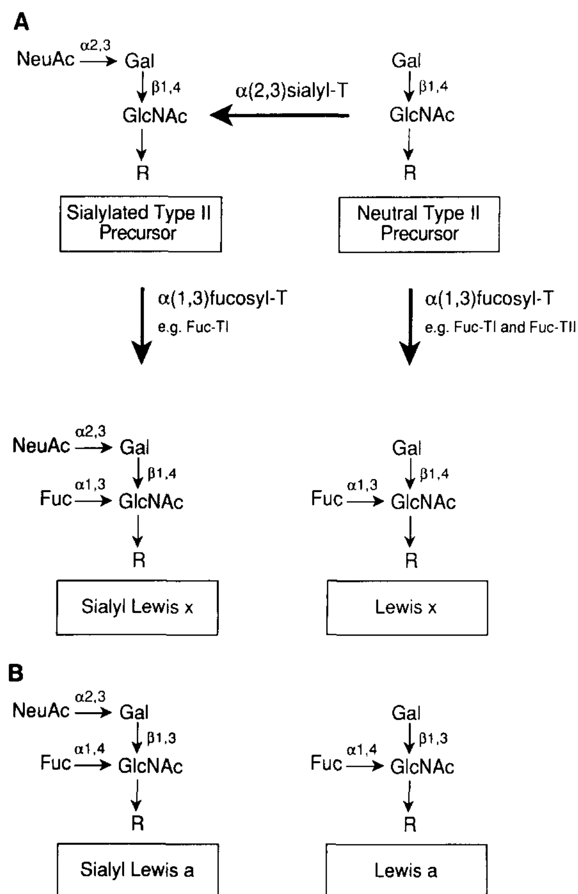

Figure 1. Structures and Biosynthesis of Lewis Oligosaccharide Determinants

(A) Structures and biosynthesis of type II-based sLex and Lex molecules. Terminal galactose residues on neutral type II precursors may be substituted with α(2,3)-linked sialic acid via the action of an α(2,3)sialyltransferase (α(2,3)sialyl-T [Weinstein et al., 1982]). The subterminal GlcNAc residues on this sialylated precursor molecule, or on its neutral predecessor, may then each be substituted with α(1,3)-linked fucose residues to form the sLex and Lex determinants, respectively. This occurs via the action of α(1,3)fucosyltransferases that can operate exclusively on neutral precursors (e.g., Fuc-TII [Howard et al., 1987]), or that operate on both neutral and sialylated precursors (e.g., Fuc-T1 [Howard et al., 1987]). R = glycoprotein or glycolipid moieties that may contain one or more additional lactosamine (Galβ1,4GlcNAcβ1,3) repeat units.

(B) Structures of the type I-based sLea and Lea determinants. The sLea and Lea molecules are thought to be constructed from sialylated and neutral type I precursors, respectively, by the action of α(1,4)fucosyltransferases, in a manner strictly analogous to the biosynthesis of the type II structures shown in (A) (Hansson and Zopf, 1985). R = glycoprotein or glycolipid moieties that display the type I precursor oligosaccharide.

the added cells attached in the presence of the control antibody IgG2b. This adhesion was largely ELAM-1 dependent since only 11% of the cells adhered in the presence of the anti-ELAM-1 antibody BB11 (Benjamin et al., 1990). By contrast, the line designated HL-60 A exhibited a lower absolute level of adhesion (mean of 16%) that was not significantly reduced by treatment of the HUVECs with BB11 (14%). Thus, HL-60 A showed virtually no ELAM-1-dependent adhesion under conditions that optimized detection of this interaction on HUVEC monolayers.

Flow cytometric analysis revealed markedly different levels of the sLex and Lex determinants at the cell surface of HL-60 A and HL-60 B (Figure 2B). Both cell lines expressed the Lex epitope as has been previously described for the HL-60 cell line (Symington et al., 1985). By contrast, the sLex epitope was detected on the adhesion-competent HL-60 line B cells exclusively. The resistance of the ELAM-1 ligand(s) to paraformaldehyde fixation and the persistence of its functional activity at reduced temperature mirror the behavior of the endogenous, sialylated ligand for LEC-CAM1 (Stoolman, 1989; Yednock and Rosen, 1989) and thus are consistent with the hypothesis that the CRD of ELAM-1 interacts with an oligosaccharide on the surface of HL-60 cells. Moreover, the coordinate loss of both ELAM-1-dependent adhesion and the sLex determinant suggested that the ligand(s) belongs to the family of sialylated, fucosylated polylactosaminoglycans expressed at high levels on myeloid cells.

ELAM-1-Dependent Cell Adhesion of COS-1 Cells Determined by Transfection of a Cloned Fucosyltransferase cDNA

The profound differences in sLex expression observed in the HL-60 variants may have resulted from differences in the activities of specific fucosyltransferases (Figure 1A), by analogy to the Chinese hamster ovary (CHO) glycosylation mutants Lec11 and Lec12 (Howard et al., 1987). The Lec11 mutant exhibits de novo expression of a specific α(1,3)fucosyltransferase, termed Fuc-TI, that determines the synthesis of surface-localized neutral and α(2,3)sialylated polylactosaminoglycans substituted with α(1,3)-linked fucose residues (Figure 1A). By contrast, the Lec12 mutant expresses a distinct α(1,3)fucosyltransferase, FucTII, that determines surface display of neutral, α(1,3) fucosylated polylactosaminoglycans, but not the α(2,3) sialylated analogs (Figure 1A). The penultimate step in the biosynthesis of these structures, which include the sLex moiety, is thought to be catalyzed by a widely distributed sialyltransferase that attaches sialic acid in α(2,3)linkage to terminal galactose residues in the polylactosamine substrate (Weinstein et al., 1982). These sialylated molecules can then serve as acceptors for some (Holmes et al., 1986; Howard et al., 1987), but not all (Howard et al., 1987), α(1,3)fucosyltransferases. These enzymes may add fucose residues in α(1,3)-linkage to the terminal GlcNAc residue, thus forming the sLex structure, and/or to one or more GlcNAc residues within internal lactosamine units (Holmes et al., 1986; Howard et al., 1987). Likewise, α(1,3) fucosyltransferases can generate the Lex moiety and its polyfucosylated analogs from neutral polylactosamine precursors. These neutral fucosylated molecules are not, however, substrates for any known α(2,3)sialyltransferases. These considerations suggest a critical, regulatory role for expression of specific α(1,3)fucosyltransferases in the biosynthesis of the family of sialylated, fucosylated lactosaminoglycans that represent putative ELAM-1 ligands.

