L-Selectin Mediates Neutrophil Rolling in Inflamed Venules Through Sialyl Lewis^X-Dependent and -Independent Recognition Pathways

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The glycoprotein (GP) L-selectin initiates adhesive interactions between leukocytes and endothelial cells (EC). It functions as a lymphocyte-lectin homing receptor recognizing carbohydrate determinants of the peripheral lymph node addressin on high endothelial venules. It also mediates neutrophil rolling, the earliest interaction of neutrophils with acutely inflamed venules. Neutrophil L-selectin presents sialyl-Lewis^X (sLe^X) as a ligand to P- and E-selectin in vitro, and we have proposed that this is a major mechanism of L-selectin–mediated rolling in vivo. In contrast, the contribution of neutrophil L-selectin as a receptor protein recognizing one (or more) ligand(s) on inflamed EC is unclear. To address this question, an sLe^X-negative murine pre-B cell line, L1-2, that can neither bind vascular selectins nor roll in inflamed rabbit venules, was transfected with human L-selectin cDNA. L-selectin expression in stable transfectants was sufficient to confer significant rolling in vivo. Rolling was unaffected by neuraminidase treatment but completely blocked by anti-L-selectin monoclonal antibody (MoAb) DREG-56. Thus, L-selectin can initiate leukocyte interactions with EC determinants potentially through recognition of endothelial carbohydrates. In contrast, when human neutrophils were tested, rolling was reduced, but not abolished, by MoAb DREG-56. Likewise, treatment with neuraminidase or anti-sLe^X MoAbs decreased, but did not abrogate, neutrophil rolling, consistent with residual EC recognition via L-selectin. Combination of MoAb DREG-56 and neuraminidase resulted in almost complete loss of rolling, as did removal of glycosylated L-selectin by chymotrypsin. Together with the demonstrable rolling of L-selectin transfectants, our results support the concept of a bidirectional interaction between L-selectin bearing sLe^X on neutrophils and activated EC in vivo. These findings also suggest that L-selectin may mediate rolling of lymphocytes that lack carbohydrate ligands for E- or P-selectin, although probably less efficiently than through bidirectional recognition.

THE L-SELECTIN is an adhesion glycoprotein (GP) that is constitutively expressed and functional on the surface of leukocytes, including granulocytes, monocytes, and most lymphocytes.[1,2] It was first recognized as a molecule that mediates lymphocyte homing to peripheral lymph nodes[1] by binding to carbohydrate ligands of the peripheral node addressin (PNAd) on high endothelial venules (HEV[3-6]). It has also been implicated in neutrophil recruitment to inflammatory sites; application of anti-L-selectin antibodies[2,7] or an L-selectin/IgG chimeric molecule[8] effectively decreases neutrophil accumulation in inflammatory sites in animal experiments. Furthermore, L-selectin may also be involved in activation-induced neutrophil aggregation.[9] Thus, therapeutic interference with molecular mechanisms that mediate L-selectin–dependent adhesion may be a suitable approach to block or attenuate neutrophil-mediated tissue damage in a number of pathologic conditions such as ischemia-reperfusion injury, shock, or adult respiratory distress syndrome (ARDS).

L-selectin was shown to mediate rolling of rabbit,[10] rat,[11] and human neutrophils[12] in mesenteric venules in vivo. The rolling phenomenon allows neutrophils to marginate in inflamed postcapillary or collecting venules and move much more slowly through these vessels than the main blood stream. Rolling was shown to be an essential feature of neutrophil function in the presence of shear force both in vitro[13-15] and in vivo.[12] The role of L-selectin as a neutrophil 'rolling receptor' may be facilitated by its conspicuous spatial distribution to the tips of microvillous-like projections on the cell surface.[16] The in vivo ligand(s) or counter-receptor(s) for L-selectin in venules of nonlymphoid tissues have not been identified yet. However, recent in vitro findings have suggested that the L-selectin on neutrophils (but not on lymphocytes) can interact with endothelial adhesion molecules by presenting sialyl-Lewis^X (NeuNAcα2-3Galβ1-4[Fucα1-3]GlcNAc, sLe^X) and possibly related carbohydrates, as ligands to the vascular selectins,[16] P- and E-selectin, which are induced on endothelial cells (EC) in response to inflammatory mediators.[17,18] L-selectin may also be able to interact with an unidentified ligand(s) expressed on cytokine-activated human umbilical vein EC in culture.[19] Furthermore, L-selectin, like the vascular selectins, has been shown in vitro to be able to bind to sLe^X and related structures through its lectin domain.[20,21]

Based on the findings listed above, we hypothesized that the L-selectin may initiate neutrophil adhesion to inflamed

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venular EC by two parallel mechanisms: carbohydrate ligand presentation to endothelial selectins and, simultaneously, recognition mediated by direct binding of L-selectin to an EC counterpart. These studies were undertaken to test this concept in vivo.

MATERIALS AND METHODS

Neutrophils. Human neutrophils were freshly isolated from whole blood of healthy donors as described.[12] Purity and viability of isolated cells was routinely found to be greater than 95%. Neutrophils were labeled for 30 minutes with 20 μg/mL carboxy fluorescein diacetate (CFDA; Molecular Probes, Eugene, OR). This procedure allows intracellular carboxy fluorescein entrapment and does not alter neutrophil adhesiveness or reactivity to activation in our model.[12] Labeled neutrophils were washed, resuspended in phosphate buffered saline (PBS) + 0.01% glucose to a concentration of 1 × 10^7 cells/mL, and kept at room temperature (RT) in the dark until use. An aliquot (1 × 10^7 cells) was treated identically except that no CFDA was added; these cells were used for quantitation of receptor expression by flow cytometry (see below).

Transfectants. L1-2 cells, murine pre-B lymphoma cells that express neither L-selectin nor E-selectin–binding carbohydrates,[6,16] were used. Cells were transfected with either the pMRB101 vector alone (L1-2^{Vector}) or a vector incorporating the gene for human L-selectin (L1-$2^{L\text{-}selectin}$) as described.[6,20] Transfected cells expressing different levels of L-selectin were sorted for high or low staining with anti–L-selectin monoclonal antibody (MoAb) using a fluorescence-activated cell sorter (Becton Dickinson, Mountain View, CA) as reported.[6] L1-2^{Vector} cells and sorted L1-$2^{L\text{-}selectin}$ cells were grown in standard medium and used for experiments within 1 week after sorting. Cells were harvested and the viability was assessed using the Trypan blue exclusion method. Dead cells were removed by centrifugation over Ficoll (Pharmacia, Piscataway, NJ) before labeling if the viability was found to be less than 90%. Thus, all preparations of transfectants used were at least 90% viable. Subsequently, cells were labeled with CFDA as described for neutrophils. Unlabeled aliquots were treated identically and used for quantitative flow cytometric analysis of L-selectin expression (see below).

