Soluble L-selectin Is Present in Human Plasma at High Levels and Retains Functional Activity

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Abstract. L-selectin expressed by granulocytes, lymphocytes, and monocytes is responsible for initial leukocyte attachment to inflamed endothelium and high endothelial venules of peripheral lymph nodes. After leukocyte activation in vitro, L-selectin is rapidly shed from the cell surface. In this study, shed L-selectin (sL-selectin) from both lymphocytes and neutrophils was demonstrated to be present in high levels in human plasma by Western blot analysis and using a quantitative ELISA. In serum from normal human blood donors, a mean sL-selectin level of 1.6 ± 0.8 μg/ml (n = 63) was found by ELISA. In addition, semi-purified sL-selectin from plasma inhibited L-selectin-specific attachment of lymphocytes to cytokine-activated endothelium in a dose-dependent manner. L-selectin-dependent leukocyte attachment was completely inhibited at sL-selectin concentrations of 8–15 μg/ml, while physiological concentrations of sL-selectin caused a small but consistent inhibition of lymphocyte attachment. sL-selectin in plasma also inhibited anti-L-selectin mAb (2–5 μg/ml) binding to the surface of leukocytes. Interestingly, one epitope present within the EGF-like domain of L-selectin was lost in sL-selectin, suggesting a conformational change in the structure of the receptor after shedding. The presence of serum sL-selectin with functional activity indicates a potential role for sL-selectin in the regulation of leukocyte attachment to endothelium.

THE ability of leukocytes to leave the circulation and to migrate into tissues is a critical feature of the immune response. Several adhesion molecules are involved in the process of adhesion and transmigration of leukocytes through vascular endothelium at sites of inflammation (45). One molecule responsible for the initial attachment of leukocytes to endothelium is L-selectin (Leukocyte Adhesion Molecule-1 [LAM-1][1] MEL-14) (24, 30, 43, 44). L-selectin is a member of the selectin family of adhesion molecules (3, 11, 27, 36, 37, 49) that includes E-selectin (Endothelial-Leukocyte Adhesion Molecule-1 [ELAM-1]) (1, 2, 31, 32) and CD62 (P-selectin, PADGEM, GMP-140) (13, 18, 25, 26). The selectins are derived from evolutionarily related genes (7, 9, 18, 33, 52), and are characterized by a NH_2-terminal, Ca-dependent lectin domain, an epidermal growth factor (EGF)-like domain followed by multiple short consensus repeat (SCR) domains, a transmembrane region, and a cytoplasmic tail.

L-selectin is expressed on the surface of most leukocytes, including lymphocytes, neutrophils, monocytes, eosinophils, hematopoietic progenitor cells, and immature thymocytes (14, 51). L-selectin is a highly glycosylated protein of 95–105 kD on neutrophils and 74 kD on lymphocytes (14, 50). Human and mouse L-selectin mediate the binding of lymphocytes to high endothelial venules (HEV) of peripheral lymph nodes through interactions with a constitutively expressed ligand (16, 23, 40, 46, 51), and are also involved in lymphocyte, neutrophil, and monocyte attachment to endothelium at sites of inflammation (15, 20, 29, 39, 43, 44, 54). In vitro, endothelial cell surface expression of the L-selectin ligand(s) is induced only after exposure of the endothelial cells to inflammatory cytokines, and the endothelial ligand shares many functional features with the L-selectin ligand(s) expressed by HEV (39, 43). Sulfated carbohydrates and mAbs which bind to the lectin domain of L-selectin, inhibit L-selectin-specific adhesion (16, 21, 43, 47, 56, 57). Thus, the lectin domain of L-selectin seems to mediate ligand binding, while the EGF-like and SCR domains may somehow regulate the affinity of this interaction (21, 38, 41, 42, 55).

A unique feature of L-selectin is that it is shed from the cell surface after cellular activation in vitro (14, 19, 22, 23, 40). It has been proposed, at least in the mouse model, that shedding of MEL-14 from leukocytes might be necessary to enable leukocytes to transmigrate through endothelium into sites of inflammation in vivo (20, 22). This would provide a rapid means for the regulation of leukocyte adhesion and deadhesion to endothelium. Although the subsequent fate

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1. *Abbreviations used in this paper*: ELAM-1, endothelial-leukocyte adhesion molecule; HEV, high endothelial venule of peripheral lymph nodes; LAM-1, leukocyte adhesion molecule-1; SCR, short consensus repeat; TNF, tumor necrosis factor; sL-selectin, shed L-selectin.

and possible function of the shed L-selectin (sL-selectin) molecule is not known, elegant studies by Woodruff and colleagues have previously demonstrated the presence of a soluble factor in rat thoracic duct lymph capable of inhibiting lymphocyte binding to HEV (4). Furthermore, they demonstrated that this factor was antigenically related to a structure(s) present on lymphocytes and speculated that this cell-surface molecule might play a role in adherence of lymphocytes to HEV of lymph nodes (4, 5, 6).

A number of surface molecules present on cells of various lineages are now known to be shed and thereby released into the extracellular milieu (48). These include the receptors for interleukin-1, interleukin-2 (CD25), transferrin (CD71), insulin, growth hormone, tumor necrosis factor (34), colony-stimulation factor-1 (10), and nerve growth factor (8), as well as CD8 and CD14. These proteins are quite diverse in structure and amino acid sequence and have no unifying functional characteristics that are currently appreciated. In most cases, proteases cleave the receptor near the membrane, releasing a nearly intact extracellular domain with ligand-binding activity (8, 10, 22, 40). Thus, receptor function may not only be regulated by proteolytic cleavage of the receptor from the cell surface, but also by the presence of shed receptor in the extracellular environment. The present study shows that sL-selectin from neutrophils and lymphocytes is found in plasma at high levels and that it may retain functional activity. Since sL-selectin inhibited L-selectin–mediated adhesion in vitro, it may modulate leukocyte binding to endothelium in vivo.

Materials and Methods

Antibodies

L-selectin directed mAbs were the anti-LAM1-3, -4, -6, -7, -8, -10, -11, and -12 mAbs directed against epitopes within the lectin domain, anti-LAM1-1, -5 and -15 reactive with epitopes within the EGF-like domain and anti-LAM1-14 which reacts with the SCR regions of L-selectin, all of the IgG_1 isotype (42). The anti-L-selectin mAbs were purified by salt fractionation followed by anion exchange chromatography, with the mAb concentration determined by light absorption. The anti-LAM1-3 mAb was bound to CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) at 2.5 mg of mAb bound per 1 ml of beads (anti-LAM-Sepharose) using the methods of the manufacturer.