To investigate this possibility, and to test the roles of these molecules in ELAM-1-dependent cell adhesion, we effected their de novo surface expression on mammalian host cells, via modification of their glycosylation pheno-

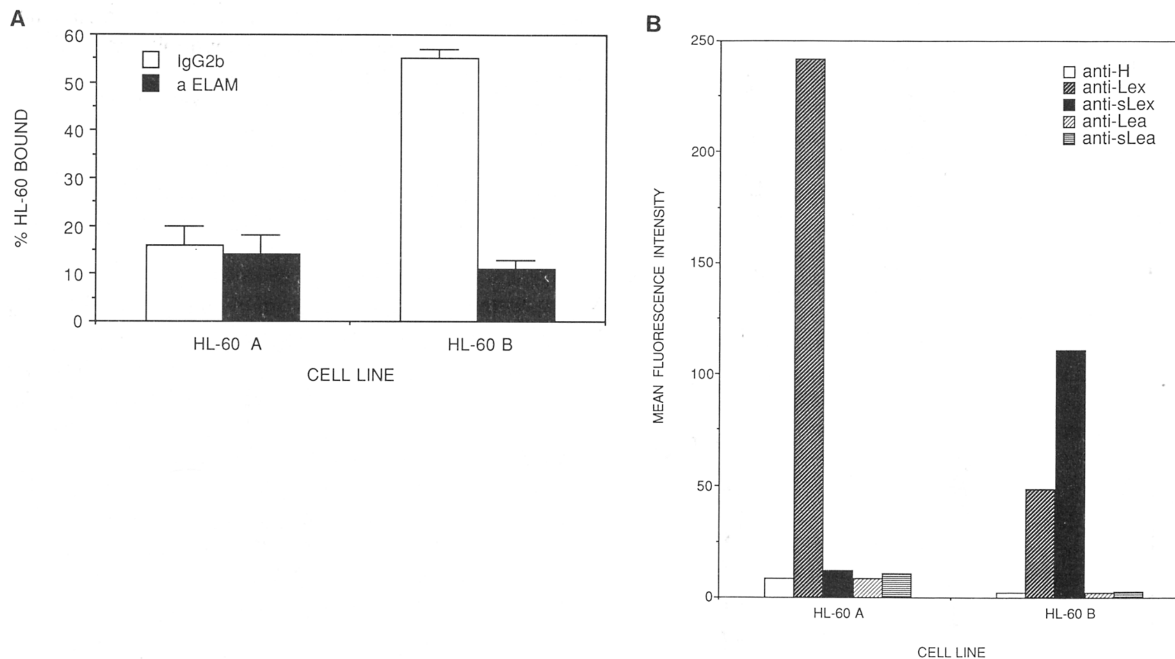

Figure 2. HL-60 Cell Lines Show Differential Expression of ELAM-1-Dependent HUVEC Adhesion and Surface-Localized sLex Determinants

(A) Binding of HL-60 cell lines to human endothelial cell monolayers. HL-60 lines A and B were tested for adherence to TNFα-activated HUVEC, using an adhesion assay that isolates the ELAM-1-dependent component of this interaction (Method II, Experimental Procedures). Cells were allowed to adhere to TNFα-treated HUVEC monolayers, at 7°C–10°C, in the presence of either the anti-ELAM-1 antibody BB11 (solid bars) or the control antibody IgG2b (open bars). Nonadherent cells were removed after 45 min; the fraction of remaining adherent cells are shown (%HL-60 Bound) and are mean determinations, ± standard errors, representing a total of 14 (HL-60 A) or 22 (HL-60 B) separate determinations from five independent experiments.

(B) Flow cytometry analysis of cell surface oligosaccharide determinants. HL-60 cell lines A and B were subjected to indirect immunofluorescence using the monoclonal antibodies directed against carbohydrate determinants detailed in the inset. Analyses were performed as described in Experimental Procedures, using a Coulter Epics V instrument equipped with a three-decade scale.

types with transfected α(1,3)fucosyltransferase cDNAs or gene segments (pCDM7-α(1,3/1,4)FT [Kukowska-Latallo et al., 1990], pCDNA1-α(1,3)FT [J. B. L. et al., unpublished data]). Nonmyeloid hosts were chosen for these experiments in order to isolate the role of the oligosaccharide molecules in ELAM-1-dependent adhesion from other myeloid-specific molecules known to participate in leukocyte-endothelial cell interactions (Springer, 1990). COS-1 cells were used for the first of these experiments since these cells do not express detectable α(1,3)fucosyltransferase activity nor cell surface oligosaccharides that contain the cognate α(1,3)fucose linkages (Kukowska-Latallo et al., 1990). Moreover, COS-1 cells do not exhibit ELAM-1-dependent adhesive properties. They do, however, express the oligosaccharide substrates necessary for α(1,3) fucosyltransferase-dependent biosynthesis of the family of oligosaccharides represented by the Lex and sLex molecules (Fukuda et al., 1988; Kukowska-Latallo et al., 1990).

