Identification of a Soluble Form of a Ligand for the Lymphocyte Homing Receptor

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Summary

Lymphocytes are engaged in constant trafficking from the blood into secondary lymphoid tissues, such as peripheral lymph nodes (PLN), mesenteric lymph nodes (MLN), and Peyer's patches (PP). The initial step in this process is the binding of lymphocytes to high endothelial venules (HEV), and in the case of trafficking of cells to the PLN, it is required that they bear the L-selectin surface receptor. Using a chimeric protein, combining the extracellular domains of L-selectin with a human immunoglobulin (Ig) G1 Fc region (L-selectin-IgG), we have probed the expression of ligands for this receptor on HEV and in cell lysates. Two sulfated glycoproteins of 50 and 90 kD have been identified in lysates from PLN and MLN, but not PP. Here we show that the 50-kD molecule is secreted in organ cultures in vitro and is present in the blood of normal animals. Indeed, normal serum inhibits lymphocyte binding to HEV by approximately 50% in an in vitro assay. This inhibitory activity can be removed by passage of the serum over an L-selectin-IgG column and has a molecular mass of approximately 50 kD. We speculate on the possible reasons for secretion of a homing receptor ligand.

Lymphocytes are engaged in constant trafficking from the blood into secondary lymphoid tissues, such as peripheral lymph nodes (PLN),[1] mesenteric lymph nodes (MLN), and Peyer's patches (PP). This constant screening of the immune environment is essential for the efficiency of the antigenic response. The initial step in this process is the binding of lymphocytes to high endothelial venules (HEV), and in the case of trafficking of cells to the PLN, it is required that they bear the L-selectin surface receptor (1, 2). Until recently the ligand(s) present on the HEV surface have not been clearly defined, but the production of antibodies to murine HEV (3, 4), the construction of a chimeric protein that combines the extracellular domains of L-selectin with a human IgG1 Fc (L-selectin-IgG) region (5), and the recent cloning of a potential ligand (6) have allowed for greater understanding of these adhesive molecules. Using such a chimeric molecule, Imai et al. (7) identified two sulfated glycoproteins of 50 and 90 kD as ligands in lysates from PLN and MLN, but not PP. Here we show that the 50-kD molecule is secreted in organ cultures and that this is not an artifact of cell culture, as this 50-kD molecule is functionally present in the serum of normal mice.

[1] *Abbreviations used in this paper:* HEV, high endothelial venules; MLN, mesenteric lymph nodes; NMS, normal mouse serum; PLN, peripheral lymph nodes; PP, Peyer's patches.

Materials and Methods

Mice. 6–8-wk-old ICR female mice (Charles River Breeding Laboratories, Inc., Wilmington, MA) were used throughout these studies.

L-Selectin-IgG Chimera. This protein was grown in human 293 cells, purified, and assayed as previously described (5).

In Vitro Organ Culture. Mesenteric lymph nodes were removed aseptically from five 6–8-wk-old ICR female mice, excess fatty tissue was removed, the tissue sliced into 1–2-mm pieces, and cultured in 1 ml Dulbecco's PBS containing 1 mg/100 ml glucose and antibiotics in the presence of 250 μCi sodium 35 sulfate (ICN Radiochemicals, Irvine, CA) at 37°C in 5% humidified CO_2 in air. After 3 h the supernatant was removed and the organ slices were washed extensively with Dulbecco's PBS and then lysed in 1 ml Dulbecco's PBS, 2% Triton X-100, 0.1% sodium azide, and aprotinin with the aid of a Tissuemizer. The lysis was allowed to continue for a further 30 min at 4°C, and then both the supernatant and the lysate were spun at 5,000 *g*. The resulting materials were precleared overnight at 4°C by the addition of protein A–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ). To radioimmune precipitate the ligand, 75 μl of either supernatant or lysate was added to 30 μg L-selectin-IgG chimera and incubated for 1 h at 4°C. The complex was precipitated by the addition of 25 μl protein A–Sepharose. Total radiolabeled material was precipitated by adding an equal volume of ice-cold acetone to either supernatant or lysate. To determine that the precipitated material seen in the supernatant was behaving in the same manner as that in the lysate, immunoprecipitations were carried out in the presence of 10 mM

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EGTA, 50 μg Fucoidan (Sigma Chemical Co., St. Louis, MO), and 100 μg MEL-14 antibody (cells obtained from American Type Culture Collection, Rockville, MD), grown as ascites and purified on protein G (Pharmacia Fine Chemicals).

