Characterization of a Functionally Important and Evolutionarily Well-conserved Epitope Mapped to the Short Consensus Repeats of E-Selectin and L-Selectin

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Summary

Selectins represent a new family of adhesion molecules, expressed by leukocytes and endothelial cells, that are involved in the regulation of leukocyte traffic. Here we have characterized a new monoclonal antibody (mAb) (EL-246) that recognizes both human leukocyte L-selectin (previously called LAM-1, LECAM-1, or gp90MEL-14) and endothelial cell E-selectin (previously called ELAM-1). EL-246 recognized a 110-kD protein expressed on cells transfected with E-selectin cDNA and stained many postcapillary venules in inflamed human tonsil. EL-246 also stained human peripheral blood leukocytes and showed identity with anti-L-selectin mAb in two-color flow cytometric analysis. The expression of the leukocyte EL-246 antigen was regulated in the same manner as L-selectin and EL-246 recognized anti-L-selectin mAb affinity-purified antigen in SDS/PAGE Western blot analysis. Further, L-selectin cDNA transfectants were specifically stained by EL-246. EL-246 blocked >95% of lymphocyte adhesion to peripheral lymph node high endothelial venules and >90% of neutrophil adhesion to E-selectin transfectants. In addition to the EL-246 epitope being expressed on two different human selectins, it was detected on L-selectin from a variety of different animals. Interestingly, domain mapping studies localized the EL-246 epitope to the short consensus repeat (SCR) domains of L-selectin. EL-246 is the first mAb that recognizes two different selectins and potentially defines a functional epitope encoded by the SCR domains. Inhibitors of selectin function targeted to this region would be expected to have the added advantage of simultaneously blocking the activity of two distinct adhesion proteins involved in inflammation.

Selectins, previously called LEC-CAMs, represent a new family of adhesion proteins that regulate leukocyte entry into lymphoid tissues and sites of inflammation (1, 2). Three members of this family have been identified: E-selectin and P-selectin (originally termed ELAM-1 and GMP-140/PADGEM, respectively), expressed by endothelial cells; and L-selectin (also called LAM-1, LECAM-1, Leu-8, TQ-1, or peripheral lymph node homing receptor), expressed by virtually all PBLs. P-Selectin is a cytoplasmic glycoprotein in endothelial cells and platelets that can be rapidly (within minutes) translocated to the cell surface upon activation with thrombin (3-5). E-Selectin is also an inducible endothelial cell surface glycoprotein, but requires 2-4 h for expression, reflecting the requirement for de novo protein and RNA synthesis (6, 7). Both P- and E-selectin are adhesion proteins for neutrophils and monocytes (3, 4, 6, 7). A subpopulation of memory T cells has also been shown to bind E-selectin (8, 9). In contrast to the vascular selectins, L-selectin is con-

stitutively expressed by leukocytes and mediates lymphocyte adhesion to peripheral lymph node high endothelial venules (HEV)[1] by binding the peripheral vascular addressin (10, 11), and neutrophil adhesion to cytokine-activated endothelial cells (12-14). Recently, neutrophil L-selectin has been shown to be a potential counter-receptor for E-selectin (15, 16).

At the molecular level, all three selectins exhibit a unique mosaic structure consisting of an NH_2-terminal type-c lectin domain, an epidermal growth factor (EGF)-like domain, and multiple short consensus repeat (SCR) domains homologous to those found in complement regulatory proteins (4, 6, 17-20). Overall these proteins share 40-60% identity at the nucleotide and amino acid level, and may have arisen by gene

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[1] Abbreviations used in this paper: EGF, epidermal growth factor; HEV, high endothelial venule; SCR, short consensus repeat.

duplication of an early ancestral gene. The lectin domains of each selectin are believed to be critical to the adhesive functions of the proteins, and the carbohydrate binding specificities of all three selectins have been partially defined. P- and E-selectin both recognize sialylated Lewis × (sLex), which decorates glycoproteins and glycolipids expressed by myeloid cells although differences in their binding properties exist (21–25). L-Selectin function is blocked by certain simple sugars, such as mannose-6-PO4, and certain complex polysaccharides, such as the mannose-6-PO$_4$-rich phosphomannan (PPME) from the yeast *Hansenula holstii* (26, 27, and reviewed in reference 1). Furthermore, many antibodies that block L-selectin function recognize epitopes encoded by the lectin domain (28–30).

Other spatially separate and distinct functional domains of the selectins may also exist. Antibodies against the mouse or human L-selectin EGF domain block lymphocyte adhesion to HEV but have little effect on carbohydrate binding (29, 31). Studies of chimeric L-selectin/Ig constructs suggest that the SCR domains also have important functional roles for selectins (32), but, in contrast to the lectin and EGF domains, no function blocking antibody has been shown to recognize this region. In addition, it is thought that the functional role of the SCRs is restricted to maintenance of proper molecular conformation, which is distinct for each selectin (32).

Here we characterize a new mAb (EL-246) that recognizes a common epitope expressed on both human E- and L-selectin. EL-246 blocks the function of both proteins, recognizes selectins from a variety of different animals, and its epitope is encoded or at least controlled by the selectin SCR domains. These results show that antibodies against the SCR domains can inhibit the adhesive function of two distinct selectins. Insight into the generation of novel therapeutics, designed to inhibit the function of two adhesion proteins involved in inflammation, may be gained by further study of the EL-246 epitope.