As shown in Figure 3A, 26%–31% of the population of COS-1 cells transfected with a plasmid encoding an α(1,3/1,4)fucosyltransferase (pCDM7-α(1,3/1,4)FT [Kukowska-Latallo et al., 1990]) express sLex and Lex molecules (Figure 3A). As expected from the properties of the enzyme encoded by pCDM7-α(1,3/1,4)FT (Prieels et al., 1981; Palcic et al., 1989; Kukowska-Latallo et al., 1990), the sialyl Lewis a (sLea) and Lea determinants are also expressed (Figures 1B and 3A). By contrast, COS-1 cells transfected with pCDNA1-α(1,3)FT, which encodes a distinct α(1,3)fucosyltransferase (J. B. L. et al., unpublished data), express new Lex determinants exclusively (Figure 3A). Cells transfected with the control vectors (pCDM7 and pCDNA1), as well as with vectors encoding two other mammalian glycosyltransferases (pCDM7-αGT [Larsen et al., 1989b] and pH3.4 [Rajan et al., 1989]), remained negative for all four surface carbohydrate determinants (Figure 3A; data not shown). Using ^{51}Cr-labeled cells, only pCDM7-α(1,3/1,4)FT transfectants, expressing the four new fucosylated oligosaccharide determinants, exhibited adhesion to TNFα-treated HUVECs (Figure 3B). This binding was substantially inhibited with the anti-ELAM-1 antibody BB11, but not with its isotype control antibody IgG2b, nor with antibodies that detect other polypeptides expressed on TNFα-treated HUVECs (VCAM1, ICAM1, and HLA class I determinants; Figure 3C).

In these experiments, approximately 7% of the pCDM7-α(1,3/1,4)FT transfectants adhered to TNFα-treated endothelium, whereas 35% of normal neutrophils adhered under the same conditions (Figure 3B, right). This apparent difference in relative adhesion "activity" can be accounted for by the fact that only a minority (26%–31%) of the radiolabeled COS-1 cells actually express new cell surface oligosaccharide determinants and by the fact that

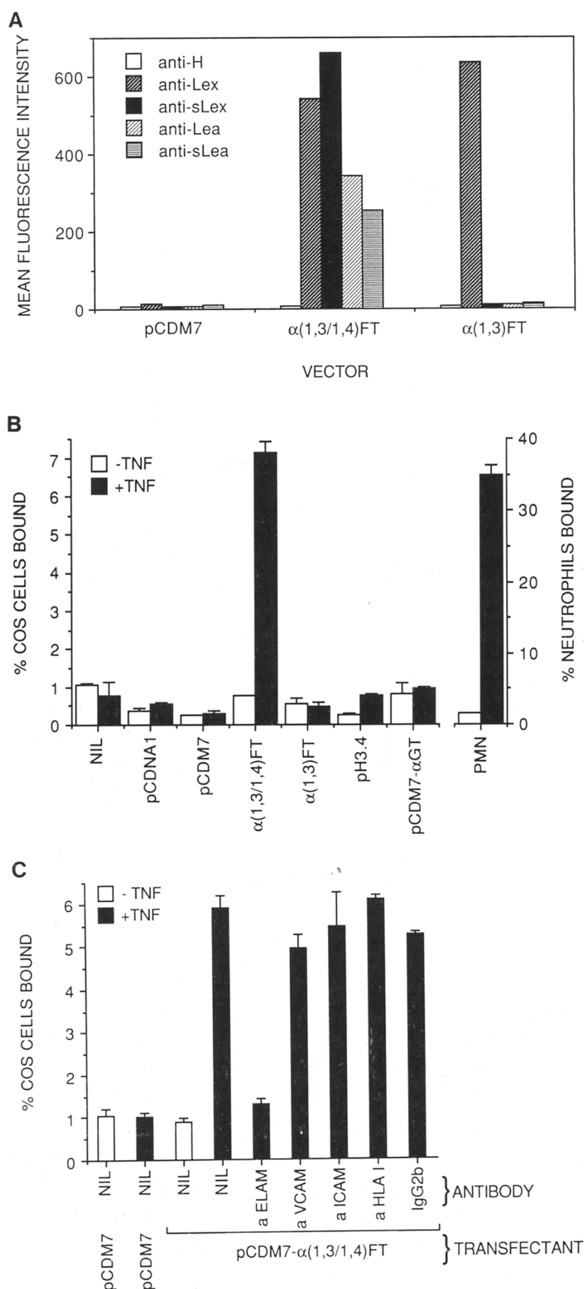

Figure 3. Differential Expression of Cell Surface Oligosaccharide Antigens, and of ELAM-1-Dependent HUVEC Adhesion, Determined in COS-1 Cells by Transfected Glycosyltransferase Expression Vectors

(A) Flow cytometry analysis of transfected COS-1 cells. Cells were transfected with two fucosyltransferase expression vectors, pCDM7-α(1,3/1,4)FT or pCDNA1-α(1,3)FT (labeled, respectively, α(1,3/1,4)FT and α(1,3)FT), or with the control vector pCDM7. Transfected cells were then subjected to flow cytometry analysis with the monoclonal antibodies detailed in the inset, as described in Experimental Procedures. Between 26% and 31% of the cells transfected with pCDM7-α(1,3/1,4)FT or with pCDNA1-α(1,3)FT expressed, respectively, all four Le determinants, or Lex determinants only, relative to background staining with anti-H antibody (data not shown). The data presented here are the mean (linear) fluorescence intensities of the antigen-positive population of transfected cells.

(B) Adhesion of transfected COS-1 cells to endothelial cell monolayers. Untransfected COS-1 cells (NIL), or COS-1 cells transfected with glycosyltransferase expression vectors or their control vectors, were radiolabeled with ^{51}Cr, harvested, and tested for adhesion on HUVEC monolayers using Method I, as detailed in Experimental Procedures. Transfected cells were applied to either TNFα-treated (+) or untreated (−) HUVEC monolayers and allowed to adhere for 20 min prior to washing. Plasmids pCDM7-α(1,3/1,4)FT and pCDNA1-α(1,3)FT (labeled as in [A]) encode distinct α(1,3)fucosyltransferases as described in the text. The corresponding control vectors for these plasmids (pCDM7 and pCDNA1) lack cDNA inserts. Plasmid pCDM7-αGT encodes a murine α(1,3)galactosyltransferase (Larsen et al., 1989b). Plasmid pH3.4 encodes a human α(1,2)fucosyltransferase (Rajan et al., 1989). COS-1 cells transfected with these latter two plasmids do not express Lex, sLex, Lea, or sLea determinants (data not shown). The adhesion of normal neutrophils (PMN) to HUVEC within the same experiment is also shown on a separately scaled ordinate.