Isolation of Blood Mononuclear Cells

Heparinized blood was obtained according to protocols approved by the Human Protection Committee of Dana-Farber Cancer Institute, Boston, MA. Mononuclear cells were isolated by Ficoll Hypaque density gradient centrifugation. Cells were immediately resuspended in RPMI 1640 (Gibco-BRL, Gaithersburg, MD) containing 10% FCS and kept at 4°C until use. In most instances, these cells will be referred to as lymphocytes since 85–95% of the cell population was lymphocytes as determined by morphology (Wright's stain) and flow cytometry analysis. Neutrophils were purified by centrifugation on a cushion of Mono-Poly Resolving Medium (Flow Laboratories, Inc., McLean, VA) followed by lysis of the red cells with ice-cold hypotonic 0.2% (wt/vol) NaCl solution. Neutrophils were finally resuspended in HBSS containing 5% FCS (Sigma Chemical Co., St. Louis, MO). When sL-selectin was obtained for Western blot analysis, the mononuclear cells were first incubated in plastic dishes for 30 min in RPMI/10% FCS at 37°C to remove adherent monocytes. Nonadherent cells retained surface L-selectin and were predominantly lymphocytes (~99%).

Cell Cultures

Lymphocytes were cultured in 24-well plates (Costar Corp., Cambridge, MA) at 10^6/ml in RPMI 1640 medium containing 10% FCS, 2% L-glutamine, penicillin, and streptomycin. The cells were cultured with PMA (100 ng/ml) for 60 min before the culture medium was harvested and tested for sL-selectin by ELISA. Neutrophils were incubated in polypropylene tubes at 8×10^6 cells/ml for 60 min at 37°C either in HBSS/5% FCS medium alone or containing granulocyte/monocyte-colony stimulating factor (25 ng/ml; a gift from Drs. Steven Clark and Gordon Wong, Genetics Institute, Cambridge, MA), monocyte-colony stimulating factor (100 ng/ml; Genetics Institute), tumor necrosis factor-α (TNF-α; 100 U/ml; Genzyme Corp., Cambridge, MA), lipopolysaccharide (1 μg/ml; *Escherichia coli* 011:B4; Sigma Chemical Co.), formyl-methionyl-leucyl-phenylalanine (10^{-8} M; Sigma Chemical Co.), interferon-γ (1,000 U/ml; Genzyme Corp.), or interleukin-1β (10 U/ml; Genzyme Corp.). After culture, the supernatant fluid was tested for the presence of sL-selectin by ELISA. The human erythroleukemia cell line, K562, was transfected with the pLAM-1 cDNA as previously described (51) and will be called K562-LAM throughout. These cells were cultured in RPMI 1640/10% FCS and were kept at cell numbers between 0.2 and 1×10^6 cells/ml. All cells were incubated at 37°C in 5% CO_2 with 100% humidity.

Indirect Immunofluorescence Analysis

Indirect immunofluorescence analysis was carried out after washing the cells three times. Cells (1×10^6 cells) were resuspended in 100 μl of media containing various concentrations of the indicated mAb, and incubated for 60 min at 4°C. After washing, the cells were treated with FITC-conjugated goat anti-mouse Ig antibodies (Southern Biotechnology Associates, Birmingham, AL) for 20 min at 4°C. The cells were washed, and fixed (1% paraformaldehyde in PBS), and single color fluorescence was determined on a flow cytometer (ELITE™; Coulter Immunology, Hialeah, FL). 10,000 cells were analyzed for each sample and the relative mean fluorescence intensity of L-selectin$^+$ cells was determined on a linear scale.

In some experiments, cells were stained in the presence of human plasma. sL-selectin was precleared from an aliquot of the same plasma by immunoprecipitation with anti-LAM-Sepharose (1 ml of beads per 4 ml of plasma). The efficiency of the immunoprecipitations was tested by ELISA (<20 ng/ml). Lymphocytes (1×10^6) were resuspended either in 100 μl of plasma or precleared plasma containing various concentrations of the purified mAb (added 1:100) as indicated. The cells were washed twice, stained, and analyzed as above.

sL-selectin Purification

sL-selectin was semipurified from plasma obtained from heparinized human blood. Plasma was salt-fractionated with Na_2SO_4 (18% wt/vol) before the sL-selectin containing supernatant fraction was dialyzed against 0.02 M Tris buffer, pH 8.0, 0.5 M NaCl. The sL-selectin preparation was further purified by affinity column chromatography using anti-LAM-Sepharose. sL-selectin was eluted from the column with 0.1 M Na acetate buffer, pH 3.5, 0.15 M NaCl, and the low pH of the eluate was immediately raised by the addition of 2.0 M Tris buffer, pH 9.0. The pooled fractions of the eluate peak were concentrated and transferred into PBS by ultrafiltration. The concentration of sL-selectin was determined by ELISA and subsequently adjusted to ~15 μg/ml in PBS. At this sL-selectin concentration, the total protein concentration of the samples varied between 130 and 220 μg/ml, and sL-selectin represented ~6–10% of total protein. In general, this procedure gave a 2,200–3,700-fold enrichment for sL-selectin. For use in lymphocyte-endothelial adhesion assays, semipurified sL-selectin was transferred into RPMI 1640/10% FCS by further ultrafiltration.

Western Blot Analysis

sL-selectin was semipurified from plasma as described above and further purified by immunoprecipitation using anti-LAM-Sepharose with repeated washing of the beads in alternating high salt (0.5 M NaCl, 0.2% Na-deoxycholate) and low salt (0.125 M NaCl, 0.05% Na-deoxycholate) RIPA buffer (100 mM Tris, pH 8.0, 1% [vol/vol] Triton X-100, 10 mM EDTA, 10 mM EGTA, 10 mM NaF, 1 mg/ml BSA). Proteins were eluted from the beads with 0.1 M acetate buffer, pH 3.5, 0.15 M NaCl. Supernatant fluid from PMA-stimulated cells was also analyzed. Cells (1×10^7/ml), including neutrophils (10 ng/ml PMA in RPMI 1640 for 10 min at 37°C), lymphocytes (10 ng/ml PMA in RPMI 1640 for 25 min at 37°C), and K562-LAM transfectants (100 ng/ml PMA in PBS for 120 min at 37°C), were induced to shed essentially all detectable cell surface L-selectin, before being pelleted by centrifugation (4°C, 400 *g*, 10 min). The supernatant fluid was

saved and concentrated 10-fold by ultrafiltration (Amicon Corp., Danvers, MA). Protein samples (100 μl) were applied to a 7.5% SDS-polyacrylamide gel, electrophoresed, and blotted onto nitrocellulose. Western blot analysis was performed using the anti-LAM1-14 mAb (ascites, 1:2,000) as the antigen-detecting antibody. The blot was developed using alkaline phosphatase conjugated goat anti-mouse IgG_1 antibody (Southern Biotechnology Associates) and NBT/BCIP as substrate (Promega Corp., Madison, WI). In preliminary experiments, the anti-LAM1-14 mAb was the most sensitive of the 12 anti-L-selectin mAbs tested, of which anti-LAM1-3, -4, -8, -10, and -15 mAb were also found to give positive staining.