Materials and Methods

Animals. The animals as sources of blood (see below) were randomly selected from the large animal facilities at Montana State University. No large animal experiments were performed. Both BALB/C and C57BL/6 mouse strains were used. The mice ranged in age from 6 to 12 wk and were used primarily for the generation of mAbs or as sources of lymphoid tissues. The mice were housed in the small animal facility at Montana State University, which is AAALAC approved.

Leukocyte Cell Suspensions. Leukocytes were harvested from the peripheral blood of humans, goats, sheep, cattle, horses, pigs, rats, and chickens. For routine immunofluorescence staining, RBCs (except chicken RBCs) were lysed in a hypotonic solution. Human blood was used as a source of leukocytes for the functional assays described below. We used previously described methods to isolate both mononuclear cells and neutrophils (30, 33–35). Briefly, blood was collected into citrate anticoagulent tubes, diluted 1:2 with warm HBSS, underlayed with Histopaque 1077, and centrifuged at 2,300 RPM for 30 min at room temperature. Mononuclear cells were collected from the Histopaque/plasma interface. The pellet, which

included RBCs and neutrophils, was resuspended to its original volume in HBSS and the neutrophils isolated by Dextran sedimentation. Residual RBCs in both the mononuclear cell and neutrophil preparations were lysed by hypotonic treatment.

mAbs. Leu-8 (purchased from Becton Dickinson & Co., Mountain View, CA) and the DREG series of mAbs (DREG 56, DREG 200, and DREG 152), which are mouse IgGs that have been shown to recognize human L-selectin (19, 30), were used in the flow cytometric and Western blot analysis described below. Leu-8 was used as a PE conjugate, and the DREG mAbs were used as unconjugated mAbs followed by appropriate second stage or as FITC conjugates. The DREG mAbs were partially purified by ammonium sulphate precipitation. Other mAbs, SH43 (mouse IgG1 anti-sheep platelet; M. A. Jutila, unpublished observations) and EL-81 (mouse IgG1 anti-ELAM-1), were used as negative controls in many of the experiments described below.

Immunization and mAb Generation. Mouse L1-2 lymphoma cells stably expressing human E-selectin cDNA (L1-2 ELAM) (16) were used as immunogen for the generation of anti-E-selectin antibodies. Briefly, L1-2ELAM cells (2 × 10^7) were injected intraperitoneally into C57BL/6 mice at biweekly intervals (a total of three injections) in the absence of adjuvant. Serum titers were tested by flow cytometry for staining of the L1-2ELAM and E-selectin cDNA-transfected L cells (see below). The last boost was done 4 d before the fusion. The SP2/0 myeloma cell line was used as a fusion partner, and previously described procedures were followed in the generation of hybridomas (30). The fusion was screened on day 10 by flow cytometry using E-selectin-transfected and mock-transfected L1-2 cells. A total of 279 cells were screened and 15 selected for further analysis. Secondary screens included SDS/PAGE Western blot analysis, immunohistology, and staining of PBLs. As described below, EL-246, which is a mouse IgG1, was found to stain both E-selectin transfectants and human leukocytes.

Immunofluorescence Staining and Flow Cytometric Analysis. Immunofluorescence staining of leukocytes was carried out as described (33–35). Briefly, 10^6 cells were initially incubated in 2% rabbit serum for 10 min on ice to block Fc receptors. The cells were washed and then incubated with primary antibody at 50 µg/ml (or undiluted culture supernatant) for 20 min on ice. After washing, bound antibodies were revealed by incubation with PE- or FITC-conjugated F(ab')$_2$ goat anti–mouse Ig (Tago Inc., Burlingame, CA) at a 1:80 dilution in 5% FCS in DMEM. Flow cytometric analysis was performed on a FACScan® (Becton Dickinson & Co.) as described (33–35). For two-color analysis, PE-conjugated Leu-8 (Becton Dickinson & Co.) or a FITC-conjugated DREG mAbs were used in combination with EL-246. The cells stained with second stage were treated with 10% mouse serum to block any available anti–mouse Ig binding sites, and negative control mouse mAbs were used to evaluate the level of background staining. Data were collected from 1–5 × 10^4 cells and are presented as histograms or contour plots.

In Vitro PMA Treatment of PBLs. Isolated PBMC from the animals listed above were incubated with PMA (10 ng/ml; Sigma Chemical Co., St. Louis, MO) for 20 min at 37°C in HBSS. After the incubation period, the cells were washed and stained for flow cytometric analysis.

Peripheral Lymph Node HEV Assay. The in vitro assay of lymphocyte binding to HEVs in frozen sections (36) has been extensively described (recently reviewed in reference 10). We have previously shown that HEV in mouse peripheral lymph nodes bind human lymphocytes well, and this binding is dependent upon L-selectin (30). Purified human lymphocytes were incubated with culture supernatants of EL-246, a blocking anti-L-selectin mAb (DREG 56), or different isotype controls, and the effect on adhe-

sion to peripheral lymph node HEV determined. Cell binding was quantitated by first identifying HEVs in each field by their characteristic autofluorescence or unique plump morphology and then counting cells bound to HEV, as described (30). Data were calculated as number of cells bound per individually scored HEV. For each data point, >150 HEVs in more than three sections were counted and are from four independent experiments. Values are presented as percentage of medium control.