(C) Specific anti-ELAM-1 blocking of adhesion of pCDM7-α(1,3/1,4)FT transfected cells to TNFα-treated HUVEC monolayers. ^{51}Cr-labeled COS-1 cells transfected with pCDM7-α(1,3/1,4)FT, or the pCDM7 control, were assessed in the adhesion assay as outlined in (B), except that the HUVEC monolayers had been pretreated with antibody directed against ELAM-1 (BB11), with an isotope control (IgG2b), or with antibodies against other endothelial-expressed polypeptides (anti-VCAM1 4B9, anti-ICAM1, and anti-HLA class 1), as described in Experimental Procedures.

Error bars equal one standard deviation.

ELAM-1 interactions represent only one of several mechanisms responsible for adhesion of neutrophils to cytokine-stimulated endothelium (Luscinska et al., 1989). Indeed, anti-ELAM-1 antibody BB11 pretreatment of the TNFα-activated HUVECs in this experiment incompletely blocked neutrophil adhesion, reducing it to approximately 17% (data not shown). These considerations indicate that ELAM-1-dependent adhesion of the two cell types is of a substantially similar magnitude.

The neutral and sLea isomers (Figure 1B) displayed by the pCDM7-α(1,3/1,4)FT transfected, adhesion-competent COS-1 cells are generally absent from blood cells of the myeloid lineage (Fukuda et al., 1984, 1985; Spooncer et al., 1984; Dunstan, 1986) and thus would not normally participate in leukocyte-ELAM-1 adhesive interactions. We therefore tentatively concluded that the ELAM-1-dependent adherence of cells transfected with pCDM7-α(1,3/1,4)FT is mediated by the surface-localized sLex tetrasaccharide molecule and/or its alternately and poly-α(1,3)fucosylated analogs, but not by the neutral family of molecules represented by the Lex determinant.

Expression of a Cloned Fucosyltransferase cDNA in Stably Transfected CHO Cells Determines ELAM-1-Dependent Cell Adhesion and sLex Expression

To isolate and further confirm the contribution of the sLex-type oligosaccharide molecules to ELAM-1-dependent binding, we generated a transfected mammalian cell line (CHO-FT) that expresses surface-localized sLex and Lex determinants but not the Lea or sLea determinants. The parental CHO cell line Ade$^-$ C (Oates and Patterson, 1977; Van Keuren et al., 1986) chosen for these experiments expresses no detectable α(1,3)fucosyltransferase activity (Experimental Procedures) nor the corresponding Lex and sLex molecules (Smith et al., 1990). It does, however, express polylactosaminoglycan precursors for these molecules but not for the Lea precursors (Smith et al.,