Endothelial-Leukocyte Attachment Assay

Lymphocyte adhesion to cytokine-activated endothelium under nonstatic conditions was determined in a test system adapted from the Stamper/Woodruff assay for frozen tissue sections (46) exactly as described (43). Briefly, human umbilical vein endothelial cells (HUVEC) were isolated from cord veins, and grown in M199 medium supplemented with 10% FCS, endothelial cell growth factor (50 μg/ml, Biomedical Technologies, Inc., Stoughton, MA) and porcine intestinal heparin (50 mg/ml; Sigma Chemical Co.) as described (43). Endothelial cells were grown to confluence on gelatin (0.1%)-coated glass slides and stimulated with TNF-α (100 U/ml) at 37°C for the times indicated. The monolayers were carefully washed and incubated at 4°C for 15 min with 75 μl of RPMI/10% FCS alone or containing semipurified sL-selectin. As a control, in some instances media containing semipurified sL-selectin were precleared by immunoprecipitation with anti-LAM-Sepharose. Without further washing, 5×10^6 lymphocytes in 75 μl of the respective media were added. After 20 min of incubation at 4°C with rotation at 64 rpm, the slides were fixed overnight in glutaraldehyde (1% [vol/vol] in PBS; Polysciences, Warrington, PA), and stained with hematoxylin. The number of adherent leukocytes was determined by counting six microscopic fields (0.09 mm^2/field) and the results were expressed as means ± SD.

Production of the L-selectin and IgG Chimera cDNA and Protein

Generation of the L-selectin and IgG chimeric cDNA will be described in detail elsewhere (44a). Basically, the 1400-bp BanII fragment from a cDNA encoding the CH1 through CH3 domains of the human IgG_1 constant region was inserted at a BanII site introduced into pLAM-1 cDNA (49) (amino acid number 370 in the membrane proximal region of the mature protein) by oligonucleotide directed mutagenesis. The recombinant DNA was sequenced and the conservation of L-selectin and IgG_1 restriction sites in the pLAM-IgG DNA was confirmed by restriction mapping. The LAM-IgG DNA was subcloned into the Ap^{r}M8 expression vector (provided by Dr. Lloyd Klickstein, Center for Blood Research, Boston, MA) and used to transiently transfect COS cells by the DEAE dextran method. The transfected COS cells were cultured in AIM-V serum-free media (Gibco-BRL) and the supernatant fluid containing the chimeric LAM-IgG fusion protein was harvested after 3 d.

sL-selectin ELISA

The ELISA used to quantitate sL-selectin levels in biological fluids will be described in detail elsewhere (44a). Briefly, wells of microtiter plates (96 well, flat bottom, E.I.A./R.I.A. plate, Costar, Cambridge, MA) were coated with anti-L-selectin mAb as indicated. After washing with TBS, the wells were blocked with 2% BSA and 1% gelatin in TBS. The wells were washed, and the test samples were added to triplicate wells. Each assay included the titration of a previously quantified plasma sample that was used to generate a standard curve. After washing, the plates were incubated with biotinylated anti-LAM1-3 mAb (1 μg/ml) for 60 min at 20°C. The wells were washed, and avidin-HRP (0.1 μg/ml, Pierce Chemical Co., Rockford, IL) was added for 30 min at 20°C. Again, the plates were washed and finally developed using *o*-phenylenediamine (0.125% wt/vol, Sigma Chemical Co.) as a substrate in 0.1 M citrate buffer, pH 4.5, in the presence of H_2O_2. The OD of the reaction mixture was quantitated using an ELISA-reader (Vmax kinetic microplate reader; Molecular Devices, Menlo Park, CA). Results were obtained when the OD for the well containing the highest concentration of standard plasma was ~0.8 at 495 nm. The relative concentration of sL-selectin in individual samples was calculated by comparing the mean OD obtained for triplicate wells with a semilog standard curve of titrated plasma using linear regression analysis ($r \geq 0.97$).

The amount of sL-selectin present in the standard plasma was quantitated in two ways. First, K562-LAM-1 cells (~11 liters of cultured cells, $\sim 1.1 \times 10^{10}$ cells) were resuspended in PBS (1×10^7 cells/ml) and were stimulated with PMA (100 ng/ml) for 2 h at 37°C. The supernatant fluid was collected, concentrated by ultrafiltration, and affinity purified by column chromatography using anti-LAM-Sepharose as described above. The semipurified sample was electrophoresed on a 10% SDS-polyacrylamide gel that was subsequently stained by Coomassie blue to reveal a prominent 71-kD band and additional bands of ~180, 57, 47, and 22 kD. The gel was scanned using a Hewlett Packard Desk Scanner and the density of the 71-kD band was quantitated against a standard curve generated with BSA using the Enhance™ program (Microsystems, Des Moines, IA) on an Apple Macintosh IIcx computer. The concentration of sL-selectin in the standard plasma was calculated to be ~1.3 μg/ml by comparing the signal from semipurified sL-selectin to the L-selectin-ELISA titration curve of standard plasma with linear regression analysis. The detection limit of the L-selectin-ELISA was determined to be ≥5 ng/ml. In a second set of experiments, COS cells were grown in serum-free medium after transient transfection with the LAM-IgG chimera cDNA. Supernatant fluid was collected from the cells and run over a Protein A-Sepharose (Pharmacia LKB Biotechnology) affinity chromatography column and the fusion protein was eluted from the column by high salt-low pH buffer. The purified fusion protein was quantitated after SDS-PAGE analysis by comparison of the stained protein band with a standard curve of BSA. From this analysis, it appeared that OD values for standard plasma would be equivalent to ~1.9 μg/ml of LAM-IgG fusion protein. Since the dimeric nature of the fusion protein might double the intensity of staining in our sandwich ELISA, the amount of sL-selectin in the standard plasma may be half the value of ~1.9 μg/ml.

Statistics

Statistical analysis used the paired or unpaired *t* test appropriately.

Results

Detection of sL-selectin in Plasma by Western Blot Analysis

In vitro, leukocytes and L-selectin cDNA-transfected cells shed L-selectin from the cell surface that can be detected in the culture supernatant fluid. To determine whether this process also occurs in vivo, the anti-LAM1-3 mAb, which identifies an epitope within the lectin domain, was used to immunoprecipitate reactive materials from normal human plasma. The precipitated materials were then analyzed by SDS-PAGE, transferred to nitrocellulose, and the presence of a soluble form of L-selectin was visualized by Western blot analysis using the anti-LAM1-14 mAb that binds to a L-selectin epitope located within the SCR region. Two predominant isoforms of sL-selectin were observed, a ~62-kD isoform and a 75–100-kD isoform (Fig. 1). Analysis of the culture supernatant fluid from PMA-stimulated lymphocytes, neutrophils, and K562 cells transfected with L-selectin cDNA (K562-LAM) revealed different, cell-specific isoforms of sL-selectin (Fig. 1). Neutrophils shed a 75–100-kD isoform of sL-selectin that traveled as a broad band with an ill-defined upper border most likely due to heavy glycosylation (33). Lymphocytes generated a 62-kD isoform and K562-LAM cells shed a 71-kD isoform of sL-selectin. No specific proteins were isolated from the supernatant fluid of untransfected K562 cells using the above assays, and preclearing the plasma or supernatant fluid with the anti-LAM1-3 mAb eliminated subsequent Western blot results (data not shown). Therefore, it is likely that the two predominant isoforms of sL-selectin identified in plasma derived from both lymphocytes and neutrophils. Furthermore, sL-selectin in serum contained the lectin, EGF, and SCR domains as it was visualized in these experiments using mAb reactive with the lec-