Some transfectants were fixed in prewarmed (37°C) 0.05% glutaraldehyde in PBS after removal of dead cells on a Ficoll 1077 gradient (30 minutes at RT) and washing in serum-free Dulbecco's modified Eagle's medium (DMEM) at 37°C. Fixed cells were washed once at RT with PBS and residual glutaraldehyde activity was blocked with 0.1% lysine in PBS (15 minutes at RT). Subsequently, cells were incubated for one hour with MoAb DREG-56 (20 μg/mL) or isotype-matched controls and washed twice. Second stage gold-labeled (particle size 10 nm) goat–antimouse Ig (Sigma #g-2272; St Louis, MO), diluted 1:5, was added for 30 minutes. Stained cells were washed twice, fixed, and embedded for transmission electron microscopy (TEM) on a Philips (Mahwah, NJ) transmission electron microscope using standard techniques. To rule out unspecific effects of the fixation and labeling procedure, L1-2 cells transfected with cDNA for human E- and P-selectin were used as additional controls. These cells were treated identically and labeled with anti–E-selectin MoAb CL2 and anti–P-selectin MoAb 512, respectively. Transfectants from two independent staining experiments were further analyzed for the spatial distribution of colloidal gold binding to the plasma membrane. Staining with gold particles on microvillous-like processes (defined as membrane projections with a length:diameter ratio of >2) and more proximal parts (grooves) of the cell surface was assessed and expressed relative to the total number of gold particles bound to each cell.

Antibody treatment. Fluorescently labeled neutrophils (1 × 10^7) or transfectants in 1 mL PBS were incubated at RT for at least 20 minutes either with anti–L-selectin MoAb DREG-56 that blocks the lectin function of L-selectin[22] or, as a control, with anti-CD45 MoAb L3B12,[23] a generous gift from Dr R. Levy, Stanford University. Both MoAbs were purified on protein A columns from serum-free culture supernatants and used at a concentration of 50 μg/mL. Mouse IgG1 was purchased from Sigma and used in control experiments with transfectants after azide was removed by overnight dialysis. Anti-sLe^X MoAb CSLEX-1 (mouse IgM^{24}) was a gift from Dr J. Paulson (Cytel, La Jolla, CA). Anti-CD18 MoAb 17 (mouse IgM), a gift from Dr D. Witt (Repligen, Cambridge, MA), was used as a control. Purified MoAb HECA-452 (rat IgM), which detects both sLe^X and sLe^a as well as the cutaneous lymphocyte antigen, an E-selectin ligand expressed on a subset of memory T cells,[25] and anti-PNAd MoAb MECA-79 (rat IgM^{26}) were also tested. Neutrophils (1 × 10^7) were incubated at RT with IgM MoAbs at a concentration of 10 μg/mL for at least 30 minutes.

Enzyme treatment. Labeled neutrophils (2 × 10^7 [2 mL]) or L1-2 cells were washed, centrifuged (5 minutes at 800*g*), and resuspended in 10 mL ice-cold PBS containing 2 mmol/L $CaCl_2$. Twenty-five μL of a neuraminidase (Calbiochem, La Jolla, CA) stock solution (1 U/mL, final concentration 5 mU/mL) or PBS (sham) was added to 5 mL of cells and the mixtures were incubated for 30 minutes on ice. Incubation was stopped by washing cells twice in excess ice-cold PBS containing 1% bovine serum albumin (BSA). Treated cells were resuspended in ice-cold PBS with 0.01% glucose to 1 × 10^7 cells/mL and kept in the dark on ice until use. Aliquots of unlabeled neutrophils were treated identically and used for flow cytometric evaluation of the surface expression of sLe^X, L-selectin, and CD18. Some neutrophils were treated for 5 minutes at 37°C with α-chymotrypsin (Sigma), 1 U/10^6 cells, as described previously.[12] Subsequently, treated cells were washed and kept in the dark at RT until use.

Fixation. Labeled untreated, sham-treated, and neuraminidase-treated neutrophils or L1-2 cells were cooled to 0°C by transfer for 5 minutes to ice. Subsequently, ice-cold paraformaldehyde (Sigma) in PBS was added to a final concentration (wt/vol) of 0.5%. After 5-minute fixation, cells were washed twice in excess PBS and kept in the dark at RT until use. Unlabeled cells were treated accordingly for use in flow cytometry.

Flow cytometry. Both CSLEX-1 and HECA-452 were conjugated with fluorescein succinimidyl ester (Molecular Probes) and used for flow cytometric determination of cell-surface sLe^X. Fluorescein isothiocyanate (FITC)-conjugated anti–L-selectin MoAb DREG-200 and anti-CD18 MoAb IB4 were used for quantitation of L-selectin and β2 integrin expression. A saturating concentration of fluorescent MoAb IB4 (5 μg/mL on ice), MoAb DREG-200 (5 μg/mL on ice), MoAb CSLEX-1 (10 μg/mL at RT), or MoAb HECA-452 (10 μg/mL at RT) was added to 50 μL aliquots (5 × 10^5 cells) of cells. Fixed or unfixed neutrophils treated with or without enzyme were analyzed on a FACScan flow cytometer (Becton Dickinson) after 30 minutes incubation to allow antibody binding. Surface expression of CD18, L-selectin, and sLe^X was determined after gating for neutrophils. L-selectin expression on L1-$2^{L\text{-}selectin}$ cells before and after treatment with neuraminidase was assessed accordingly. Results were expressed as median fluorescence channel number from data collected on 5,000 cells. Staining intensities of transfected cells with FITC-DREG–200 were compared with those of neutrophils used during the same experiment under identical conditions (after subtraction of background fluorescence).