Neutrophil-E-Selectin Transfectant Binding Assay. L cells stably expressing human E-selectin cDNA (80% ELAM-1 positive determined by flow cytometry), previously described by Kishimoto et al., (15), were grown on plastic eight-well Lab Tek slides (Miles Scientific, Naperville, IL). Neutrophils isolated from human peripheral blood were resuspended at 10^6 cells/ml in cRPMI, and 400 μl was added to the wells of the transfected L cell cultures. The neutrophils were allowed to adhere at room temperature for 15 min under constant rotation, as previously described (15). After the incubation, the medium in each well was aspirated, slide chambers were removed, and the slides were placed in a coplin jar with 1.0% glutaraldehyde in HBSS. Adhesion was measured by counting the number of neutrophils/L cell. The effect of mAb treatment of the L cells was determined as follows. In all experiments the neutrophils were precoated with 10% rabbit serum to block available Fc binding sites. The E-selectin transfectants were treated with EL-246 (culture supernatant) or an isotype-negative control mAb for 20 min at room temperature, washed, and then used in the adhesion assay.

SDS-PAGE Western Blot Analysis. Lysates of human PBL or L1-2ELAM cell suspensions were prepared by incubating 3×10^7 cells in 1.0 ml of NP-40 lysis buffer (3% NP-40, 150 mM NaCl, 1 mM MgCl$_2$, 5 mM EDTA, 0.02% NaN$_3$, and 10 μg/ml of the following protease inhibitors: pepstatin A, antipain, leupeptin, chymostatin, benzamidine, and PMSF; all in 50 mM Tris-HCl, pH 7.5) for 30 min on ice. Lysates were clarified by centrifugation at 10,000 *g* for 10 min at 4°C and either used in affinity purification or directly in SDS-PAGE Western blot analysis.

For affinity isolation, CNBR-activated Sepharose 4B beads (Pharmacia Fine Chemicals, Piscataway, NJ) were coupled to the appropriate mAb (4 mg of mAb/ml of beads) according to manufacturer's instructions (Pharmacia Fine Chemicals) using poly preparatory chromatography columns (Bio-Rad Laboratories, Richmond, CA). 1 ml of lysate containing the antigen of interest was mixed with 3 ml of wash buffer (150 mM NaCl, 1 mM MgCl$_2$, 0.1% NP-40, 5 mM NaN$_3$, 20 mM Tris buffer, pH 7.5) and combined with the beads described above on a rotator for 2 h at 4°C. After the incubation, the beads were washed with 10 ml of wash buffer to remove any unbound antigen. Bound antigen was eluted with 3 ml of elution buffer (500 mM NaCl, 0.1% NP-40, 5 mM NaN$_3$, 200 mM acetic acid) and eluents collected in 0.5-ml fractions and neutralized with 100 μl of 1 M Tris buffer, pH 8.0. Fractions containing proteins of interest were determined by dot-blot analysis.

For SDS/PAGE Western blot analysis, crude lysates or affinity-purified antigen were mixed with equal volumes of 2× nonreducing SDS-solubilization buffer (samples were not boiled), run on an 8% SDS/PAGE gel, and transferred to nitrocellulose at 4°C with a transblot apparatus per manufacturer's directions (Bio-Rad Laboratories). The mild nondenaturing conditions (no boiling and most procedures done at 4°C) were required in the analysis of L-selectin. Filters were incubated with 50% horse serum in TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween-20) for 30 min. Using a 25-lane mini-blot apparatus (Immunonetics, Cambridge, MA), the filters were then incubated for 60 min with ei-

ther specific or negative control mouse mAb at 50-μg/ml concentrations or as culture supernatant fluid. The nitrocellulose filters were then washed in TBST, incubated with goat anti–mouse Ig-alkaline phosphatase conjugate (A-9654; Sigma Chemical Co.), diluted 1:200, and then washed again. The blots were developed by addition of substrate solution (Promega Biotech, Madison, WI).

Immunoperoxidase Staining. Acetone-fixed 6-μm frozen sections were incubated with antibodies in PBS (50 μg/ml) for 30 min at room temperature in a humidified chamber, and then washed in PBS. Using a histochemical kit (Histoprobe; Tago Inc., Burlingame, CA), a three-stage immunoperoxidase stain using an avidin biotin system was done per manufacturer's instructions. Sections were lightly counterstained with hematoxylin.

Domain Mapping of the EL246 Epitope. The epitope defined by the EL-246 mAb was localized using previously described (29) L-selectin/P-selectin chimeras. Stable transfectants of the 300.19 mouse pre-B cell (37) expressing either: (*a*) native L-selectin; (*b*) L2P, which contains the lectin domain from L-selectin and the remainder of the protein from P-selectin; or (*c*) L2P3L, in which the selectin SCR domains have been substituted for the P-selectin SCR domains in L2P, were produced as described elsewhere (Kansas et al., manuscript in preparation). 5×10^5 cells of each type were incubated on ice for 15 min in 100 μl of culture supernatants or PBS/1% FCS containing diluted ascites of the indicated mAb, washed, and incubated with FITC-conjugated goat anti–mouse Ig (Tago Inc.). The cells were then washed and analyzed by flow cytometry on an EPICS Profile (Coulter Immunology, Hialeah, FL).