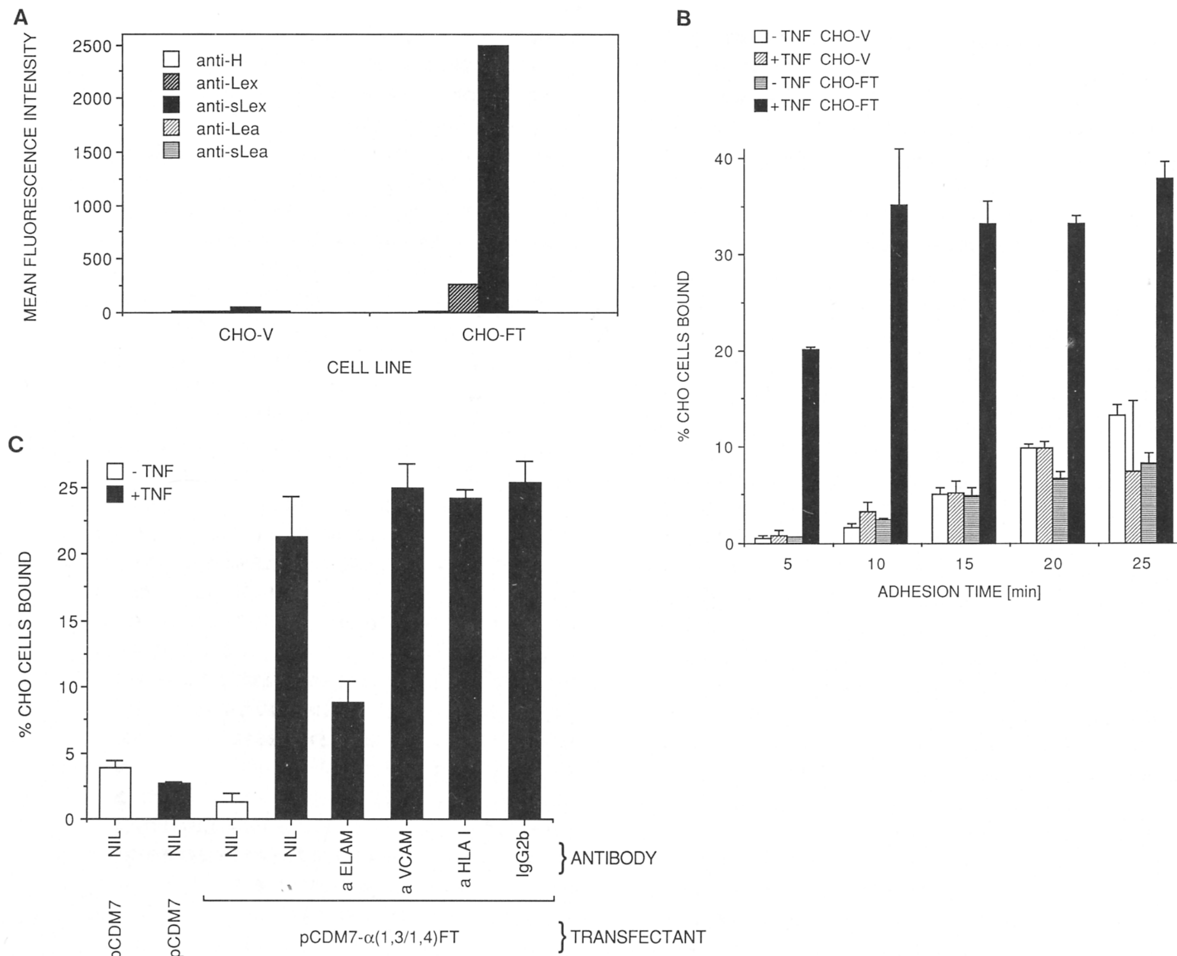

Figure 4. CHO Cells Stably Transfected with a Fucosyltransferase Expression Vector Exhibit ELAM-1-Dependent Adhesion to TNFα-Stimulated HUVECs

(A) Flow cytometry analysis of transfected CHO cells. CHO cells stably transfected with the fucosyltransferase expression vector pCDM7-α(1,3/1,4)FT (CHO-FT) or with the control vector pCDM7 (CHO-V) were subjected to flow cytometry analysis with the monoclonal antibodies detailed in the figure and as described in Experimental Procedures. The data presented here are the mean fluorescence intensities of the entire population of these transfected cells; virtually 100% of the CHO-FT cells stain with anti-sLex and anti-Lex antibodies, but not with the other three antibodies (data not shown). Likewise, the entire population of CHO-V cells do not stain with any of the antibodies.

(B) Time course of adhesion of CHO cells stably transfected with pCDM7-α(1,3/1,4)FT (CHO-FT) or vector alone (CHO-V). Transfected cell lines were labeled with ^{51}Cr, harvested, and tested for adhesion on untreated (−) or TNFα-treated (+) HUVEC monolayers. Nonadherent cells were removed after the times shown, and adhesion was determined by Method I as described in Experimental Procedures.

(C) Specific anti-ELAM-1 blocking of adhesion of CHO-FT cells to TNFα-treated HUVEC monolayers. ^{51}Cr-labeled CHO-FT cells were subjected to adhesion for 10 min, as outlined in (B), except that the HUVEC monolayers had been pretreated with the anti-ELAM-1 antibody BB11, the isotype control IgG2b, or with antibodies against VCAM1 and HLA class 1 molecules, at the same concentrations used in Figure 3C. Pretreatment with anti-ICAM1 fails to inhibit binding to TNFα-treated HUVEC monolayers (data not shown).

Error bars equal one standard deviation.

1990). Expression of stably transfected pCDM7-α(1,3/1,4)FT in this host determines surface display of substantial amounts of the sLex determinant, and to a lesser extent the Lex determinant, but not the Lea isomers (Figure 4A).

Each CHO transfectant line was assessed for adhesion to untreated and TNFα-treated HUVECs (Figures 4B and 4C). Cells were allowed to adhere for between 5 and 25 min, before washing off unbound cells. CHO-FT cells exhibited marked adhesion to TNFα-treated HUVECs, but not to untreated endothelium (Figures 4B). Adhesion of CHO-FT cells was well-established within 5 min, plateaued at 10 min, and was resistant to repeated and vigorous washing (Experimental Procedures). By contrast, control transfectants (CHO-V) that do not express significant amounts of the Lex or sLex molecules (Figure 4A) did not exhibit significant binding to either TNFα-treated or untreated HUVECs (Figure 4B) at early time points, and minimal, nonspecific adhesion at later ones. Adhesion of CHO-FT cells was ELAM-1 dependent since it was inhibited by pretreatment of the HUVECs with the BB11 anti-ELAM-1 antibody, but not by the control antibody IgG2b, nor with antibodies directed against other adhesion receptors (VCAM1 and ICAM1), or to HLA class I determinants (Figure 4C; data not shown). The very rapid onset

of the ELAM-1-dependent adhesion to vascular endothelium exhibited by the CHO-FT cells is physiologically consistent with the speed of the analogous interaction between neutrophils and ELAM-1, which arrests these latter cells as they pass over activated endothelium.

Discussion

We have demonstrated here that expression of an α(1,3/1,4)fucosyltransferase results in the appearance of ligands for ELAM-1 on the surface of transfected COS-1 and CHO cells. In COS-1 transfectants the sLex, Lex, Lea, and sLea oligosaccharides appeared at the surface, while in CHO transfectants only new sLex and Lex structures were detected. Thus, oligosaccharides terminating in the Lea and sLea structures are not necessary for expression of ELAM-1-dependent cell adhesion. Moreover, ELAM-1-dependent adhesion was not manifested either by COS-1 cells transfected with pCDNA1-α(1,3)FT or by the variant of the HL-60 cell line when expression of the Lex determinant occurs in the absence of the sLex structure. We conclude that expression of α(1,3)fucosyltransferases capable of modifying acceptors containing α(2,3)sialic acid-substituted lactosaminoglycans is a critical step in the synthesis of the ligand(s) for ELAM-1. Therefore, one or more members of the family of sialylated, fucosylated lactosaminoglycans constructed by such enzymes are the most likely ligands for the CRD of ELAM-1.