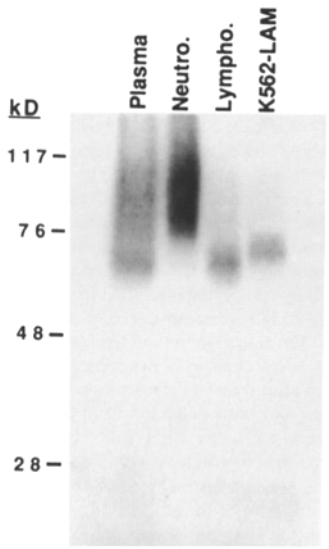

Figure 1. Different isoforms of sL-selectin detected by Western blot analysis. Plasma from a normal donor was salt fractionated, purified by affinity chromatography, and immunoprecipitation using anti-LAM1-3 mAb bound to Sepharose beads. Lymphocytes, neutrophils, and K562-LAM cells (1×10^7/ml) were incubated with PMA, and the culture supernatant fluids were isolated and concentrated tenfold by ultrafiltration. The harvested materials were analyzed by SDS-PAGE (7.5%), blotted onto nitrocellulose, and stained with the anti-LAM1-14 mAb for Western blot analysis.

tin (anti-LAM1-3) and SCR (anti-LAM1-14) domains, but the relative molecular mass of sL-selectin is smaller than that for L-selectin isolated from detergent solubilized cells as previously described (27, 40).

Quantitation of sL-selectin in Plasma and Serum by ELISA

The amount of sL-selectin found in human plasma was quantitated with a sandwich ELISA using the anti-LAM1-5 mAb as a capture antibody and biotinylated anti-LAM1-3 mAb as a detecting antibody. This specific combination of mAb provided the highest level of sensitivity so that ~5 ng of sL-selectin could be easily detected. The level of sL-selectin determined by ELISA in the sera of a population of healthy normal blood donors was found to be 1.6 ± 0.8 μg/ml, $n = 63$ (Fig. 2). In some donors, sL-selectin levels were simultaneously quantitated in plasma and serum with similar results obtained (1.9 ± 1.0 μg/ml, $n = 18$). It is unlikely that sL-selectin in plasma or serum might still be membrane bound, as the same levels of sL-selectin were found in serum and plasma before and after ultracentrifugation. sL-selectin was stable in whole blood left to stand at 20°C before separation of serum or plasma for at least 24 h. Storage of serum or plasma at 4°C for up to three months in the presence of azide or repeated thawing and freezing (up to 10 times) did not affect the ability to detect sL-selectin in serum. In conclusion, it appears that sL-selectin is stable and present at relatively high levels in human plasma and serum.

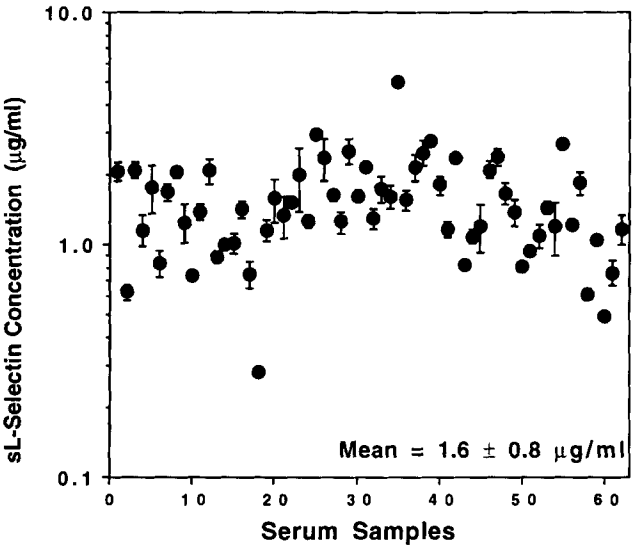

Figure 2. Quantitation of sL-selectin levels in sera of normal individuals. Serum was examined for sL-selectin by a sandwich ELISA using the anti-LAM1-5 mAb as catching antibody. Biotinylated anti-LAM1-3 mAb was used as detecting antibody, with avidin-HRP and *o*-phenylenediamine used as substrate for visualization of anti-LAM1-3 mAb binding. Standardization of the ELISA is described in detail in Materials and Methods. Values represent the mean values ± SD obtained in triplicate determinations for each sample.

Quantitation of sL-selectin in Supernatants of Activated Leukocytes

Stimulation or the culturing of leukocytes has been associated with shedding of L-selectin from the cell surface. Therefore, experiments were carried out to determine if sL-selectin accumulated in culture supernatant fluid. Culture supernatant fluid obtained from an erythroleukemia cell line transfected with the pLAM-1 cDNA (K562-LAM) was found to contain detectable sL-selectin, in contrast to supernatant

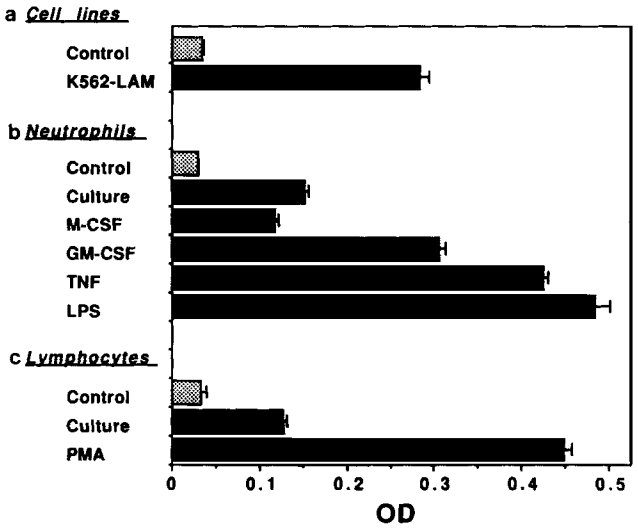

Figure 3. Neutrophils and lymphocytes shed L-selectin after activation. Culture supernatant fluid from pLAM-1 cDNA-transfected K562 cells or untransfected cells (*Control*, ▨) were analyzed. Neutrophils and lymphocytes (8×10^6/ml) were incubated in 24-well flat-bottom culture plates for 1 h at 37°C in the presence of medium alone (*culture*) or medium containing monocyte-colony stimulating factor (*M-CSF*), granulocyte/monocyte-colony stimulating factor (*GM-CSF*), formyl-methionyl-leucyl-phenylalanine (*FLMP*), TNF or lipopolysaccharide (*LPS*). Culture media were harvested, centrifuged, and supernatant fluids were tested for sL-selectin by ELISA. Culture medium alone served as controls (▨). Values represent the mean OD values ± SD obtained in triplicate determinations for each sample. These results are representative of 12 K652-LAM experiments, two neutrophil experiments, and three lymphocyte experiments.