Results

EL246 Recognizes Human E-Selectin. EL-246 was initially screened on human E-selectin cDNA-transfected mouse L1-2 cells by flow cytometry and SDS/PAGE Western blot. As shown in Fig. 1, E-selectin-transfected, but not the mock-transfected, L1-2 cells stained brightly with EL-246 in flow cytometric analysis. The molecular mass of the antigen expressed by the transfectants recognized by EL-246 was ~110 kD under nonreducing SDS/PAGE Western blot (Fig. 2). EL-246 also recognized E-selectin cDNA-transfected L cells, but did not recognize P-selectin cDNA-transfected cells (data not shown, and see below). As shown in Fig. 3, EL-246 stained many venules in inflamed human tonsil (Fig. 3), indicating the antibody did not recognize an artifact of the transfectants. Therefore, EL-246 clearly recognized human E-selectin.

EL246 Recognizes Human L-Selectin. Flow cytometric analysis showed that EL-246 also stained human PBL (Fig. 4).

Figure 1. EL-246 stains L1-2 cells transfected with human E-selectin cDNA. Human E-selectin cDNA-transfected and mock-transfected mouse L1-2 cells were stained with EL-246 for flow cytometric analysis. Arrows point to histograms that represent (*1*) EL-246 staining of L1-2ELAM and (*2*) L1-2 transfectant controls, and (*3*) background staining (second stage control) of the L1-2ELAM transfectants.

1 2 3
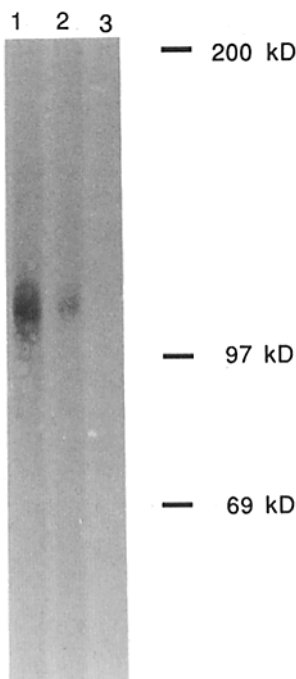
— 200 kD

— 97 kD

— 69 kD
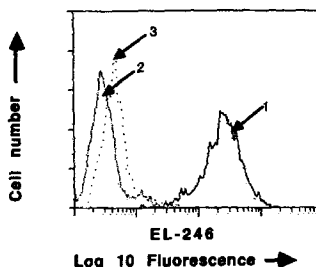
Figure 2. EL-246 recognizes a 110-kD antigen expressed by L1-2ELAM cells. L1-2ELAM NP40 lysates were run on a nonreducing 8% SDS gel and transferred to nitrocellulose. The blots were probed with EL-81 (anti-E-selectin, lane *1*), EL-246 (lane *2*), and negative control antibody (lane *3*). The distance of migration of the molecular mass markers is as indicated.

EL-246 stained all circulating human neutrophils and a subpopulation of lymphocytes, which is the same distribution pattern described for L-selectin (30, 38). In two-color flow cytometry, all EL-246-positive cells were shown to be DREG 56 (anti-L-selectin mAb [30]) positive, and the staining patterns of the two antibodies were similar (Fig. 5). The human leukocyte EL-246 antigen was lost from the cell surface after activation of neutrophils and lymphocytes with PMA (Fig. 4), which is also characteristic of L-selectin. Cells transfected with human L-selectin cDNA, but not the transfectant controls, were specifically stained with EL-246 (see below), providing direct evidence that EL-246 stains L-selectin. Furthermore, EL-246 recognized affinity-purified L-selectin in Western blots (Fig. 6). Therefore, by biochemical and molecular criteria, EL-246 reacted with L-selectin.