Animal preparation. A total of 12 male New Zealand White rabbits weighing 0.9 to 1.4 kg were used. Animals were sedated with an intramuscular (im) injection of 0.1 mL solution containing 1% fluanison and 0.02% fentanyl (Hypnorm, Janssen, Belgium) 4 hours before the experiment. Five milliliters PBS containing 1,000

U (or 3,000 U) recombinant human interleukin-1β (IL-1) were given as an intraperitoneal (IP) injection. Two different batches of recombinant IL-1β were used for IP injection in these studies. One batch that was used for most experiments (nine animals) was a gift from Dr R. Thomas (Genentech, San Francisco, CA) and, as reported previously,[12] effectively induced increased neutrophil rolling at a dose of 1,000 U in 5-mL PBS. The second batch, a gift from Dr D. Witt (Repligen, Cambridge, MA) had inconsistent effects on rolling at 1,000 U, but showed reproducible results when the dose was increased to 3,000 U. Therefore, the second batch of IL-1 was used at 3,000 U in 5-mL PBS (three animals). Consistent with our previous observations,[12] neither batch or dose had a significant influence on systemic parameters such as body temperature, total or differential white blood cell (WBC) counts, hematocrit, or blood pressure.

Two hours after IL-1 injection, animals were again sedated and given orally 2 mg loperamide hydrochloride (Imodium, Janssen, Belgium) in 1.5-mL PBS. Subsequently, surgical anesthesia was induced by intravenous (IV) injection of a 20% urethane solution. Rabbits were prepared for microscopic observation of the inflamed mesentery microcirculation adjacent to the terminal ileum as described previously.[12] Briefly, after placement of a tracheal tube and catheters in the left jugular vein and the right carotid artery, the terminal ileum was exteriorized through a midline abdominal incision and spread over a heated glass window on the stage of a Leitz (Germany) intravital microscope. The mesentery was continuously superfused with endotoxin-free, isotonic saline solution (pH 7.4) heated to 37.5°C and all parts of the intestine that were not observed microscopically were covered with soaked cotton pads. A polyethylene catheter was inserted into a side branch of the terminal superior mesentery artery through which fluorescent cells could be injected. This method allows analysis of the behavior of injected cells without serious alterations of local microhemodynamics.[12] Because of the small distribution volume of injected cells during their first passage through the mesentery injection of relatively few (0.05 to 1×10^7) cells is sufficient. Injected cells do not recirculate systemically probably because they are retained in the animal's liver or lung. This allows us to study consecutively multiple samples of differently treated neutrophils and/or transfectants in a venular network.

Intravital microscopy. Fluorescently labeled cells were made visible during their passage through a venular segment by stroboscopic epi-illumination using a video-triggered Xenon lamp (Chadwick Helmuth, Mountain View, CA) and a Leitz Ploemopak epi-illuminator with an I2 filter block as described.[12] All scenes were recorded through a silicon-intensified tube camera (SIT68, Dage mti, Michigan City, IN) using a Nikon (Melville, NY) X10/0.13 water immersion objective. Recordings were stored on video tape employing a SVHS video recorder (JVC HC-6600, Japan).

Image analysis. A total of 51 venules with diameters ranging from 15 to 60 μm were analyzed. Rolling of control and treated neutrophils as well as transfectants was quantitated from video recordings as described.[12] Briefly, the total flux of fluorescent cells passing a venular segment during one injection was counted. Subsequently, the scene was again analyzed to determine the flux of rolling cells in the same segment. The rolling fraction (RF) was then calculated as the number of rolling cells per 100 cells passing the segment.

The velocity profiles of neutrophils and L1-2$^{\text{L-selectin}}$ cells (high expression) were determined in 6 venules (47 ± 12 cells/venule) of two animals by frame-by-frame analysis using a personal computer (PC)-based interactive video image analysis system.[27] The centerline velocity, V_{CL}, in the blood stream of each venule was determined from the fastest cell in each sample according to Ley and Gaethgens.[28] Velocities of individual cells were normalized to V_{CL}, which was given a value of 1. The relative frequencies of cell velocities were determined after assigning normalized velocities to classes from 0 to < 0.1, 0.1 to < 0.2, and so on. The critical velocity, ie, the lowest velocity a freely flowing cell can assume in a parabolic flow profile in a microvessel, was calculated for each venule as described elsewhere.[28] Cell velocities below the critical velocity indicate retardation of the cell caused by adhesive interaction with the vessel wall (ie, rolling).

Statistics. The Friedman test was used for nonparametric analysis of multiple samples of paired data. P values less than .05 were considered statistically significant. All results in text and figures are given as arithmetic mean ± 1 SD unless otherwise indicated.

RESULTS

Expression and distribution of L-selectin on transfectants. L1-2$^{\text{L-selectin}}$ cells were sorted for high or modest (low) expression of human L-selectin and grown separately. Quantitative flow cytometry showed that the average expression level of L-selectin on transfectants with high expression, assessed by staining with FITC anti–L-selectin MoAb DREG-200, was 80.2% of that of control neutrophils used in the same experiment. The staining intensity on L1-2$^{\text{L-selectin}}$ cells with low expression was 48.6% of isolated neutrophils.

Immunogold staining with anti–L-selectin MoAb DREG-56 and TEM (Fig 1) showed that L-selectin on L1-2$^{\text{L-selectin}}$ cells was preferentially localized on the tips of microvillous-like protrusions of the plasma membrane of transfectants as reported previously for human neutrophils.[16] Analysis of TEM pictures from 62 L1-2$^{\text{L-selectin}}$ cells stained with anti–L-selectin MoAb showed that 85% to 90% of the colloidal gold was bound to microvillous-like cell projections. In contrast, L1-2 cells transfected with cDNA for human E-selectin (19 cells) or P-selectin (41 cells) displayed a homogenous surface distribution of these receptors; 49% to 58% of the anti–E-selectin and 48% to 52% of the anti–P-selectin MoAbs localized to microvilli. This corresponds well to the relative surface area of microvilli that was assessed by morphometry and was found on all transfectants to be in the range of 37% to 65% (mean ± SD: 49% ± 9.5%) of the total cell-surface area. These results suggest that the conspicuous expression pattern of L-selectin on neutrophils as well as transfectants may be an exceptional feature of this adhesion molecule.