The precise molecular nature of the ELAM-1 ligand(s) determined by the transfected fucosyltransferase cDNA remains to be elucidated. Likewise, the identities of the protein or lipid molecules that display the candidate oligosaccharide ligand(s) are as yet unknown. It is possible that the core structure of such underlying molecules may participate directly in the adhesion process. For example, the composite molecule might engage both the carbohydrate and putative complement regulatory domains of ELAM-1 and thereby possibly modulate complement-mediated leukocyte-endothelial adhesion (Marks et al., 1989). However, one can conclude that polylactosamine-type ganglioside precursors contained in normal myeloid cells (Fukuda et al., 1985) are not essential to the generation of ELAM-1 binding activity since the CHO host used for the stable transfection experiments does not construct such molecules (Smith et al., 1990). As this observation suggests, the nonhematopoietic COS-1 and CHO cells are unlikely to express unique, myeloid-specific protein or lipid structures. In light of these considerations, we propose that the oligosaccharide moieties determined by the transfected cDNA are themselves sufficient to mediate ELAM-1-dependent adhesion.

The sLex structure and its mono- and polyfucosylated analogs are candidates for such molecules. Our attempts to assign binding activity to the sLex tetrasaccharide determinant by antibody blocking approaches have been inconclusive because of the agglutinating nature of the IgM anti-sLex antibody (R. M. M. and L. M. S., unpublished data). Detailed structural analyses of the oligosaccharide determinants at the surfaces of the ELAM-1 binding CHO transfectants should facilitate identification of the new oligosaccharide structures specified by the transfected fucosyltransferase cDNA. Once identified, these molecules may be isolated and tested individually for their ability to bind specifically to ELAM-1 and to inhibit its interaction with neutrophils. Alternatively, testing may be done on candidate molecules already purified by others from myeloid cells (Fukuda et al., 1984; Symington et al., 1985). Recent advances in enzyme-catalyzed in vitro synthesis of oligosaccharides (Wong, 1989; Toone et al., 1989), coupled with the emerging availability of cloned glycosyltransferase cDNAs (Paulson and Colley, 1989), should also facilitate the availability of these molecules for such analyses.

The fucosyltransferase encoded by pCDM7-α(1,3/1,4FT), and used here to generate the ligand(s) for ELAM-1, represents the product of the human Lewis blood group locus (Kukowska-Latallo et al., 1990). It exhibits a uniquely broad spectrum of acceptor substrate requirements that encompasses the requirements exhibited by members of two other general classes of α(1,3)fucosyltransferases (Prieels et al., 1981; Palcic et al., 1989). Expression of this enzyme is thought to be restricted to secretory epithelium, except in Lewis blood group negative individuals who inherit an inability to express this enzyme in any tissue (Watkins, 1980). This enzyme is thus probably not responsible for synthesis of the ELAM-1 ligand(s) in the myeloid lineage. The α(1,3)fucosyltransferase encoded by pCDNA1-α(1,3)FT is likewise not responsible since it is representative of a second distinct class of α(1,3)fucosyltransferases (Howard et al., 1987) unable to utilize α(2,3)sialic acid-substituted lactosamine molecules, which are the apparent precursors for the ELAM-1 ligand(s). It therefore seems likely that a member of a third class of α(1,3)fucosyltransferases, capable of constructing the Lex and sLex moieties but not the Lea isomers (Potvin et al., 1990), represents the enzyme that determines expression of the ELAM-1 ligand(s) in myeloid cells. Confirmation of this hypothesis will await the isolation and characterization of cloned gene segments or cDNAs that encode such an enzyme.

The observations presented here suggest the possibility that a leukocyte adhesion deficiency-like phenotype (Anderson and Springer, 1987) might occur in individuals with a genetic defect in the ability to express the appropriate α(1,3)fucosyltransferase in the myeloid lineage. In this context, it may be noted that many patients with adenocarcinoma, but not healthy individuals, maintain circulating mucin-like molecules containing sLex moieties (Kannagi et al., 1986). Such molecules are thought to be shed from malignant tumors in part as a consequence of the "aberrant" expression of the sLex determinant frequently seen in association with malignant transformation (Fukushi et al., 1984; Fukushima et al., 1984; Kannagi et al., 1986). Should such molecules participate in specific interactions with ELAM-1, it seems possible that they might act to inhibit normal interactions between leukocytes and the vascular wall, and thereby act to blunt inflammation-induced leukocyte recruitment in these patients. Given that adenocarcinoma cells have also been shown to exhibit ELAM-1-dependent HUVEC adhesion (Rice and Bevilacqua, 1989), it is also interesting to consider the possibility that

its oligosaccharide ligand(s) may participate in the metastatic process.

The extensive homology between the CRDs of the LEC-CAM/SELECTIN family raises the possibility that the endogenous ligands for GMP140/PADGEM and LEC-CAM1 are structurally related to the sLex family of oligosaccharides. The former receptor, in particular, appears to interact with the same spectrum of normal cells and cell lines that adhere to ELAM-1 (Larsen et al., 1989a). Rosen and colleagues have demonstrated that the vascular ligand for $gp90^{MEL14}$ is neuraminidase sensitive, suggesting that terminally linked sialic acid may represent a component of this ligand (Yednock and Rosen, 1989). Of potential interest with respect to a fucosylated ligand for this molecule are studies implicating selective inhibition of functional activity with L-fucose, and fucoidan, a heteropolysaccharide rich in fucose-4-sulphate (Stoolman and Rosen, 1983; Stoolman et al., 1984). In recent studies, a direct interaction between two fucose-sulphate–containing polysaccharides, fucoidan and the egg-jelly fucan, and purified $gp90^{MEL14}$ has been demonstrated (Yasuyuki et al., 1990). Perhaps these synthetic ligands mimic key structural features or the charge distribution of an endogenous fucosylated sialyl-lactosamine. Thus, the identification of an endogenous carbohydrate determinant capable of interacting with ELAM-1 may provide important clues to the structure and synthesis of ligands for other members of the LEC-CAM/SELECTIN family of adhesion molecules. Following their identification, these molecules, or their structural analogs, may find utility as specific therapeutic agents in inflammatory disease, in ischemia-reperfusion injury that often occurs as a concomitant of myocardial injury and stroke, and in other conditions in which LEC-CAMs contribute to leukocyte recruitment.