The EL-246 Epitope Is Expressed on Selectins from a Variety of Different Animals. To evaluate the level of evolutionary conservation of the EL-246 epitope, peripheral blood cells from a variety of different animals were screened for EL-246 staining by flow cytometry. As shown in Table 1, EL-246 stained leukocytes isolated from humans, goats, sheep, cattle, and pigs. Chicken and rat leukocytes were EL-246 negative by flow cyto-
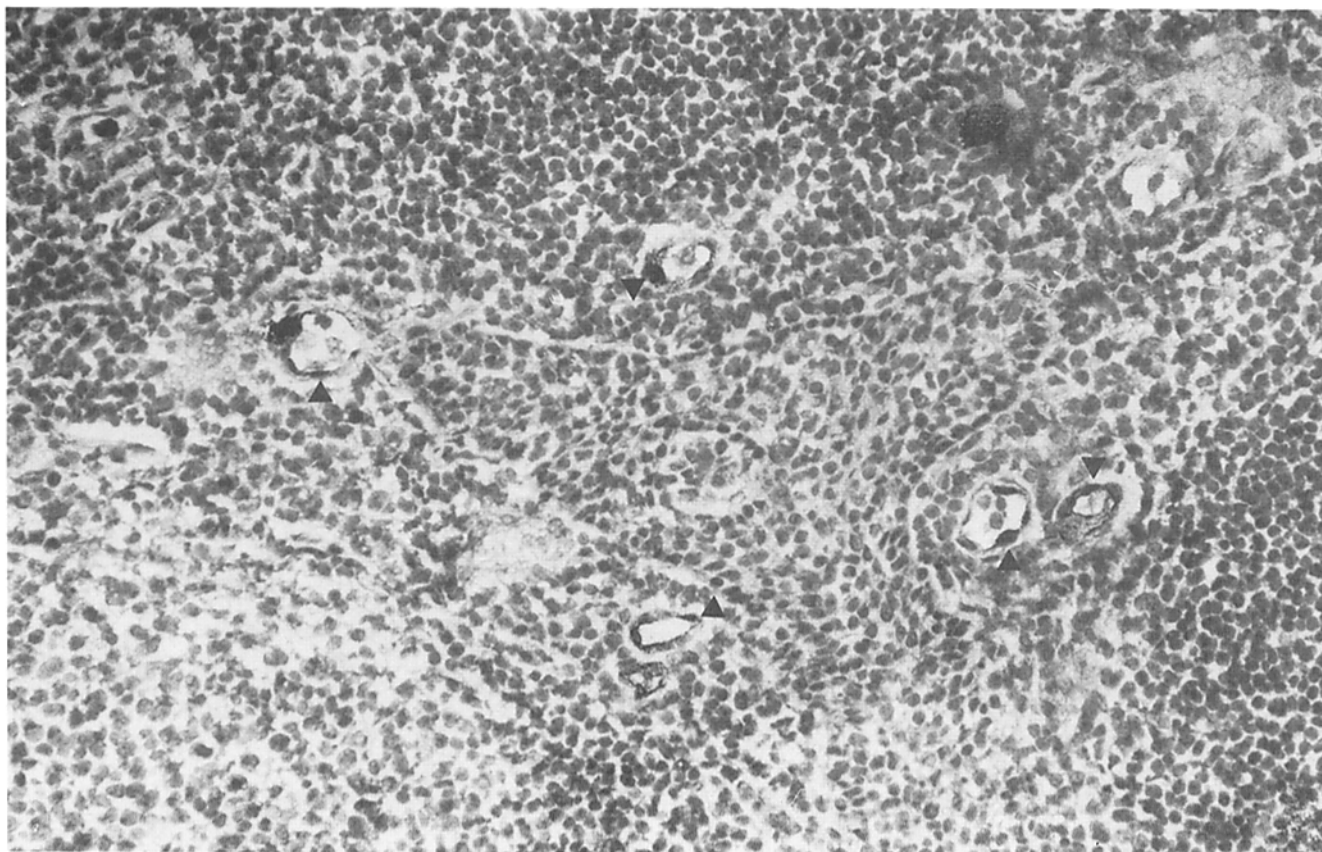

Figure 3. EL-246 recognizes inflamed venules in frozen sections of human tonsil. Frozen sections of human tonsil were prepared as described in Materials and Methods and stained by immunoperoxidase with EL-246. Arrows point to positive staining endothelial cells. ×200.

Neutrophils **Lymphocytes**
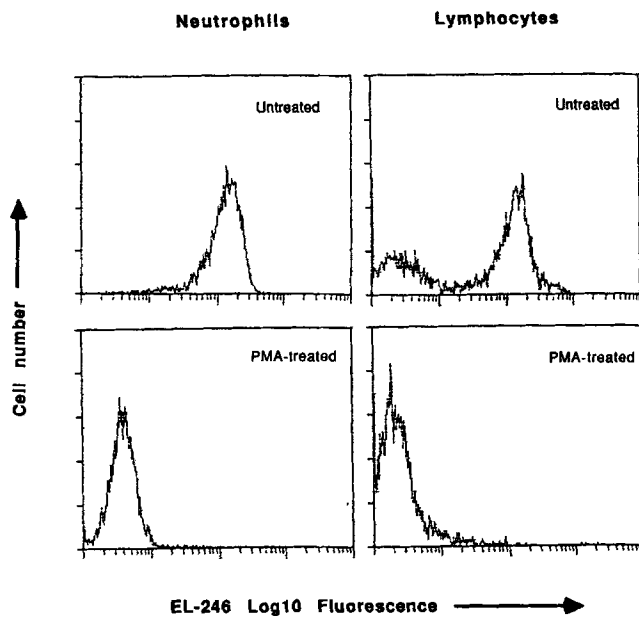
DREG purified antigen

EL-246 Log10 Fluorescence ⟶

Figure 4. EL-246 recognizes a human PBL surface antigen that is down-regulated after treatment with PMA. Human PBL were isolated as described in Materials and Methods and stained with EL-246 for flow cytometric analysis. The expression of the EL-246 antigen on neutrophils and lymphocytes, which were identified by their distinctive forward and side light scatter profiles, is shown in the representative histograms. A comparison of the staining before (untreated) and 20 min after PMA activation (PMA-treated) is shown. Background fluorescence with an isotype control or second stage alone gave a mode fluorescence value of <10 in each analysis.

metric analysis, which was also confirmed by lack of staining of cytospin preparations. The antigen recognized by EL-246 in these other animals had the characteristic distribution of L-selectin, with lymphocytes exhibiting a bimodal distribution and its surface expression lost after the cells were treated with PMA (data not shown).