fluid obtained from untransfected cells cultured at the same density (Fig. 3 *a*). Similarly, medium from freshly isolated neutrophils cultured at 37°C for 60 min also contained detectable sL-selectin (Fig. 3 *b*). Incubation of the neutrophils with stimuli that do not affect cell surface L-selectin expression, granulocyte-colony stimulating factor, interleukin 1, monocyte-colony stimulating factor, interleukin 6, interferon γ, and interleukin 4, did not induce an increase in sL-selectin in these experiments. However, stimulation with formylated methionine-leucine-phenylalanine, lipopolysaccharide, granulocyte/monocyte-colony stimulating factor, interleukin 8, and tumor necrosis factor (TNF) induced L-selectin shedding corresponding to their potency to stimulate cell surface shedding (Fig. 3 *b* and data not shown). Culturing lymphocytes for 60 min at 37°C caused some shedding of L-selectin, which was greatly enhanced by PMA treatment (Fig. 3 *c*). In similar experiments, quantitation of the amount of sL-selectin found in the supernatant fluid after activation of neutrophils and lymphocytes (1×10^7 cells/ml) varied between ~10–30 ng/ml. However, PMA-activation in PBS of lymphocytes for >25 min, and neutrophils for >10 min, resulted in the gradual degradation of sL-selectin. Degradation was not observed in the supernatant from K562-LAM cells, where sL-selectin was consistently found at concentrations of 22 ± 6 ng/ml (1.7 ± 0.5% of standard plasma; $n = 14$). In experiments not shown, elevated levels of sL-selectin were also detected in the culture supernatant fluid of lymphocytes cultured with phytohemagglutinin, concanavalin A, and pokeweed mitogen for 3–6 d at 37°C. These mitogens are known to cause cell surface loss of L-selectin (51). Thus, loss of L-selectin from the cell surface directly correlates with an increase of sL-selectin in the culture medium.

Inhibition of Lymphocyte Binding to Activated Endothelium by sL-selectin

sL-selectin was semipurified from plasma by salt fractionation followed by affinity chromatography with the anti-LAM1-3 mAb. The column eluate was concentrated, and the level of sL-selectin present was quantitated by ELISA and adjusted to ~15 μg/ml (6–10% of total eluate protein). sL-selectin, at different concentrations, in RPMI 1640/10% FCS was incubated (15 min, 4°C) with cytokine-activated endothelial cells before examining lymphocyte attachment to endothelium through L-selectin. While sL-selectin at physiological concentrations caused only partial inhibition of lymphocyte attachment (32.1 ± 15.9%, $n = 10$), it was found to inhibit most L-selectin-dependent lymphocyte binding at concentrations of 8 μg/ml (52–100%) and caused almost total inhibition of L-selectin-mediated binding at 12–15 μg/ml (93–100%; Fig. 4 depicts a typical dose response curve). Lymphocyte binding mediated by L-selectin was calculated as the difference between total binding at any given concentration of sL-selectin and lymphocyte binding found in the presence of anti-LAM1-3 mAb, which blocks all L-selectin mediated adhesion (43). Importantly, sL-selectin at 15 μg/ml caused no additional inhibition of lymphocyte binding beyond that obtained with anti-LAM1-3 mAb alone. When the sL-selectin samples were precleared with the anti-LAM1-15 mAb (which does not block L-selectin adhesion) before the assays, the preparation was not able to inhibit lymphocyte attachment, demonstrating that sL-selectin mediated the inhibition (Fig. 4). Thus, it appears that sL-selectin is capable of inhibiting L-selectin-specific lymphocyte adhesion to endothelium by binding to its putative endothelial ligand.

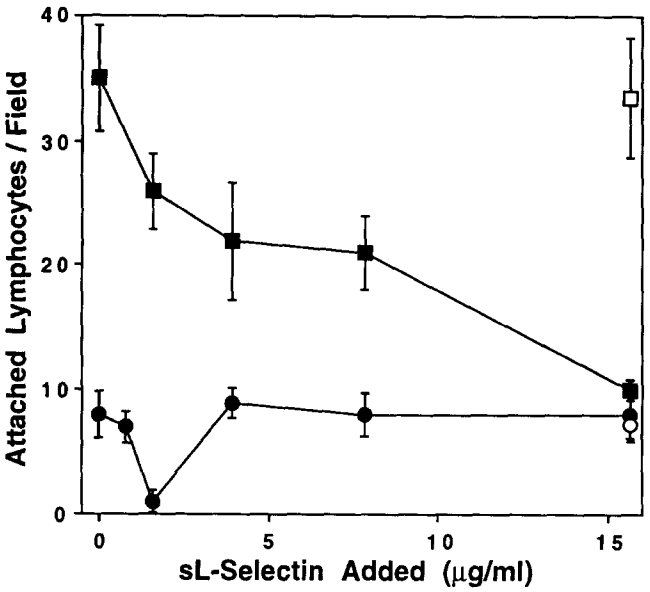

Figure 4. Inhibition of lymphocyte binding to activated endothelium by sL-selectin. Confluent monolayers of endothelial cells grown on slides were activated with TNF (100 U/ml) for 3 h, followed by incubation (4°C, 15 min) with medium containing various concentrations of sL-selectin (in 75 μl) that was semipurified from plasma and resuspended in RPMI 1640/10% FCS. Blood lymphocytes (5×10^6) in 75 μl of medium (■) or medium containing anti-LAM1-3 mAb (15 μg, ●) were layered onto the slides for 20 min at 4°C with rotation at 64 rpm. Attachment of lymphocytes in the presence of anti-LAM1-3 mAb indicates L-selectin-independent binding. Results obtained with a portion of the sL-selectin preparation (equivalent to that used at 15.7 μg/ml) that was first precleared with the anti-LAM1-15 mAb are shown as open symbols. Values represent the means ± SD of six counted fields, and are typical for three experiments.

The inhibitory capacity of sL-selectin at normal physiological concentrations (1.5 μg/ml) was further evaluated to determine if it could alter the interaction of lymphocytes with endothelium. Endothelium was activated with TNF (100 U/ml) for different periods of time to induce the L-selectin ligand in a manner similar to what might happen during the initiation of an inflammatory response. During the course of induction of the L-selectin ligand, sL-selectin treatment of the endothelium caused a consistent, but small, inhibition of L-selectin-dependent lymphocyte attachment (30% at 2 h, 43% at 3 h, 41% at 4 h, 15% at 5 h, and 2% at 6 h) (Fig. 5). Again, the combination of sL-selectin with the anti-LAM1-3 mAb did not cause a greater inhibition than that observed with the mAb alone. Thus, during the development of an inflammatory response, it is likely that sL-selectin will be able to alter the course of leukocyte attachment, although the influence appears to be small in this in vitro assay.