Pulse-Chase Experiments. Organ cultures were set up as described above, but radiolabeled sulfate was only present for the first 30 min of incubation. Then the radiolabel was washed out and fresh medium containing 1 mM cold sodium sulfate was added back to the cultures. Samples were removed at increasing time intervals.

In Vivo Labeling Experiments. Mice were injected with 1 mCi $Na_2{}^{35}SO_4$ either intravenously or intraperitoneally, and 4 h later the animals were exsanguinated and the PLN, MLN, PP, thymus, and spleen removed. The blood was allowed to clot at room temperature and then placed at 4°C overnight to allow retraction of the clot, and then serum was removed. The organs were lysed as described above, and the immunoprecipitation with L-selectin-IgG was performed as described above.

Stamper-Woodruff Assays. These were performed as described by Stamper and Woodruff (8), and modified by the procedure of Butcher et al. (9). Normal PLN cells were labeled with 2 mg/ml Texas red isothiocyanate for 20 min at room temperature for use as internal control cells. To determine the effects of normal mouse serum (NMS), PLN cells were incubated with NMS diluted in RPMI 1640. The data are expressed as a specific adherence ratio (10).

Peptide Antisera. Peptides derived from the amino acid sequence predicted from the nucleotide sequence of an isolated ligand cDNA (6) were produced on a peptide synthesizer (Applied Biosystems, Inc., Foster City, CA). A peptide from the middle region of the protein (CAM02: CKEPSIFREELISKD) was coupled to KLH through the added (nonligand), underlined cysteine residue and injected into rabbits as previously described (11). Immune serum from vaccinated, boosted rabbits was collected, and shown to immunoprecipitate sulfate-labeled L-selectin ligand (6).

Results

The 50-kD Form of the Ligand Is Secreted In Vitro. It has been suggested (12) that lymphocyte trafficking via HEV may be controlled by the level of expression of ligand for the lymphocyte homing receptor, L-selectin. As a first step in examining this hypothesis, we were interested in the turnover of this molecule. Taking advantage of previous observations (7, 13) that this molecule incorporates inorganic sulfate, we labeled MLN organ cultures with $Na_2{}^{35}SO_4$ (Fig. 1 *A*). Lysates and supernatants from these cultures were then examined by immunoprecipitation with the L-selectin-IgG chimera. Both the supernatant and the lysate showed specific precipitation of a 50-kD band, whereas a 90-kD band was apparent only in the lysate, where it was far fainter. That the supernatant 50-kD band is identical to the 50-kD band in the lysate is shown by inhibition of precipitation in the presence of MEL-14 (a mAb that inhibits binding of lymphocytes to HEVs by binding to the lectin domain of L-selectin [14], or by Fucoidan [a polysulfated saccharide] binding to L-selectin [5]). Furthermore, the immunoprecipitation of both bands is dependent on the presence of calcium ions (Fig. 1 *A*). Thus, organ cultures of MLN release a soluble form of the ligand for L-selectin. Similar results (data not shown) were obtained using PLN. Precipitations performed with a CD4-IgG chimera never resulted in the precipitation of a detectable band (data not shown).

Pulse-Chase Experiment. We examined the kinetics of ligand release in a pulse-chase experiment (Fig. 1 *B*). After a 30-min pulse, all the label was incorporated into a 50-kD molecule associated with the lysate; no band was discernable in the supernatant until 30 min later. Interestingly, 3 h after the pulse, ~75% of the 50-kD molecule had been released into the supernatant. Thus, the released material comprises a very significant portion of the total amount of ligand available and can often approach 90% of the 50-kD form at later (5–6 h) time points (data not shown). The 90-kD molecule was only observed as a cell-associated molecule throughout the entire time course of this experiment.