EL-246 Blocks the Function of Both L-Selectin and E-Selectin. The ability of EL-246 to block E- and L-selectin function was tested. The function universally attributed to L-selectin is the adhesion of lymphocytes to HEV cells in peripheral lymph nodes (1, 10). Using the Stamper and Woodruff ex vivo frozen section binding assay (36), we found that EL-246 blocked lymphocyte adhesion to peripheral lymph node HEV equally as
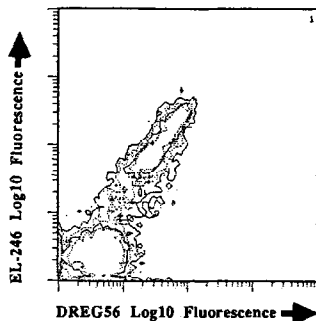

Figure 5. EL-246 costains with anti-L-selectin antibodies. Two-color flow cytometric staining was done using FITC-labeled DREG 56 (anti-L-selectin mAb (30) and EL-246 as described in Materials and Methods. A contour plot is shown that demonstrates that all EL-246 cells are also L-selectin positive.
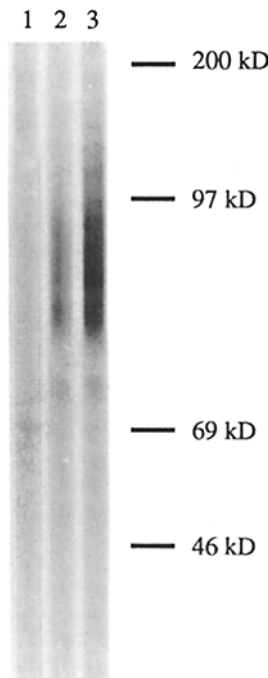
DREG56 Log10 Fluorescence ⟶

Figure 6. EL-246 recognizes affinity purified L-selectin in Western blots. L-selectin was affinity purified using the DREG 56 anti-L-selectin mAb as described in Materials and Methods, run on an 8% SDS gel under nonreducing conditions, transferred to nitrocellulose, and probed with EL-246 (lane *2*), another anti-L-selectin mAb (DREG 152) (lane *3*), or second-stage control (lane *1*). The migration of the molecular mass markers was as indicated.

well or perhaps better than our previous blocking anti-L-selectin mAb, DREG 56 (95.6 ± 4.8% vs. 88 ± 5.1% blocking, respectively) (Fig. 7). Control mAbs, including some generated in the same fusion that yielded EL-246, had no effect on lymphocyte-HEV binding (Fig. 7). EL-246 did not significantly block the binding of FITC-PPME to human lymphocytes (data not shown), another function mediated by L-selectin (1). These results are similar to the blocking activity of mAbs directed to the EGF domain of L-selectin (29, 31).

Table 1. *The EL-246 Epitope Is Conserved on Leukocytes from a Variety of Different Animals*

| | Staining of PBL* | |
Animal	DREG 56	EL-246
Human	+	+
Cow	+	+
Sheep	−	+
Goat	−	+
Pig	−	+
Horse	−	−
Rat	−	−
Chicken	−	−

* PBL were harvested from each animal as described in Materials and Methods and stained with EL-246 and DREG 56 for flow cytometric analysis. Positive reactivity was staining that was above background negative control antibody staining.
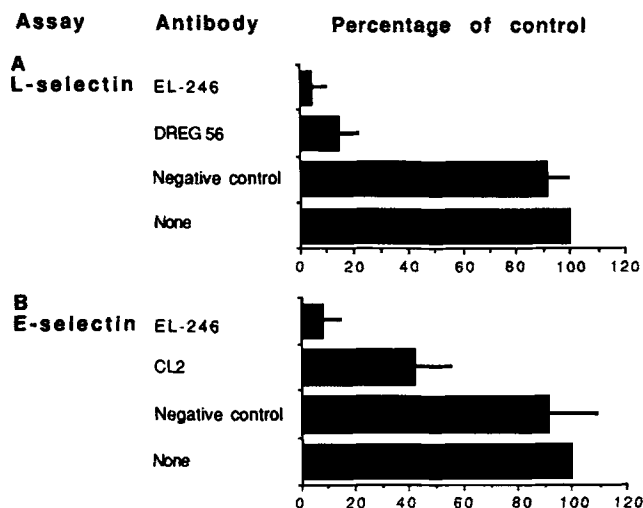
Figure 7. EL-246 blocks the function of L-selectin and E-selectin. (A) The Stamper and Woodruff ex vivo lymph node HEV binding assay (36) was used to measure L-selectin function. Human lymphocytes were treated with culture supernatants of EL-246, DREG 56, or an isotype-matched negative control (EL-81), for 20 min on ice, and the effect on binding to peripheral lymph node HEV was determined. (B) Neutrophil adhesion to E-selectin cDNA-transfected L cells was used to measure the effect of EL-246 on E-selectin function. The transfectants were treated with EL-246 (culture supernatant), CL2 (blocking anti-E-selectin mAb [25 µg/ml]), or an isotype-negative control (EL-81 culture supernatant) for 20 min, washed, and the effect on binding to human neutrophils determined. Values were recorded as percentage of control cell binding, where controls cells were incubated in assay medium alone. The experiments were repeated four times, and the means ± SEM are presented.