sL-selectin in Serum Blocks Anti-L-selectin mAb Binding

The presence of circulating sL-selectin was further verified

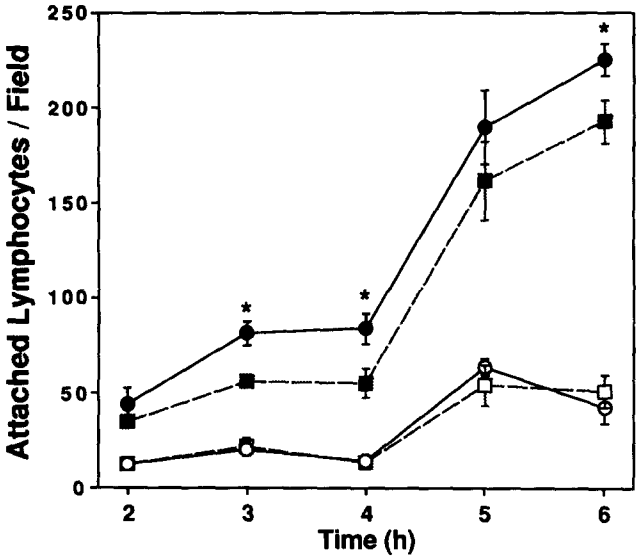

Figure 5. Inhibition of lymphocyte–endothelial binding by physiological levels of sL-selectin. Confluent monolayers of endothelial cells grown on slides were activated with TNF (100 U/ml) for various time periods as indicated. The slides were then washed and incubated (4°C, 15 min) with 75 μl of medium alone (*circles*) or medium containing 1.5 μg/ml sL-selectin that was semipurified from plasma (*squares*). Blood lymphocytes (5×10^6) in 75 μl of medium (■, ●) or medium containing the anti-LAM1-3 mAb (□, ○; 15 μg) were layered onto the slides for 20 min at 4°C with rotation at 64 rpm. Attachment of lymphocytes in the presence of anti-LAM1-3 mAb indicates L-selectin–independent binding. Values represent the means ± SD of six counted fields, and are typical for three experiments. Time points designated by asterisks indicate statistically significant differences ($P < 0.005$) between attachment with or without sL-selectin present.

by demonstrating that the reactivity of anti-L-selectin mAb with L-selectin$^+$ cells could be inhibited by plasma. When lymphocytes were stained using anti-L-selectin mAb in undiluted human plasma, no significant staining was obtained at concentrations of mAb that were saturating in RPMI/FCS (data not shown). Next, lymphocytes (1×10^6/100 μl) were incubated with various concentrations of L-selectin–directed mAb diluted (1:100) in autologous plasma or autologous plasma which had been precleared of sL-selectin by immunoprecipitation. After completion of indirect immunofluorescence staining, antibody binding was assessed by flow cytometry. In most cases, the presence of plasma inhibited anti-L-selectin mAb staining when the mAbs were used at 2 to 5 μg/ml (Fig. 6). Thus, ~13–33 pM IgG_1 (150 kD) bound to ~21 pM sL-selectin (75 kD) based on the concentration of sL-selectin present in plasma (1.6 μg/ml) determined as outlined above. sL-selectin–dependent inhibition of mAb binding was seen in plasma for mAb which recognize the lectin domain (anti-LAM1-3, -4, and -10) and the EGF-like domain (anti-LAM1-5, and -15). In contrast, binding of the anti-LAM1-1 mAb, which binds an epitope in the EGF-like domain, was not significantly inhibited by the presence of plasma (Fig. 6 *D*).

The LAM1-1 Epitope was not Detected on sL-selectin from Human Plasma

Since anti-LAM1-1 mAb binding to lymphocytes was not inhibited by plasma, the epitope identified by this mAb may not be present on sL-selectin. To examine this further, ELISA were performed in which different L-selectin–directed mAbs were bound to an ELISA plate as capture antibodies with the anti-LAM1-3 mAb as the detecting antibody. Lectin domain-specific antibodies (anti-LAM1-6, -7, -10, and -11) and EGF-like domain specific mAb (anti-LAM1-5 and -15) gave strong, easily detectable positive signals (Fig. 7). Wells coated with the anti-LAM1-3 and -4 mAb, which cross-block the binding of the detecting antibody (anti-LAM1-3), served as internal controls for background reactivity. In contrast to all other mAb, anti-LAM1-1 gave a signal that was not significantly different from background, as defined by using BSA as the capture reagent. Thus, the

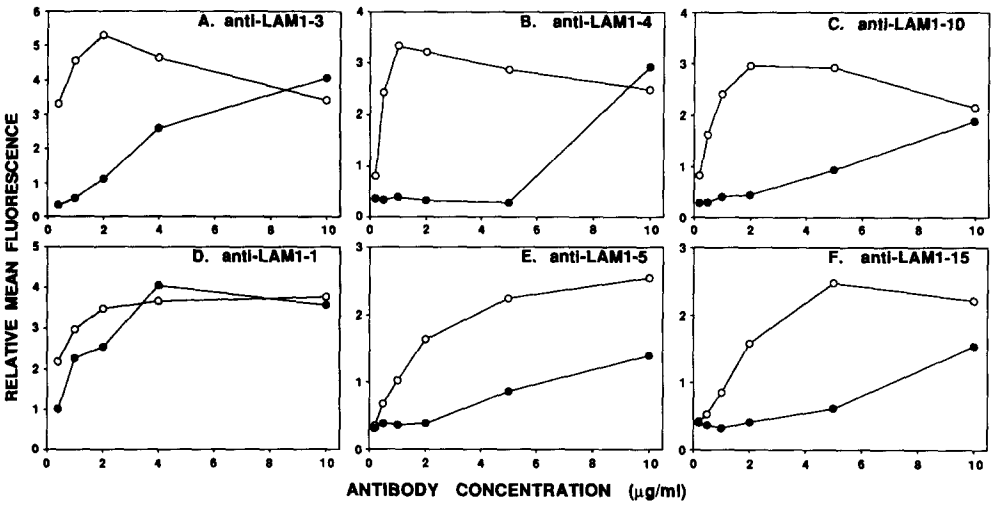

Figure 6. The influence of sL-selectin on the binding of anti-L-selectin mAb to lymphocytes. Heparinized plasma was either precleared of sL-selectin using anti-LAM-Sepharose or left on ice. Lymphocytes from the same donor were resuspended in 100 μl of plasma (●) or precleared plasma (○). Various anti-L-selectin mAbs at different concentrations were added to the plasma (1:100) and mAb binding was assessed by indirect immunofluorescence staining. Washed lymphocytes were fixed in paraformaldehyde, and the intensity of fluorescence staining was determined by flow cytometry. Values represent the relative mean intensity of fluorescence for stained cells. These results are representative of those obtained in two sets of experiments.

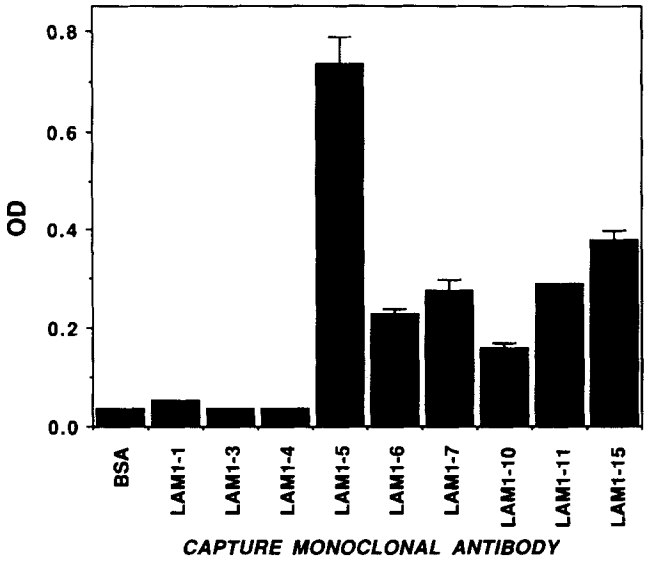

Figure 7. The epitope identified by the anti-LAM1-1 mAb is not detected on sL-selectin from plasma. Plasma was tested for detectable sL-selectin by a sandwich ELISA using the indicated anti-L-selectin mAb as catching antibody, and biotinylated anti-LAM1-3 mAb as detecting antibody. Anti-LAM1-3 mAb binding was visualized using avidin-HRP and *o*-phenylenediamine as substrate. BSA bound to the wells of the ELISA plate served as a control. Antibodies anti-LAM1-3 and -4 cross-block the binding of the detecting antibody (anti-LAM1-3). Values represent mean ± SD of OD values obtained in triplicate wells. These results are representative of three similar experiments.