Rolling of L1-2$^{\text{L-selectin}}$ cells in inflamed venules. The ability of transfected cells to interact with venular EC in vivo was tested in 14 mesenteric venules of three rabbits. As shown in Fig 2, L1-2$^{\text{Vector}}$ cells were unable to interact with IL-1–treated EC; the RF was 0.96% ± 0.78% (mean ± SD). In contrast, both samples of L1-2$^{\text{L-selectin}}$ cells had acquired a significant ability to roll. RF of L1-2$^{\text{L-selectin}}$ cells with high expression was 13.6 ± 6.83% ($P < .001$ v L1-2$^{\text{Vector}}$ cells), whereas 5.5% ± 3.2% of L1-2$^{\text{L-selectin}}$ cells with low expression rolled (n = 9 venules in two rabbits; $P < .01$ v L1-2$^{\text{Vector}}$, $P < .01$ v high expression); this suggests a positive correlation between RF and the surface density of L-selectin. Rolling of transfectants was completely L-selectin dependent (Fig 3); preincubation of highly expressing L1-2$^{\text{L-selectin}}$ cells with anti–L-selectin MoAb DREG-56, but not with control

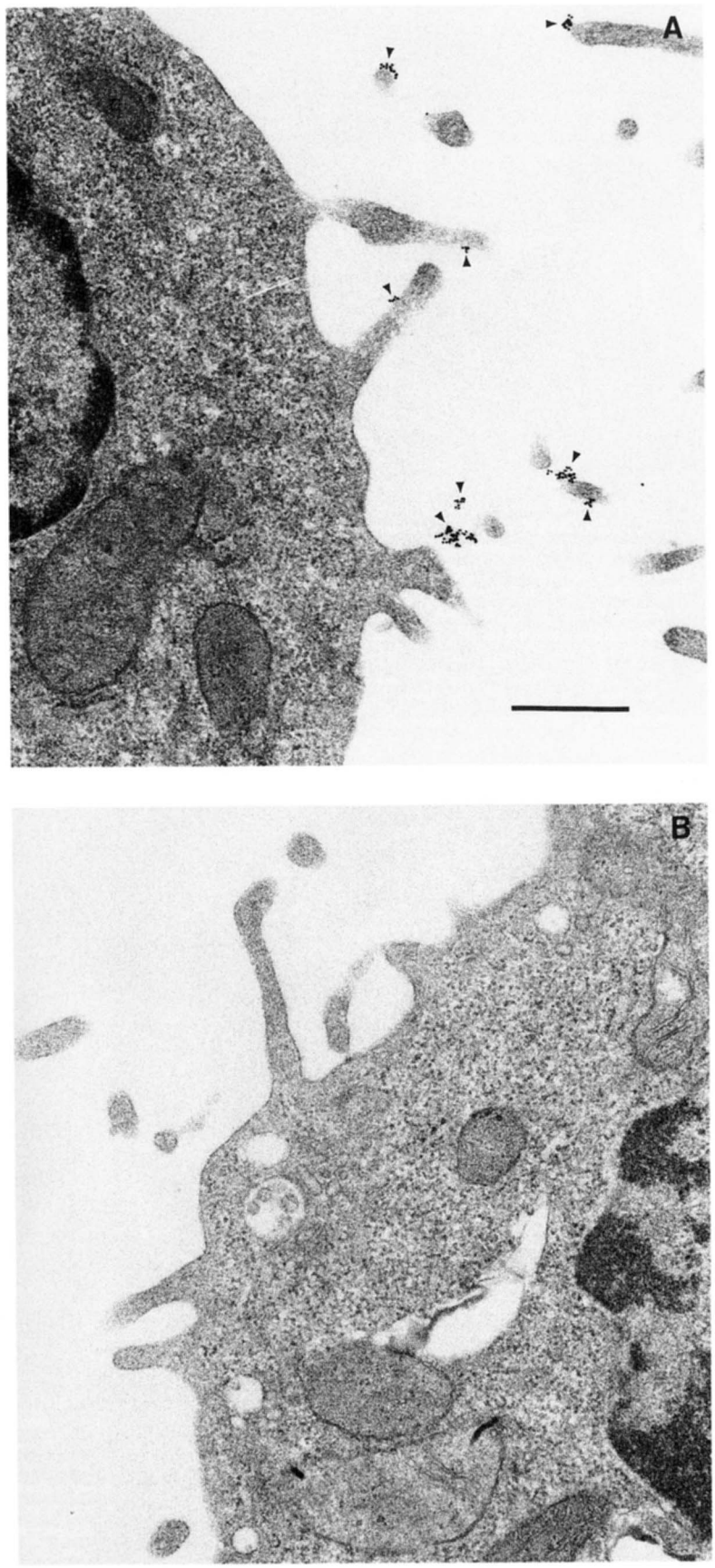

Fig 1. Immunolocalization of L-selectin on L1-2$^{L\text{-selectin}}$ cells. Transfectants were sorted for high expression of L-selectin and incubated with anti–L-selectin MoAb DREG-56 (A) or an isotype-matched nonbinding control MoAb (B). Unbound MoAb was removed by washing and second-stage gold-labeled goat antimouse IgG was added. Stained cells were washed, fixed, embedded, and prepared for TEM. (A) Anti–L-selectin MoAb localized preferentially to the tips of microvillous-like processes on L1-2$^{L\text{-selectin}}$ cells (arrowheads), whereas (B) no specific staining was detected with control MoAb. The scale bar represents 1 μm.

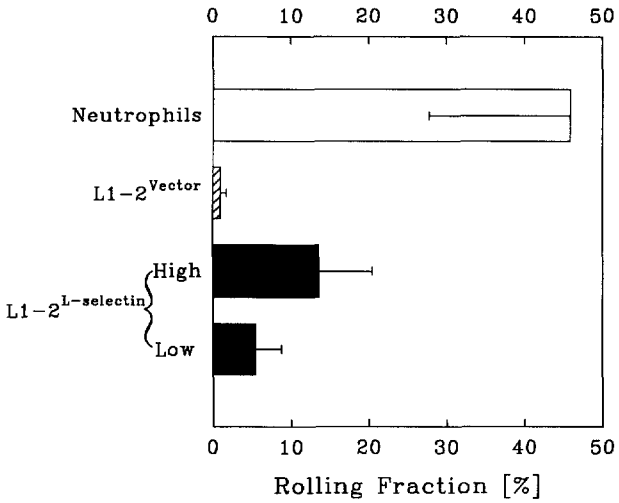

Fig 2. Rolling of neutrophils and transfected L1-2 cells in IL-1–stimulated mesenteric venules. Carboxy fluorescein diacetate (CFDA)-labeled cells were injected into the mesenteric circulation and RF was determined as described. L-selectin expression relative to neutrophils (100%) was 80% on L1-2$^{L\text{-selectin}}$ cells with high expression (High) and 49% on L1-2$^{L\text{-selectin}}$ cells with low expression (Low). L1-2^{Vector} cells did not stain with anti–L-selectin MoAb above background. $P < .001$ for neutrophils versus L1-2^{Vector} or L1-2$^{L\text{-selectin}}$ High and Low; $P < .001$ for L1-2$^{L\text{-selectin}}$ High versus L1-2^{Vector}; $P < .01$ for L1-2$^{L\text{-selectin}}$ Low versus L1-2^{Vector}; $P < .01$ for L1-2$^{L\text{-selectin}}$ High versus L1-2$^{L\text{-selectin}}$ Low. Bars and error bars represent mean ± SD of data obtained from 14 venules in three rabbits (nine venules in two rabbits for L1-2$^{L\text{-selectin}}$ Low).