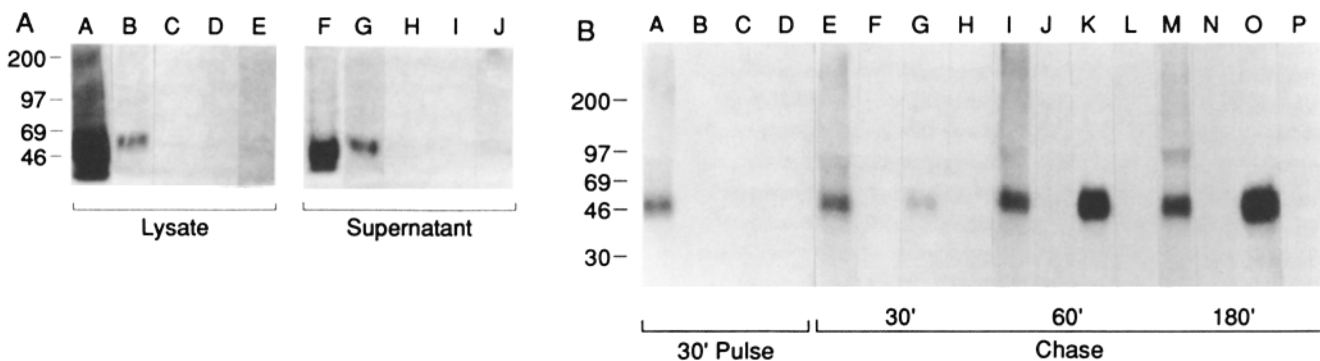

Figure 1. Demonstration that the 50-kD sulfated ligand for L-selectin-IgG is secreted. (*A*) Immunoprecipitation of material from MLN organ culture lysates (lanes *A–E*) and supernatants (lanes *F–J*). Lanes *A* and *F* represent total acetone precipitable material. Lanes *B* and *G* represent material precipitated by L-selectin-IgG. Lanes *C* and *H* are immunoprecipitations that were carried out in the presence of 10 mM EGTA, 50 μg Fucoidan (lanes *D* and *I*), and 100 μg MEL-14 (lanes *E* and *J*). (*B*) Pulse-chase experiment. Lane *A* shows bands precipitated by L-selectin-IgG from the lysate after a 30-min pulse of $Na_2{}^{35}SO_4$. Lane *B* is the immunoprecipitation done in the presence of 10 mM EGTA. Lanes *C* and *D* show the lack of a precipitable band in the supernatant at the same time point. Lanes *E* and *F* show the bands that are precipitated in the presence and absence of calcium ions in the lysate material 30 min after the removal of the pulse. Lanes *G* and *H* show the band in the supernatant in the presence but not the absence of calcium ions. Lanes *I–L* follow the same sequence at 60 min, and lanes *M–P* at 180 min. Lanes *I* and *M* show the bands precipitable from lysate, and lanes *K* and *O* from the supernatant by L-selectin-IgG. The effect of EGTA is shown in lanes *J*, *L*, *N*, and *P* for the respective time points.