To examine the effect of EL-246 on E-selectin function, the ability of neutrophils to bind L cells stably expressing E-selectin was tested. In this binding assay the adhesion of neutrophils to the transfectants is clearly E-selectin dependent (15). The transfectants were treated with EL-246 for 30 min and washed before addition of purified human neutrophils, as described in Materials and Methods. Fc receptors were saturated by pretreating the neutrophils with 10% RBS for 20 min before the assay. As shown in Fig. 7, EL-246 nearly completely blocked the binding of neutrophils to the trans-

fectants (>90%), whereas another mAb (EL-81) generated in the same fusion as EL-246 and recognized E-selectin had little effect on binding. CL2 (anti-E-selectin) had similar blocking effects to those seen in our previous report (15) (Fig. 7). Therefore, EL-246 is an effective blocker of E-selectin function.

Mapping of the EL-246 Epitope to the SCR Domains. The pattern of binding of EL-246 mAb to L-selectin/P-selectin chimeras was used to determine the domain of L-selectin in which the EL-246 epitope resides. As controls, we used the LAM1-3, LAM1-1, and LAM1-14 mAbs, which define epitopes within the lectin, EGF, and SCR domains of L-selectin, respectively (29, 39), and the AC1.2 mAb (40), which identifies an epitope in the SCR domains of P-selectin (1). EL-246 specifically recognized the native L-selectin transfectants (Fig. 8), confirming that EL-246 recognizes L-selectin. Replacement of the EGF and SCR domains of L-selectin with those of P-selectin (L2P) abolished EL-246 binding (Fig. 8), demonstrating that the EL-246 epitope is not within the lectin domain of L-selectin. Furthermore, substitution of only the L-selectin SCR domains back into the L2P construct (L2P3L) restored EL-246 binding (Fig. 8). These data indicate that at least part of the EL-246 epitope is within, or requires, the SCR domains of L-selectin. Additional data in support of these results are that EL-246 does not block the lectin activity of L-selectin or cross-block the binding of four mAbs (DREG 200, DREG 55, DREG 56, and Leu-8) that recognize the L-selectin domain (data not shown).

Discussion

Selectins represent a new family of adhesion proteins that are important for leukocyte migration into tissues. These proteins mediate neutrophil and monocyte adhesion to inflamed endothelial cells (1–7, 12–16) and migration into inflammatory sites in vivo (33, 35, 41–44), and lymphocyte homing into peripheral lymphoid tissues (reviewed in reference 10). P- and E-selectin, are expressed by endothelial cells, while L-selectin is restricted to leukocytes. Here we provide the characterization of a mAb (EL-246) that recognizes a common epitope
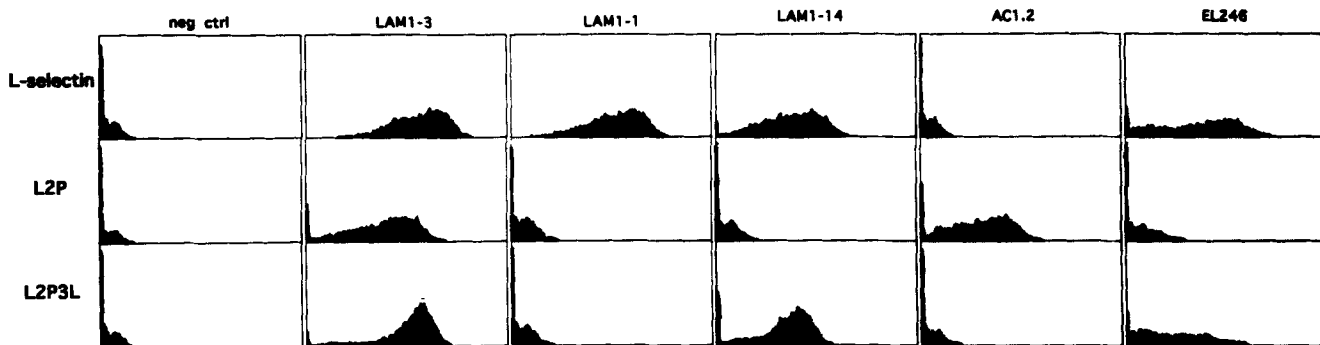

Figure 8. The EL-246 epitope is located in the SCR domains of L-selectin. Expression of the EL-246 epitope was assessed on native L-selectin and on chimeric selectins expressing the lectin domain of L-selectin with the remainder of the protein as P-selectin (L2P); or L-selectin with the EGF domain of P-selectin substituted for that of L-selectin (L2P3L). Analysis was carried out by flow cytometry as described in Materials and Methods. Included for comparison are the LAM1-3, Lam1-1, and LAM1-14 mAbs, which are directed against the lectin, EGF, and SCR domains of L-selectin, respectively; and AC1.2 mAb, directed against the SCR domains of P-selectin. EL246 stained only those selectins containing the SCR domains of L-selectin.

expressed on both E- and L-selectin. EL-246 specifically stains cells transfected with E- and L-selectin cDNAs as well as inflamed endothelial cells and PBLs. The leukocyte EL-246 epitope is regulated like L-selectin and EL-246 shows identity with another anti-L-selectin mAb in two-color flow cytometric staining. Analysis of crude lysates and purified EL-246 antigen confirmed the reactivity of EL-246 at the protein level. These data clearly establish that EL-246 recognizes both E- and L-selectin.