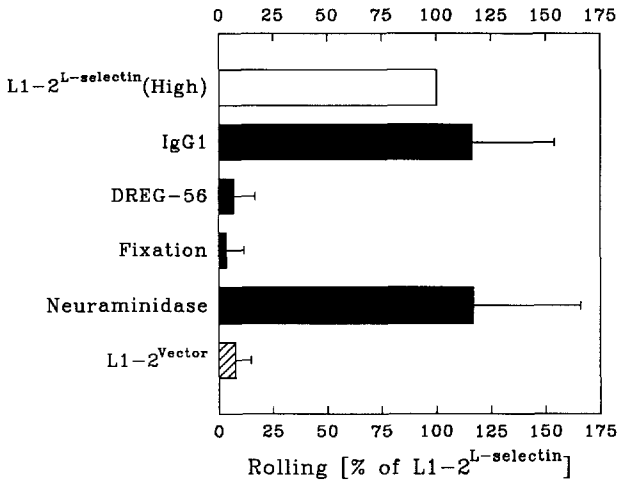

Fig 3. Rolling of L1-2$^{L\text{-selectin}}$ cells in IL-1–treated venules is L-selectin dependent. L1-2$^{L\text{-selectin}}$ cells sorted for high expression of L-selectin were incubated with 50 μg/mL anti–L-selectin MoAb DREG-56 or control mouse IgG1. Some cells were fixed in 0.5% paraformaldehyde or treated with neuraminidase. L1-2^{Vector} cells were used as a negative control. Results are expressed relative to RF of untreated or sham-treated L1-2$^{L\text{-selectin}}$ cells determined in the same venules. Mean ± SD from 14 venules in three preparations are shown, fixed cells were tested in nine venules of two animals. $P < .001$ for L1-2$^{L\text{-selectin}}$ control cells versus DREG-56, or Fixation, or L1-2^{Vector}; $P < .001$ for IgG1 versus DREG-56.

mouse IgG1, reduced rolling by 93% ± 9.7% to RF of 1.1% ± 1.6% ($P < .001$ *v* IgG1, not significant *v* L1-2^{Vector}). Further, paraformaldehyde fixation that caused a complete loss of immunoreactivity with FITC-anti–L-selectin MoAb DREG-200 on L1-2$^{L\text{-selectin}}$ cells (but preserves sLe^{X} on neutrophils[16]) when tested by flow cytometry, also reduced RF (analyzed in nine venules of two mesenteries) to 0.8% ± 2.0% corresponding to 96.3% ± 7.9% inhibition of rolling ($P < .001$ *v* untreated). Rolling of transfectants did not involve neuraminidase-sensitive glycoconjugates as pretreatment with neuraminidase had no effect; the relative ability of neuraminidase-treated L1-2$^{L\text{-selectin}}$ cells to roll was 117% ± 49% of that of untreated L1-2$^{L\text{-selectin}}$ cells. Thus, expression of L-selectin even in the absence of carbohydrate ligands for the vascular selectins was clearly sufficient to confer the ability to roll. Furthermore, when expressed on the same host cell, higher levels of L-selectin expression conferred significantly higher levels of rolling.

Interestingly, L1-2$^{L\text{-selectin}}$ cells were considerably less efficient at rolling than control neutrophils tested in the same preparations (Fig 2) and the rolling velocity of interacting transfectants (high receptor expression) was markedly higher than that of neutrophils as well (Fig 4). Mean velocities of freely flowing cells determined in six venules (34.5 ± 6.7 μm diameter) of two rabbits were not different between neutrophils and L1-2$^{L\text{-selectin}}$ cells (2072 ± 399 μm/s and 1,987 ± 606 μm/s respectively), whereas the rolling velocities of neutrophils were with 238 ± 217 μm/s (median 170 μm/s) significantly lower than those of L1-2$^{L\text{-selectin}}$ cells with 501 ± 189 μm/s (median 468 μm/s). These findings indicate that transfectants may interact much more loosely with venular EC than neutrophils despite a nearly comparable

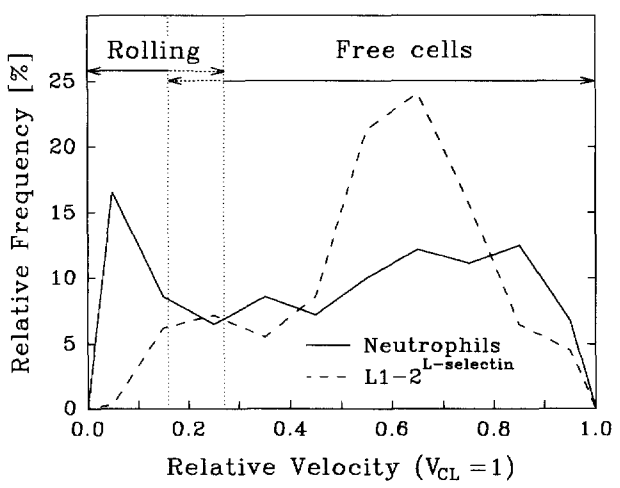

Fig 4. Velocity profile of neutrophils (solid line) and L1-2$^{L\text{-selectin}}$ cells (dashed line) determined in six venules of two rabbits. Velocities of individual cells were normalized to the V_{CL} in each venule. Lines were drawn after assessment of the relative frequency of normalized cell velocities in classes from 0 to less than 0.1, 0.1 to less than 0.2, and so on. The range of relative critical velocities that depend on the venular diameter (22 to 40 μm) is indicated by dotted lines.

surface expression of L-selectin. This discrepancy may reflect additional pathways of selectin-mediated adhesion on neutrophils. However, differences in the functional state, topographical distribution of the expressed L-selectin, or other aspects such as differences in cell size and deformability or the very different host-cell environments may also be important.