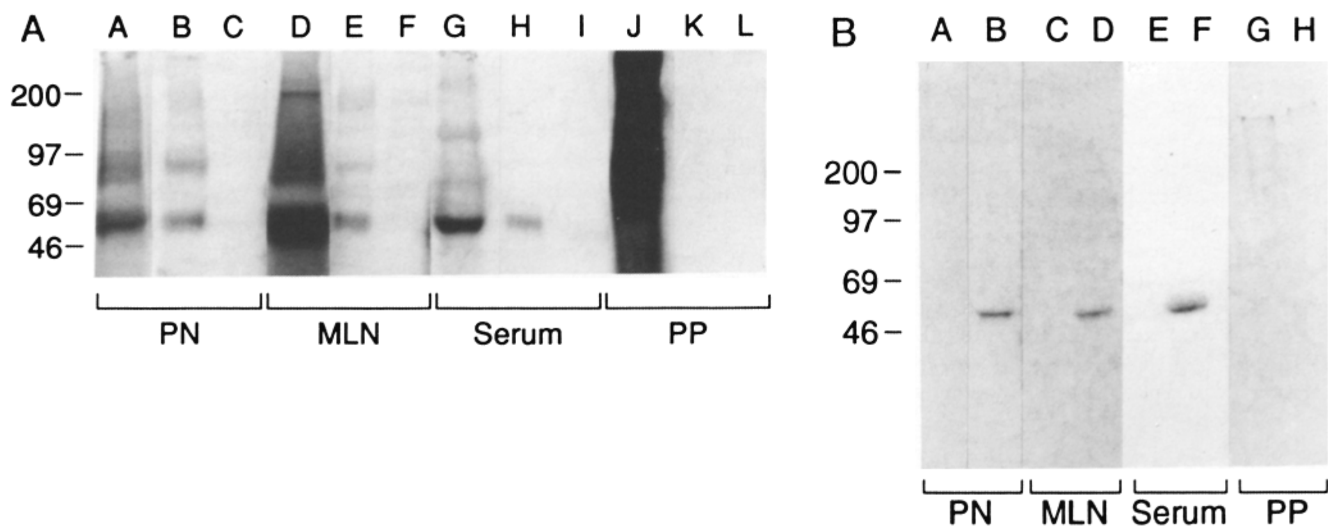

Figure 2. (*A*) In vivo labeling experiments. Mice were injected with 1 mCi $Na_2{}^{35}SO_4$ intravenously or intraperitoneally, and 4 h later the animals were exsanguinated and the PLN, MLN, PP, thymus, and spleen removed. Lane *A* shows total acetone-precipitable material from peripheral lymph nodes, lane *B* shows material precipitable by L-selectin-IgG, and lane *C* shows the precipitation performed in the presence of EGTA (10 mM). Lanes *D–F* are the same sequence performed for MLN, lanes *G–I* are the results from serum, and lanes *J–L* are the results with material from PP. (*B*) Only the 50-kD band was precipitated from the PLN, MLN, and serum with a rabbit polyclonal antiserum raised against the middle region of a recently cloned ligand for L-selectin (lanes *B*, *D*, and *F*). Preimmune serum did not precipitate a band (lanes *A*, *C*, and *E*), and no band was precipitated from PP with either preimmune or immune sera (lanes *G* and *H*).

In Vivo Labeling Experiments. Given the conditions of organ culture it was possible that the apparent secretion of the 50-kD molecule was an in vitro artifact. To examine this question, we injected mice with 1 mCi $Na_2{}^{35}SO_4$ either intravenously or intraperitoneally, and 4 h later removed samples of blood, thymus, spleen, MLN, PLN, and PP. Lysates were made from the organ samples and these, together with the serum samples, were immunoprecipitated using the L-selectin chimaera. Fig. 2 *A* shows that bands at 50 and 90 kD could be observed in lysates from PLN and MLN. A 180-kD band was also observed, which is also observed in some organ culture experiments (not shown). Serum samples contained at least four distinct labeled bands in total acetone-precipitable protein, but of these only the band at 50 kD was precipitable with the chimeric protein in a Ca^{2+}-dependent fashion. Furthermore, as shown in Fig. 2 *B*, the 50-kD band in PLN, MLN, and serum was immunoprecipitated by a rabbit polyclonal antiserum raised to the middle region of the recently cloned mucin-like ligand for L-selectin (6). Preimmune rabbit serum had no reactivity in this precipitation. Parenthetically, it should be noted that only the 50-kD, and neither the 90-kD or the 180-kD, band is recognized by the peptide antiserum. Thus, while all the molecules are sulfated and precipitable with L-selectin-IgG, they are immunologically distinct as determined by the use of antipeptide antiserum. It is possible that the band at 130–150 kD may be a complex of soluble L-selectin with secreted ligand, since L-selectin is also released from leukocytes (15). Spleen did not incorporate label well in vivo (data not shown), and no bands were seen that could be precipitated by L-selectin-IgG, as expected since spleen does not contain HEVs. PP incorporated labeled sulfate but generally no band was precipitable with L-selectin-IgG ligand. However, in one experiment, overexposure of the gel did show a very faint 50-kD band (data not shown). Thus, it is possible that PP can express this molecule but that the expression is regulated by extrinsic factors.

NMS Inhibits Lymphocyte Binding to HEV. Having observed that the 50-kD molecule was present in serum, we wished to determine whether it had biological activity. We therefore preincubated PLN cells with NMS and examined their ability to bind mouse HEV in Stamper-Woodruff assays (8). As shown in Fig. 3 *A*, lymphocyte binding was inhibited by ~50% in the presence of 50% NMS. This is the maximal inhibition that can be observed; even the addition of 100% NMS can only inhibit the binding by 50%. The inhibitory activity present in the serum could be removed using an L-selectin-IgG affinity column; material eluted from the column with 10 mM EGTA was also able to inhibit the binding assay (Fig. 3 *B*). This purification of the inhibitory factor was essentially quantitative, since when the eluate is diluted to the same volume as the starting material, the same level of inhibition is seen. Passage of NMS over a control CD4-IgG column did not remove the inhibitory activity. The inhibitory activity had a molecular mass of ~50 kD (Fig. 3 *C*). Further experiments with concentrated NMS have shown that it is possible to inhibit >80% of the binding of lymphocytes to HEV without affecting the viability of the treated cells (data not shown).