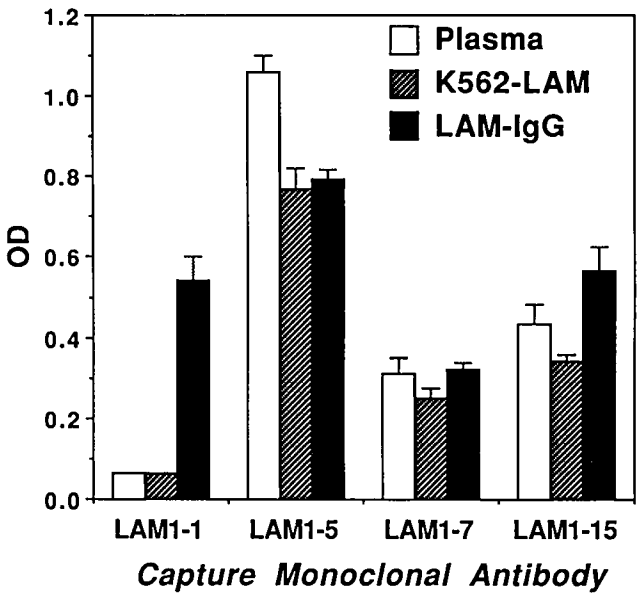

Figure 8. Detection of the LAM1-1 epitope, which is lost on sL-selectin from plasma, on a soluble LAM-IgG fusion protein. A LAM-IgG chimeric cDNA was constructed and the fusion protein was expressed in COS cells. After 3 d of culture, supernatant fluids were harvested and examined for LAM-IgG (■) content by ELISA. In parallel, supernatant fluid of PMA-stimulated K562-LAM cells (▨) and plasma (diluted 1/5) (□) were examined. The indicated mAbs were bound to the wells of an ELISA plate and the ELISA was carried out as described in Fig. 7. Bars denote mean OD values ± SD of triplicate determinations. These results are representative of those obtained in three separate experiments.

epitope located within the EGF-like region which is specifically recognized by the anti-LAM1-1 mAb appears to be lost from L-selectin upon shedding. As L-selectin was easily recognized by all L-selectin-specific mAb in immunofluorescence staining of lymphocytes and all three extracellular domains are preserved on sL-selectin found in plasma, conformational changes in the L-selectin protein may lead to the loss of the EGF-like domain related LAM1-1 epitope.

To determine whether the transmembrane or cytoplasmic regions of L-selectin are necessary to uphold the complete tertiary structure of its extracellular domains, a second form of soluble L-selectin, a LAM-IgG fusion protein was generated and epitope-mapped by ELISA. The chimeric cDNA used to generate the fusion protein was constructed so that it contained essentially the entire extracellular region of L-selectin. Culture supernatant fluid of COS cells that were transiently transfected with the chimeric cDNA were tested for the production of LAM-IgG by ELISA. The anti-LAM1-5, -7, and -15 mAb, which detected sL-selectin in plasma and in supernatant fluid from PMA-stimulated K562-LAM cells, also bound the LAM-IgG chimera at similar levels (Fig. 8). In contrast, while the anti-LAM1-1 mAb failed to generate a significant signal with sL-selectin from plasma and K562-LAM transfectants, it readily bound the LAM-IgG fusion protein (Fig. 8). Thus, it is likely that sL-selectin generated in vivo or in vitro loses a conformational determinant that is required for anti-LAM1-1 mAb binding.

Discussion

The presence of different isoforms of intact sL-selectin in plasma and serum was revealed by Western blot analysis (Fig. 1). sL-selectin in serum appeared to be derived from both lymphocytes and neutrophils since the analysis of sL-selectin isolated from neutrophils (95–105 kD) and lymphocytes (74 kd) produced bands of corresponding relative molecular mass. The differences in relative molecular mass between lymphocyte and neutrophil sL-selectin most likely result from differences in glycosylation of a single protein species since only a single L-selectin mRNA species has been identified (33), and K562 cells transfected with the pLAM-1 cDNA that is complementary to this mRNA also produced a different isoform of sL-selectin. Cleavage of L-selectin is likely to be proximal to the membrane spanning region encoded by exon VIII; cleavage in this region would account for the small difference in relative molecular mass between the intact cellular L-selectin molecule and its shed form (23, 28, 40). This is supported by the extraordinarily high degree of conservation of exon VIII between human L-selectin and the corresponding region of mouse L-selectin (9, 33). In addition, anti-L-selectin mAb binding studies demonstrated that the lectin, EGF-like, and SCR domains are retained by sL-selectin (Figs. 1 and 7). It is therefore possible that the factor in rat lymph characterized by Chin and Woodruff as being capable of inhibiting lymphocyte attachment to HEV was in part sL-selectin (4–6).

While the direct demonstration of sL-selectin in plasma and supernatant fluid from activated neutrophils and lymphocytes confirms that the in vivo and in vitro loss of L-selectin

surface expression is due to shedding, development of the sL-selectin–ELISA allowed for the first time an easy, efficient, and sensitive method for quantification of sL-selectin. Remarkably, in a population of normal healthy blood donors the mean serum level of sL-selectin was determined to be 1.6 ± 0.8 μg/ml. The concentration of sL-selectin was determined by comparison with a reference plasma sample that had been standardized by two independent means, and ranged between 0.85 and 1.9 μg/ml. The mean of this range of values of 1.3 μg/ml was therefore chosen as the basis of all calculations. Experimental data derived from shedding experiments in K562-LAM transfectants further substantiate the validity of this estimate (44a). K562-LAM cells express 30,000–43,000 L-selectin molecules on each cell as determined by Scatchard analysis. After PMA stimulation of these cells, all L-selectin molecules (71 kD) are shed. Thereby, the concentration of sL-selectin in plasma can be calculated by comparing the concentration of sL-selectin in medium (1.7% the concentration of standard plasma) shed from K562 transfectants (1×10^7 cells/ml) to a titration curve of standard plasma. According to this calculation, plasma concentrations would lie in the range of 2.1–3.0 μg/ml. Similar considerations make it highly unlikely that the amount of sL-selectin found in serum or plasma is generated from the shedding of cell surface L-selectin by blood leukocytes present during the preparation of the samples: the quantity of sL-selectin actually found in serum is 10–25-fold higher than the quantity of L-selectin that could maximally be shed from the cell surface of leukocytes ($\sim 4 \times 10^6$/ml) present in the samples, since most leukocytes express 50,000–100,000 receptors/cell as determined by Scatchard analysis (44a). Therefore, sL-selectin must be generated by the ongoing shedding of cell surface L-selectin from leukocytes in vivo.