SLe^X-dependent and sLe^X-independent contributions of L-selectin to neutrophil rolling. We next analyzed in 14 mesenteric venules of three IL-1–treated animals the relative contribution of L-selectin and sLe^X to rolling of human neutrophils. In good agreement with our previous observations[12] a significant inhibition of rolling by 68.8% ± 10.6% ($P < .001$) was seen when neutrophils were treated with anti–L-selectin MoAb DREG-56 (RF = 13.2% ± 4.5%; [Fig 5]). In contrast, anti-CD45 MoAb L3B12 (RF = 42.7% ± 10.6%) had no effect on rolling when compared with control cells (RF = 42.4% ± 16.2%). As described recently,[12] rolling was even more decreased when neutrophils were treated with chymotrypsin ($P < .001$ *v* control, $P < .01$ *v* DREG-56), which removes greater than 95% of all surface L-selectin. In agreement with previous observations by us and others[12,16] the effect of chymotrypsin treatment on neutrophil sLe^X expression was negligible; surface sLe^X levels assessed by binding of FITC-labeled MoAbs CSLEX-1 and HECA-452 were in the range of 85% to 95% of untreated control cells.

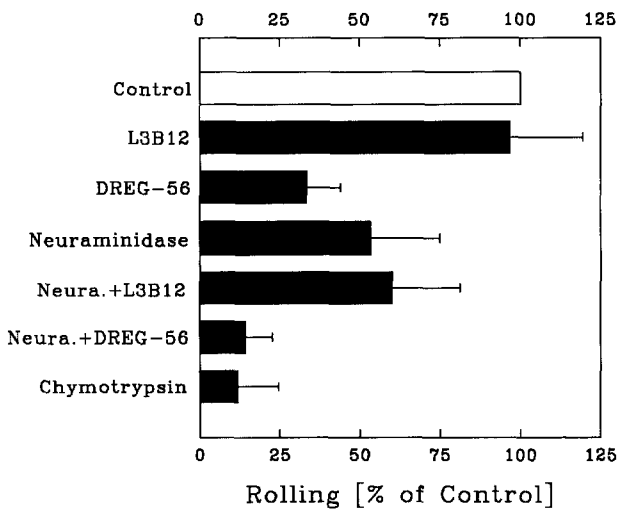

Fig 5. Anti–L-selectin MoAb DREG-56 and neuraminidase treatment have additive inhibitory effects on rolling of neutrophils in IL-1–treated venules. Neutrophils were treated with control (L3B12) or blocking anti–L-selectin (DREG-56) MoAb and with neuraminidase alone or in combination with MoAbs. Some cells were subjected to chymotrypsin treatment for complete removal of surface L-selectin. Results are expressed relative to RF of untreated or sham-treated (control) neutrophils determined in the same venules. Mean ± SD of 14 vessels in three animals are shown. Control versus L3B12 was not significant; $P < .001$ for control and L3B12 versus all other samples; $P < .05$ for DREG-56 versus neuraminidase; $P < .01$ for DREG-56 versus Neura + DREG-56 and DREG-56 versus chymotrypsin; $P < .001$ for neuraminidase or Neura + L3B12 versus Neura + DREG-56 or chymotrypsin.

In contrast to $L1\text{-}2^{L\text{-selectin}}$ cells inhibition of neutrophil rolling by anti–L-selectin MoAb DREG-56 was not complete. Thus, we investigated the role of sialylated carbohydrate determinants in neutrophil rolling (14 venules in three animals). In agreement with a recent report,[16] treatment of neutrophils with 5 mU/mL neuraminidase caused a decrease in binding of anti-sLe^X MoAbs CSLEX-1 and HECA-452 by 80% to 90%. The removal of surface sLe^X by neuraminidase was accompanied by a significant decrease in RF to 23.6% ± 16.4% ($P < .001$ *v* control, $P < .05$ *v* DREG-56) confirming that sialylated glycoconjugates on neutrophils do in fact contribute to rolling in vivo. Combination of neuraminidase treatment with control MoAb L3B12 had no additional effect on rolling (RF = 28.1% ± 12.6%), whereas addition of MoAb DREG-56 to neuraminidase-treated cells significantly reduced RF even further to 6.1% ± 3.5% ($P < .001$ *v* control and neuraminidase alone, $P < .01$ *v* DREG-56, not significant *v* chymotrypsin).

To study the role of sLe^X directly, we incubated neutrophils with anti-sLe^X MoAbs CSLEX-1 and HECA-452. Both MoAbs caused formation of large cell aggregates when used in concentrations higher than 10 μg/mL. Thus, 1×10^7 cells in 1 mL PBS were incubated for 30 minutes at RT with 10 μg/mL antibody. This dose and incubation procedure was sufficient to allow saturating binding of MoAb CSLEX-1 and nearly saturating (70% of maximum) binding of MoAb HECA-452 as determined by flow cytometry. When tested in vivo (14 venules in three rabbits), both anti-sLe^X MoAbs caused a highly significant reduction by 55.9% ± 15.2% (CSLEX-1) and 51.4% ± 22.0% (HECA-452) in RF relative to untreated control cells or neutrophils incubated with 10 μg/mL anti-CD18 MoAb 17 or MoAb MECA79 (Fig 6). Taken together, these results provide evidence that neutrophils engage both L-selectin and sLe^X to roll in inflamed venules.

DISCUSSION

It has become increasingly clear in recent years that adhesive interactions between neutrophils and EC in the presence of shear force require several sequential steps that are essential for neutrophils to be able to bind to and extravasate through inflamed venular endothelium.[10,12-14,29-32] The first link in this chain of adhesive interactions amenable for direct in vivo observation is neutrophil rolling, an event that has been recognized for more than 150 years.[33] However, only recently has some understanding emerged of the meaning and physiologic importance of this phenomenon for neutrophil function. The secondary adhesive step during which stimulated neutrophils adhere firmly ("stick") to an endothelial substrate by using β2 integrins (CD11/CD18) does not occur under conditions of flow[34] unless rolling is initiated by engagement of at least one member of a family of adhesion molecules termed selectins.[12,13]

The selectins are single-chain GPs consisting of a short intracellular tail, a single transmembrane domain, several repeating domains with sequence homology to complement regulatory proteins, an epidermal growth factor (EGF)-like domain, and an N-terminal Ca^{2+}-dependent (C-type) lectin domain in the most distal extracellular portion. All three