Even though many antiselectin mAbs have been developed, none have been shown to have the staining characteristics of EL-246. CL2, which recognizes human E-selectin, reacts with dog L-selectin, but not both proteins in the human (44; C. W. Smith, personal communication). Spertini et al. (39) provided the functional characterization and molecular localization of at least 11 different epitopes on L-selectin, but again, none of these are expressed on two different selectins. TQ-1 and Leu-8, which recognize L-selectin, also show a much more restricted pattern of staining and do not stain other selectins (M. A. Jutila, unpublished results). Finally, to our knowledge, none of the published anti-E-selectin or P-selectin mAbs have been shown to react with other selectins. It is intriguing that even though there is a significant level of identity at the amino acid level between different selectins and a large number of antiselectin mAbs have been generated, only EL-246 has been reported to recognize a shared epitope. This suggests that these sites are not very immunogenic.

In addition to recognizing E- and L-selectin, we show that EL-246 blocks the adhesive functions of both molecules. Lymphocyte adhesion to peripheral lymph node HEV was blocked by >95% with EL-246, which was greater than the DREG 56 anti-L-selectin blocking mAb (88%) (30). In contrast, the carbohydrate (PPME) binding activity of L-selectin was not apparently inhibited by EL-246. EL-246 effectively blocked (>90%) the capacity of E-selectin, expressed in adherent L cells, to bind human neutrophils. Additional evidence that the EL-246 epitope is of considerable functional importance is the conservation of this site in L-selectin from a variety of different animals, including humans, goats, sheep, cattle, and pigs. We have not examined the conservation of E-selectin in these other animals.

Insight into the diverse functional activity of E- and L-selectin, and the potential "homotypic" interaction of these proteins, may be gained by further analysis of the EL-246 epitope. Neutrophil adhesion to cytokine-activated endothelial cells can be blocked by anti-E-selectin as well as anti-L-selectin mAbs (6, 7, 12-14). Kishimoto et al. (15) showed that certain anti-E- and -L-selectin mAbs are not additive in their blocking effects on neutrophil-activated endothelial cell adhesion, suggesting that these two proteins participate in the same adhesion pathway, perhaps as receptor–counter-receptor pairs. This hypothesis was supported by the observation that neutrophil binding to E-selectin cDNA-transfected L cells is blocked by anti-L-selectin mAb treatment of the leukocyte (15). Picker et al. (16) extended these findings by demonstrating that L-selectin on neutrophils is decorated by sLex carbohydrates and may preferentially present these structures to E-selectin. In contrast, Spertini et al. (14) have also demon-

strated that neutrophil-activated endothelial cell adhesion involves E- and L-selectin, but they found that mAbs to these proteins have additive blocking effects, suggesting separate adhesion pathways. Since EL-246 is an effective blocker of E- and L-selectin function and recognizes a different molecular region (see below) than the blocking mAbs used in the above studies, it may be useful in determining the basis for some of the discrepancies in the different reports.

Preliminary domain mapping studies using L-selectin/P-selectin chimeric proteins that have been used in mapping other L-selectin epitopes (29) localized the EL-246 epitope to the SCR domains of L-selectin. Although we have not performed analogous studies with E-selectin, it seems likely that the epitope defined by EL-246 also resides within the SCR domains of this selectin. The location of the EL-246 epitope in the SCRs is supported by the inability of EL-246 to block carbohydrate (PPME) binding and cross-block antiselectin mAbs that recognize the L-selectin lectin domain. Our studies are consistent with recent reports showing that the SCR domains of L- and E-selectin are essential for optimal adhesive activity of each molecule (32, 46). As mentioned in the introduction, the lectin domain of the selectins is required for function, and many blocking antiselectin mAbs recognize epitopes encoded by this region (28-30). Previously, we and others have localized epitopes for mAbs that inhibit adhesion mediated by L-selectin to the EGF domain (25, 29, 31). The data presented here extend those observations and demonstrate that mAbs to appropriate epitopes within each selectin extracellular domain can inhibit adhesive function.

It remains unclear whether mAbs that inhibit adhesion do so as a result of direct interference with ligand binding or if binding of mAbs, especially those mAbs that define epitopes outside the lectin domain, might perturb the conformation of the protein so as to indirectly impair the functional integrity of the lectin domain. If EL-246 blocks by altering the functional conformation of the selectins, this would suggest that the role of the SCRs in adhesion is similar for E- and L-selectin. This is in contrast to Watson et al. (32), who predicted that the role of the SCRs is unique for each selectin. Since EL-246 only recognizes E- and L-selectin, this latter prediction may be true for P-selectin. Thus, in addition to having greater numbers of SCR domains (nine vs. six and two in E- and L-selectin, respectively), there may exist molecular differences in P-selectin SCRs, which contribute to the unique functions of this molecule, such as adhesion of thrombin-activated platelets.

In summary, additional molecular and functional characterization of the EL-246 epitope will likely lead to new insights into selectin function and the evolutionary conservation of this family of adhesion proteins. If this region is confirmed to be functionally important, it may provide an ideal target for treatments designed to regulate selectin activity in vivo. Importantly, new therapeutics that inhibit or alter this site would have the added advantage of blocking the activity of leukocyte-endothelial cell adhesion by simultaneously inhibiting both leukocyte as well as endothelial cell adhesion proteins.

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