Another adhesion molecule, ICAM-1, was recently shown to circulate in plasma at a concentration of 0.16 μg/ml (35), a concentration 10-fold lower than that found for sL-selectin. Functional activity of soluble ICAM-1, however, could only be demonstrated when it was immobilized to plastic (35). In contrast, sL-selectin semipurified from plasma inhibited L-selectin–mediated lymphocyte attachment at suboptimal levels of ligand expression on TNF-activated endothelium in a dose-dependent manner with complete inhibition at ~8–15 μg/ml (Fig. 4). At concentrations of sL-selectin similar to those found in the plasma of normal blood donors, a small but significant ($p < 0.005$) inhibition of leukocyte endothelial binding was observed at various levels of ligand expression (Fig. 5). Similarly, using a soluble fusion protein composed of the Fc portion of human IgG and the extracellular domains of MEL-14 at 1 μg per tissue section, Watson et al. (53) have found ~75% inhibition of lymphocyte binding to HEV. Preliminary studies suggest that circulating sL-selectin may be functional in vivo since immunohistochemical staining of tissues with anti–L-selectin mAb revealed sL-selectin specifically bound to the luminal surface of endothelial cells, both on HEV endothelium and at sites of inflammation (Munro, M., B. Schleiffenbaum, and T. F. Tedder, manuscript in preparation). This is not unexpected, as the MEL-14-IgG fusion protein also binds to HEV in vitro (53).

High levels of relatively low avidity sL-selectin circulating in plasma might serve as a biological adhesion buffer system to prevent the attachment of leukocytes at sites where low level upregulation of the L-selectin ligand has occurred on endothelial cells, similar to the function proposed for soluble P-selectin (12). This hypothesis is in keeping with the small but consistent inhibitory effect of physiological concentrations of sL-selectin on L-selectin specific lymphocyte attachment to HUVEC during the course of ligand induction (Fig. 5). The inhibitory effect of sL-selectin may be biologically most relevant at low levels of ligand expression. Future studies on the binding affinity of sL-selectin for its endothelial ligand will have to be carried out to determine whether sL-selectin can efficiently compete with cell-bound receptor for ligand. Since the number of sL-selectin molecules in blood is much higher than the number of cell-surface receptors in the same volume of blood, it is likely that sL-selectin is of a lower affinity. Alternatively, their affinities may be equivalent, but cell-surface receptors may function in a cooperative manner or the localized relative density of receptors on the cell surface may be higher than the corresponding relative density in solution. It is also possible that the increase in affinity of L-selectin which follows cellular activation may allow the competitive displacement of sL-selectin from the endothelial surface (41).

An interesting finding was that sL-selectin did not contain the epitope within the EGF-like domain identified by the anti–LAM1-1 mAb, while this epitope was readily demonstrated on the LAM-IgG fusion protein (Fig. 8) and is found on cell-surface L-selectin (21, 42, 51). This was revealed both by ELISA (Fig. 7) and by a lack of competitive inhibition in immunofluorescence staining of leukocytes (Fig. 6). Since the EGF-like domain is retained in sL-selectin, alterations in the tertiary structure of L-selectin may have occurred after shedding that result in a lack of anti–LAM1-1 mAb binding. It is possible that the Fc portion of IgG fused to the L-selectin extracellular domain was sufficient to stabilize the tertiary structure of L-selectin, so that anti–LAM1-1 mAb is still able to recognize its epitope on this soluble form of the molecule. Anti–LAM1-1 mAb binding to L-selectin not only inhibits leukocyte attachment to endothelium, but also induces an increase in the binding of PPME and fucoidin (21, 42), carbohydrates which define L-selectin lectin activity (16, 21, 43, 47, 56, 57). Since conformational changes involving the LAM1-1 epitope may be important for the increase in L-selectin affinity that follows leukocyte activation (41), it is conceivable that the loss of the LAM1-1 epitope results in a lower affinity of sL-selectin for ligand.

The levels of sL-selectin normally found in human serum competitively inhibited the in vitro binding of L-selectin–directed mAbs to leukocytes. This finding has important implications for future in vivo studies both in animals and humans. Functionally active mAbs will have to be introduced into animals in large enough quantities to saturate binding to sL-selectin in blood to allow efficient binding of mAb to leukocytes and subsequent modulation of leukocyte function. In addition, the formation of immune complexes between sL-selectin and L-selectin directed mAb will have to be considered. The use of $F(ab')_2$ or F(ab) fragments instead of whole immunoglobulin might also be preferable, especially when neutrophil functions are to be evaluated. Therefore, inhibitory mAb such as anti–LAM1-1 which recognizes epitopes on L-selectin that are lost on sL-selectin might also prove helpful.

It has been proposed that L-selectin shedding is necessary

for the transmigration of leukocytes through endothelium allowing rapid deadhesion of the leukocytes from endothelial cell surfaces. However, immunohistochemical staining of lymph nodes and inflamed tissues demonstrated variable proportions of L-selectin$^+$ extravascular lymphocytes, neutrophils, and monocytes (Munro, M., B. Schleiffenbaum, and T. F. Tedder, manuscript in preparation). Therefore, it is possible that once leukocytes localize in tissues, they continue to express L-selectin, but this receptor is continually shed. As only a small proportion of the body's leukocytes are normally found in the circulation, it might be hypothesized that tissue localized leukocytes represent a major source of sL-selectin. The in situ production of sL-selectin by tissue-localized leukocytes would help explain the unpredicted high level of sL-selectin found in the circulation. However, the L-selectin ligand expressed by HEV of peripheral lymph nodes is shed in vitro and may also be shed in vivo (28). Thereby, if the ligand is shed at high enough levels in vivo, it might adhere to sL-selectin, thus inhibiting its ability to affect cell adhesion in vivo. In addition, significant levels of the shed ligand may also serve as an inhibitor of L-selectin-mediated adhesion to endothelium. Nonetheless, the presence of a soluble isoform of L-selectin with functional activity in serum indicates a new potential mechanism for in vivo regulation of leukocyte attachment to endothelium.

The authors thank Ms. A. Penta for assistance with these experiments, Ms. B. Barett of the Blood Components Laboratory of Dana-Farber Cancer Institute for providing blood samples from normal donors, and Drs. B. Luscinskas and M. Gimbrone Jr. for providing human umbilical vein endothelial cells.

This work was supported by grants from the National Institutes of Health, CA-34183, Al-26872, and CA-54464. O. Spertini and B. Schleiffenbaum were supported by grants from the Swiss National Foundation for Scientific Research. T. F. Tedder is a Scholar of the Leukemia Society of America.

Received for publication 18 March 1992 and in revised form 6 June 1992.

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