Experimental Procedures

Cell Culture

Human umbilical vein endothelial cells were isolated and propagated according to the method of Jaffe (Jaffe, 1984). Cells were maintained in medium 199 with 20% fetal bovine serum, 100 μg/ml endothelial growth supplement (Collaborative Research), and 100 μg/ml bovine lung heparin (Sigma). Cells were used between passages 2 and 4. Endothelial identity was confirmed by the presence of typical morphology at confluence, by immunofluorescence with antibody to von Willebrand factor, and by uptake of acetylated low density lipoprotein. For adhesion assays, HUVECs were passaged with trypsin/EDTA (GIBCO) and plated in 96-well plates (Dynatech, Method I; Falcon, Method II; see HUVEC Adhesion Assays section). Cells were allowed to grow to confluence for at least 2 days prior to use in adhesion assays. HL-60 lines in spinner culture were maintained between 2.5×10^6 and 15×10^6 cells per ml in RPMI 1640 supplemented with 10% fetal calf serum and antibiotics. The CHO line Ade-C (Oates and Patterson, 1977; Van Keuren et al., 1986) was grown in α-modified Eagle's medium supplemented with 10% fetal calf serum. Transfected CHO cells were grown in media supplemented with G418 (GIBCO) at 400 μg/ml (active drug). COS-1 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

Glycosyltransferase Expression Vectors

Previous publications describe the plasmids pCDM7 and pCDM7-αGT (Larsen et al., 1989b), pCDM7-α(1,3/1,4)FT (Kukowska-Latallo et al., 1990), and pH3.4 (Rajan et al., 1989). Plasmid pCDNA1-α(1,3)FT contains a 3.6 kb PstI human genomic DNA restriction fragment that encodes an α(1,3)fucosyltransferase (J. B. L. et al., unpublished data). This fragment was isolated from a human lambda phage rescued from a genomic DNA library probed at low stringency with the insert in pCDM7-α(1,3/1,4)FT. The gene segment is cloned in the appropriate transcriptional orientation into the mammalian expression vector pCDNA1 (InVitrogen).

Antibodies

The anti-Lex antibody anti-SSEA-1 (mouse monoclonal IgM as ascites; Solter and Knowles, 1978) was provided by Dr. D. Solter (Philadelphia). Anti-sLex antibody CSLEX1 (mouse monoclonal IgM, HPLC purified [Fukushima et al., 1984]) and anti-sLea antibody CSLEA1 (mouse monoclonal IgG3, ammonium sulfate precipitate [Galton et al., 1985; Chia et al., 1985]) were provided by Dr. P. Terasaki (Los Angeles). Anti-H and anti-Lea antibodies (both mouse monoclonal IgM, antigen affinity purified) were purchased from Chembiomed Ltd. (Edmonton). Anti-ELAM-1 antibody BB11 (Benjamin et al., 1990) was the gift of Dr. Roy Lobb (Biogen, Inc., Cambridge, MA). IgG2b, anti-CD11b, anti-CD19, and anti-CD33 antibodies were purchased from Coulter Corp (Hialeah, FLA). Anti-VCAM antibody 4B9 (Carlos and Harlan, 1990) was provided by Dr. John Harlan (University of Washington, Seattle). Anti-ICAM1 antibody 84H10 (Makgoba et al., 1988) was purchased from AMAC, Inc. Anti-HLA class I antibody W6/32 (Parham et al., 1979) was purchased from Sera-Lab, Inc. (England).

Transfection, ^{51}Cr Labeling, and Harvesting of COS-1 Cells

COS-1 cells were transfected with various plasmids using the DEAE–dextran procedure (Davis et al., 1986) as previously described (Larsen et al., 1989b). Approximately 72 hr after transfection, cells (approximately 1×10^6) were labeled with ^{51}Cr by incubating them for 3–4 hr with 10 μCi/ml $Na^{51}CrO_4$ (NEN). Labeled or unlabeled, transfected COS-1 cells were harvested for HUVEC binding assays, or for flow cytometry analyses, by washing the cell monolayers with calcium, magnesium-free PBS (CMF-PBS) containing 2 mM EDTA, and then incubating the washed monolayers with CMF-PBS/2 mM EDTA. Detached cells were washed once by centrifugation through CMF-PBS/2 mM EDTA and then resuspended in buffers compatible with flow cytometry or binding analyses.

Construction and ^{51}Cr Labeling of Stably Transfected CHO Cell Lines

CHO Ade-C cells (Oates and Patterson, 1977; Van Keuren et al., 1986) were transfected (Chen and Okayama, 1987) with XhoI-linearized pCDM7-α(1,3/1,4)FT (Kukowska-Latallo et al., 1990) or with XhoI-linearized pCDM7 (Larsen et al., 1989b), each coprecipitated in a 10-fold molar excess over EcoRI-linearized pSV2-Neo (Southern and Berg, 1982). Approximately 26% of the G418-resistant transfectants generated with pCDM7-α(1,3/1,4)FT stained positively with an anti-Lex antibody (anti-SSEA-1; Solter and Knowles, 1978). A single, clonal, SSEA-1-positive cell line (CHO-FT) was derived from this population. The G418-resistant transfectants generated with pCDM7 were maintained as an uncloned, pooled population (CHO-V). Cell extracts prepared from CHO-FT contained substantial amounts of α(1,3)fucosyltransferase activity when assayed with the acceptor N-acetyllactosamine (Kukowska-Latallo et al., 1990), whereas extracts prepared from the parental cell line and from the CHO-V cells contained no detectable fucosyltransferase activity. CHO-FT or CHO-V (6×10^6) were labeled with ^{51}Cr by incubating them for 5–6 hr in 25 μCi/ml $Na^{51}Cr$. Labeled cells were then harvested using the procedure described above for COS-1 cells.

HUVEC Adhesion Assays

Two methods were used for quantitating heterotypic adhesion to HUVEC monolayers.