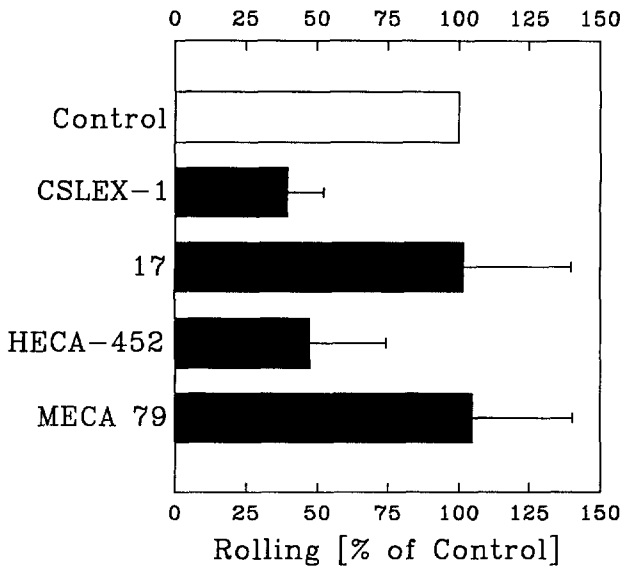

Fig 6. Anti-sLe^X MoAbs attenuate neutrophil rolling in IL-1–treated mesenteric venules. Neutrophils were incubated with PBS (Control) or 10 μg/mL anti-sLe^X MoAbs CSLEX-1 (mouse IgM) or HECA-452 (rat IgM). Anti-CD18 MoAb 17 (mouse IgM) or nonbinding MECA79 (rat IgM) were used as controls. Rolling was analyzed in 14 venules of three rabbits and expressed relative to RF of control neutrophils. Mean ± SD are shown. $P < .001$ for CSLEX-1 and HECA-452 versus Control, 17, and MECA79.

currently known selectins were shown to bind to the carbohydrate sLe^X and related structures[20,21] and were implied to play a role in adhesion initiation and rolling.

P-selectin (CD62, GMP-140, PADGEM) is stored in α-granules of platelets and in Weibel Palade bodies of EC. This molecule is rapidly translocated to the plasma membrane on stimulation with histamine, thrombin, or superoxide.[17,35] Immobilized soluble P-selectin supports neutrophil binding in static assays[36,37] and rolling of nonactivated neutrophils under conditions of flow in vitro.[13]

E-selectin (ELAM-1) is found on the surface of activated EC.[18] It is newly synthesized by stimulated EC with peak surface expression at 4 to 6 hours after treatment with IL-1, tumor necrosis factor (TNF), or endotoxin in vitro.[18] Detectable amounts of E-selectin can also be found in rabbit mesentery venules 4 hours after IP treatment with IL-1 (M. Olofsson, U.H. von Andrian, and K.-E. Arfors, manuscript in preparation). E-selectin expressed on stimulated EC or transfected L-cell monolayers can support neutrophil rolling in a flow-chamber assay.[38]

L-selectin that is expressed by most leukocytes including neutrophils differs from the vascular selectins, E- and P-selectin, in that it is constitutively present and functional on most circulating nonactivated leukocytes. This selectin was also reported to be critically involved in mediating neutrophil adhesion[2,39] and rolling in shear-dependent adhesion assays in vitro.[14,15] It also contributes to activation-induced aggregation of neutrophils in suspension.[9] L-selectin is the only molecule to date that has been shown in vivo to play a dominant role as a "rolling receptor" on rabbit,[10] rat,[11] and human neutrophils.[12]

Thus, a growing body of evidence suggests that all three selectins may participate in rolling, at least at the low shear rates applied in in vitro assays. The in vivo data presented here are consistent with the in vitro observations cited above, and provide a physiologic perspective on the role, function, and mutual interactions of selectins.

L1-2 is a murine pre-B cell line that does not express L-selectin or carbohydrate ligands for vascular selectins.[16] L1-2 cells do not roll or interact otherwise with venules in the rabbit mesentery. We show that stable transfection of these cells with human L-selectin cDNA induces the expression of functionally intact L-selectin and confers the ability to roll in IL-1 treated venules in vivo. The ability of L1-2$^{\text{L-selectin}}$ cells to roll is unlikely to be caused by a change in surface glycosylation because blocking anti–L-selectin antibodies or paraformaldehyde fixation (which may have little effect on surface carbohydrates[16]) completely abolished rolling of L1-2$^{\text{L-selectin}}$ cells. Furthermore, in contrast to neutrophils, removal of sialic acid residues by neuraminidase had no effect on rolling of L1-2$^{\text{L-selectin}}$ cells in our model. Although the L1-2$^{\text{L-selectin}}$ cells rolled less efficiently and with higher velocity than control neutrophils, we conclude that expression of the L-selectin GP, even in the absence of specific carbohydrate ligands for the vascular selectins, is sufficient to allow leukocyte rolling in inflamed venules in vivo.

The ability of L-selectin to mediate rolling, albeit perhaps weakly, has obvious implications for the potential interactions of lymphocyte subsets in sites of acute and subacute inflammation as well. Whether the levels of L-selectin expressed on naive or L-selectin positive lymphocytes are sufficient to allow rolling and effective activation through EC-surface stimuli ultimately leading to lymphocyte sticking in inflamed venules remains to be determined.

The ligand or counter-receptor on inflamed venules for L-selectin on L1-2$^{\text{L-selectin}}$ cells has not been identified yet. L-selectin binds to carbohydrate determinants of PNAd,[6] one component of which may be GLYCAM-1,[40] a mucin-like GP secreted by high endothelial venules (HEV) in peripheral lymphoid tissues. However, Northern blot analysis of the presence of mRNA for GLYCAM-1 indicates that the expression of this molecule is limited to lymph nodes and lung tissue,[40] and immunohistological studies indicate that PNAd is not expressed on acutely inflamed EC in vivo.[41] It seems likely that activated EC in inflamed venules may be induced to express carbohydrate ligands for L-selectin that are immunologically distinct from PNAd.[16,19,41] Such carbohydrate ligands may be presented to the L-selectin by mucin-like molecules related to GLYCAM-1, or perhaps even by the vascular selectins themselves, resulting in a bidirectional lectin-carbohydrate recognition at the molecular level. However, the possibility of alternative lectin-independent protein-protein interactions that might involve the EGF-domain of L-selectin[42] or the complement regulatory repeats cannot be excluded.

In contrast to the L1-2$^{\text{L-selectin}}$ cells, our data suggest that neutrophils can use both L-selectin recognition and presentation of sLe^X in interactions with activated venules. The

involvement of sLe^X and/or related neutrophil carbohydrates is supported by the ability of neuraminidase treatment and of two anti-sLe^X MoAbs to reduce rolling in our model. A critical role for sLe^X is further underscored by our recent studies on neutrophils from a patient with the newly described inherited leukocyte adhesion deficiency (LAD)-II.[43] These patients have a genetic defect in fucosylation and are unable to synthesize sLe^X. Their neutrophils roll poorly in our model,[32] consistent with a key role for sLe^X in this event; however, residual rolling albeit at higher velocity is nonetheless detected, perhaps indicating the engagement of L-selectin that is expressed at normal levels on LAD-II neutrophils but lacks fucosylated carbohydrates such as sLe^X.