Discussion

These studies show that the 50-kD ligand for the peripheral lymph node homing receptor is secreted in vitro and that this event occurs very rapidly. This form of the ligand is present

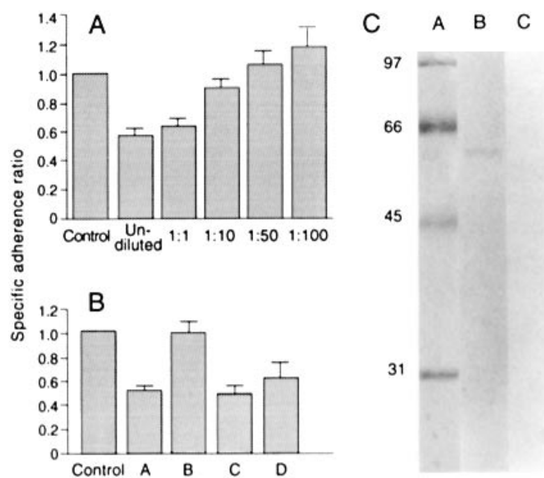

Figure 3. The effect of NMS in the Stamper-Woodruff assay. (*A*) Dose-response. Cells that were incubated in RPMI 1640 only were calculated as unity (*Control*). Dilutions of NMS were made in RPMI 1640 and PLN cells were incubated before testing in the binding assay. (*B*) Removal of inhibitory activity on an L-selectin-IgG column. Cells to be tested were incubated with NMS diluted 1:1 with RPMI 1640 (*A*), in NMS that had been passed over an Affigel column to which L-selectin-IgG had been coupled (*B*), or NMS that had been passed over a control CD4-IgG column (*D*). Cells that were incubated in RPMI 1640 only were calculated as unity (*Control*). Material bound to the L-selectin-IgG column was eluted with 10 mM EGTA and immediately dialyzed against Dulbecco's PBS. This eluted material was diluted to the same volume as NMS that passed over the column, and when added to the cells, a similar reduction in binding was seen (*C*). (*C*) Gel. Lane *A*, molecular mass markers; lane *B*, eluate from L-selectin-IgG column; lane *C*, eluate from CD4-IgG column.

in the serum of normal mice and inhibits binding of lymphocytes to HEV. These results suggest that the presence of the soluble ligand may be biologically significant. It has been previously observed that the initial binding of lymphocytes to HEV is not so tight as to prevent movement. Indeed, lymphocytes appear to experience multiple collisions with HEV before extravasation (16). Thus, it is possible to imagine a situation in which lymphocytes bind to this ligand, which is being rapidly secreted. In this way the cells can move across the surface of the HEV until they find the junction between two endothelial cells, this presumably being a site that facilitates extravasation of the lymphocyte from the blood into the lymph node. It is also imaginable that by having circulating levels of a ligand for L-selectin, it functions to further enhance the organ specificity of lymphocyte trafficking (12). Thus, cells that express high levels of L-selectin would be bound more selectively to PLN HEV than low L-selectin expressors, since the latter would be more effectively competed by the soluble 50-kD molecule. It also should be remembered that selectin-mediated events are the earliest in the adhesion cascade, and are of lower affinity than the later integrin-mediated binding. Thus, it might seem logical that such adhesive events would be mediated by molecules that could be either secreted or shed.

The recent cloning of this 50-kD molecule has shown that is has all the characteristics of a secreted protein (6). It is interesting to consider how such a molecule can function as an adhesive ligand. One possible model is that the 50-kD protein can have a loose association with the membrane by virtue of its amphipathic helix (6). A second possibility is that the L-selectin ligand is a heterodimeric molecule, comprising the 90-kD molecule, which would be expected to contain a transmembrane domain, noncovalently associated with the 50-kD molecule in a manner analogous to β_2-microglobulin's association with MHC class I (17). The 50-kD band is immunologically distinct from 90- and 180-kD forms, as it is the only band precipitated by a rabbit polyclonal antiserum raised against a peptide from the middle region of the GLYCAM-1 protein (6). Thus, there is suggestive evidence that at least two distinct protein cores may be able to serve as the scaffold for specific glycosylation, resulting in a L-selectin ligand. A final possibility is that several independent HEV ligands for L-selectin exist, some of which are tightly associated with the endothelial cell surface, such as the ~90-kD sulfated ligand described by Imai et al. (7) or the PLN addressins described by Streeter et al. (3, 18), and others, like the ~50 kD-ligand described here, that are secreted.

It should be noted that other cell surface adhesion molecules, including the selectins (15, 19, 20) and ICAM-1 (21), have been reported to be shed or secreted. Thus, it is possible that serum levels of the 50-kD molecule may fluctuate during immune and inflammatory responses, leading to alterations in lymphocyte trafficking. It has recently proved possible to obtain sequence information from purified protein of the secreted form of the L-selectin ligand, and this has allowed cloning of the GLYCAM-1 protein (6). The availability of recombinant GLYCAM-I will allow the development of assays to determine the effects of inflammatory processes on circulating levels of this molecule.

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