Method I was used for analyses with transfected cells and is a conventional HUVEC adhesion assay utilizing ^{51}Cr-labeled cells incubated at 37°C. HUVECs in 96-well plates were placed in growth media without growth factors, with or without 20 ng/ml TNFα (Genentech), for 4 to 6 hr prior to binding assays. In experiments where inhibitory antibodies were used, antibodies were added in 50 μl of PBS containing a 100 μg/ml concentration each of Ca and Mg (PBS/Ca/Mg), 0.5% human serum albumin (endotoxin-free, Cutter), and incubated with HUVEC at 4°C for 1 hr. Antibodies were added to a final concentration of 1 μg/ml, except the anti-HLA class I antibody, which was ascites used at a 1:1000 dilution. These represent saturating concentrations,

as determined by radioimmunoassay. After treating with antibodies, HUVEC monolayers were washed three times with PBS/Ca/Mg. Cells to be tested for adhesion were harvested as detailed above, resuspended in PBS/Ca/Mg, and held at 4°C for the shortest time possible until used in the assay. Cells (3 × 10^5 neutrophils [prepared as described by Marks et al., 1989], 1 × 10^5 COS-1 cells, 3 × 10^5 CHO cells) were added to HUVEC monolayers in 100 µl of PBS/Ca/Mg and incubated at 37°C. Microscopic titration was used to determine the numbers of cells added of each type (sufficient to just allow cells to form an essentially confluent monolayer overlying the HUVEC monolayer, providing the majority of added cells an opportunity to interact with the underlying endothelium without multilayering). Cells were allowed to adhere for various times (10 to 25 min; see figure legends). Unbound cells were removed by exchanging the wells three times with 150 µl of PBS/Ca/Mg. Each well was then counted in a gamma counter, and the number of bound cells was calculated based upon a previous determination of the number of cpms incorporated per radiolabeled cell.

Method II maximizes carbohydrate-dependent adhesion by adopting principles used to measure the lectin-dependent adhesion of lymphocytes to frozen sections of lymph nodes (Stoolman et al., 1987). This method was used to determine ELAM-1-dependent adhesion of the HL-60 myeloid cell lines. HUVECs were plated in 96-well tissue culture plates; 24 hr later, the confluent HUVEC monolayers were incubated with 20 ng/ml TNFα for 4-6 hr prior to initiating binding assays. HL-60 cells were washed in MEM+ (minimal essential medium buffered to pH 7.3 with Tricine [40 mM] and supplemented with 1 mg/ml bovine serum albumin) immediately prior to fixation. Immediately prior to the binding assay, cells in suspension were fixed in freshly prepared paraformaldehyde (0.5% paraformaldehyde in 0.15 M cacodylate buffer [pH 7.4]; 20 min, 4°C), washed extensively, and resuspended at 10^6 cells per ml in MEM+. In some experiments, the TNFα-treated HUVEC monolayers were preincubated at 7°C-10°C with 50 µl of either IgG2b or BB11 monoclonal antibodies at a concentration of 20 µg/ml. Fixed cells (10^5 cells in 50 µl) were generally added directly to the plates after 15-30 min of preincubation (washing the HUVEC monolayers free of unbound antibody prior to instituting the binding assays had no effect on the level of inhibition; data not shown). Added cells were allowed to settle and interact with the HUVEC for 45 min at 7°C-10°C prior to quantitation of adhesion.

Unattached cells were removed by aspirating the cell suspensions and gently washing the monolayers three times with 200 µl of MEM+ at room temperature. The wash solution was applied with an 8-channel multipipettor to the upper wall of the wells while maintaining the plates at a 30° angle. The solutions were then drawn off the lower wall without contacting the monolayer. The number of cells recovered from each well was determined by counting the pooled washes on a Coulter ZBI cell counter. The number of bound cells represents the difference between the number of cells applied to each well and the number recovered from the well.

Flow Cytometry

Transfected COS-1 cells, or CHO transfectants, were subjected to flow cytometry analysis with mouse monoclonal IgM antibodies directed against carbohydrate epitopes, using procedures described previously (Ernst et al., 1989; Kukowska-Latallo et al., 1990). Cells were stained with saturating amounts of anti-SSEA-1 monoclonal antibody (1:1000 dilution of ascites), anti-Lea monoclonal antibody (10 µg/ml), anti-H antibody (10 µg/ml), or anti-CSLEX1 (10 µg/ml). Cells were then stained with fluorescein-conjugated goat anti-mouse IgM (Sigma; 40 µg/ml) and subjected to analysis by flow cytometry on a FACScan (Becton Dickinson, Mountain View, CA) as described previously (Ernst et al., 1989; Larsen et al., 1989b). Cell staining was measured in arbitrary fluorescent intensity units and was displayed on a four-decade log scale. Analyses with the IgG3 class mouse anti-CSLEA1 antibody (10.8 µg/ml), or the pooled mouse IgG control antibody (MsIg, Coulter, 10 µg/ml), were performed in an identical manner, except that an FITC-conjugated goat anti-mouse IgG antibody (Sigma, 40 µg/ml) was used.

Flow cytometry-based indirect immunofluorescence analysis of HL-60 cells was conducted in microtiter plates. Both primary and secondary antibodies were used at saturating concentrations. Antigen-specific mouse anti-human monoclonal antibodies (50 µl) were combined with cells (2.5 × 10^5 in 50 µl) and incubated at 4°C for 30 min in PBS+ (phosphate buffered saline supplemented with 1% fetal bovine serum and 0.05% sodium azide). Cells were washed twice in PBS+ (200 µl), resuspended and combined with 50 µl of FITC-labeled goat anti-mouse IgG (Fab′ 2 fragments, Cappel Laboratories, West Chester, PA) for an additional 30 min at 4°C. After three washes in PBS+, pellets were resuspended and combined with 200 µl of 1%-2% paraformaldehyde prior to cytometric analysis. Fluorescence quantitation was performed on a Coulter Epics V Flow Cytometer equipped with a three-decade scale.

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