Involvement of L-selectin in neutrophil recognition of inflamed EC, independent of neutrophil carbohydrates, is also supported by several lines of evidence. First, neutrophil L-selectin is known to be a functional lectin, capable of supporting neutrophil binding for example to peripheral lymph node HEV[2]; it also binds to the well-characterized L-selectin ligand polyphosphomannan ester (PPME).[22] Thus, if expression of carbohydrate ligands by inflamed venules forms the basis for L-selectin–mediated rolling of $L1\text{-}2^{L\text{-selectin}}$ cells, it is likely that neutrophil L-selectin participates in rolling in the same manner. More direct support comes from our observation that neither anti–L-selectin MoAbs nor neuraminidase treatment alone could abolish rolling of neutrophils. However, a combination of both treatments had an additive effect and blocked neutrophil rolling almost completely. Likewise, paraformaldehyde fixation of neutrophils that may block protein-mediated functions of L-selectin[16] attenuated but did not abrogate rolling, whereas fixation of neuraminidase-treated neutrophils rendered cells that were almost completely unable to roll (data not shown). Thus, L-selectin appears to play a significant role in neutrophil rolling that is independent of but operates in conjunction with sialylated neutrophil carbohydrates.

We have previously shown that removal of the extracellular portion of L-selectin by treatment with low-dose chymotrypsin, which has little or no effect on overall sLe^X expression of neutrophils,[12,16] essentially abolishes rolling to a level equivalent to that obtained by combined treatment with neuraminidase and anti–L-selectin MoAb in the present study. Similarly, we have observed a comparably high inhibition of rolling when neutrophils were maximally activated with a variety of stimulating agents before injection into the mesentery circulation.[12] Neutrophil activation is known to induce rapid shedding of glycosylated L-selectin by proteolytic cleavage from the cell surface,[22,30] but causes only a moderate or undetectable reduction in sLe^X expression[9,13] or in carbohydrates able to bind soluble P-selectin.[44] This suggests that only a small fraction of the total pool of sLe^X on neutrophils participates in initiating rolling. Thus, it is likely that sLe^X on circulating neutrophils must be presented in a special way to be able to initiate contact with the endothelium under flow conditions in vivo. In this context, the unique concentration of L-selectin on microvilli may render it especially effective in presenting sLe^X to the endothelial selectins.

Relatively large particles in the blood stream such as leukocytes flowing through a microvessel are subjected to considerable dispersal hemodynamic forces that draw the leukocyte away from the vessel wall toward the axis of the blood stream.[45,46] Both neutrophils and EC carry negative surface charges[47-49] that may add a considerable repulsive effect over the short distances (about 10 to 30 nm[50]) required for molecular interactions between cells. These physical aspects of leukocyte margination in vivo are consistent with early observations that the effective surface area on neutrophils that is available to initialize contact with EC under conditions of shear in vitro primarily involves microvillous-like protrusions of the plasma membrane of neutrophils.[51] Likewise, it was postulated that microvilli are required to support adhesion of B and T cells to EC as well as other immune cells in vivo.[52] The spacial distribution of L-selectin on neutrophils (and on $L1\text{-}2^{L\text{-selectin}}$ cells used here) corresponds exactly to this pattern (see Fig 1 and the study by Picker et al[16]).

In addition to the importance of the topographic distribution, a candidate molecule that functions in presenting sLe^X on a rolling neutrophil should meet several other criteria. Firstly, neutrophil rolling does not require cell activation (even fixed neutrophils can roll) indicating that sLe^X is constitutively presented on nonactivated neutrophils. Secondly, as discussed above, anti–L-selectin or anti-sLe^X MoAbs or neuraminidase can only attenuate rolling, whereas both low-dose chymotrypsin treatment and neutrophil activation virtually abolish it.[12] This suggests that the latter two manipulations downregulate both mechanisms for rolling, L-selectin–mediated adhesion and sLe^X presentation. Therefore, sLe^X-presenting molecules involved in rolling should be highly susceptible to chymotrypsin treatment and should lose their function on activated neutrophils. To our knowledge, the only molecule reported so far to meet these requirements is L-selectin. In contrast to lymphocytes, L-selectin on neutrophils bears sLe^X determinants that it can present to P- and E-selectin in vitro.[16] Furthermore, MoAbs to L-selectin and E-selectin can both reduce binding of neutrophils to E-selectin transfected COS cells (which lack carbohydrate ligands for L-selectin), and of CD18-deficient neutrophils to cultured EC treated for 3 hours with IL-1.[53] Combination of the two MoAbs has no additive effect on neutrophil binding, suggesting that the two selectins share a common adhesion pathway.[53] In combination with the results herein, these observations are consistent with the concept that neutrophil rolling is predominantly mediated by L-selectin that acts in a dual function by recognizing an endothelial (carbohydrate?) ligand and, simultaneously, by presenting sLe^X (and/or related structures) to vascular selectins.

Other surface molecules on neutrophils have been reported to bear sLe^X-like carbohydrate ligands for the vascular selectins as well,[44,54,55] but these in vitro studies have used either soluble receptors or static adhesion assays. For example, a recent report indicates that neutrophil surface GPs detected by anti-CD66 MoAbs can present sLe^X to E-selectin on IL-1–stimulated EC under static conditions.[55] However, CD66 is upregulated on neutrophil activation[56] and CD66-mediated neutrophil adhesion to E-selectin is not al-

tered after activation-induced shedding of L-selectin.[55] As activation of neutrophils is known to cause a rapid loss of rolling in vitro[13,14] and in vivo,[12] it appears less likely that CD66 plays a dominant role in rolling. It is presently unclear whether other molecules capable of presenting carbohydrates to vascular selectins in vitro are actually involved in adhesion initiation leading to rolling in vivo. Their ability to do so may depend on their topographic distribution on the cell surface. Our observations suggest that such glycoconjugates are less important than L-selectin for the initiation of neutrophil rolling. These GPs, as well as glycolipids bearing sLe^X, may function primarily in a later phase of the adhesion cascade, eg, after rolling is well established or during or after engagement of $\beta 2$ integrins when adherent cells begin to attach and spread.

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