Demonstration that a Lectin-like Receptor (gp90<sup>MEL</sup>) Directly Mediates Adhesion of Lymphocytes to High Endothelial Venules of Lymph Nodes

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Abstract. Lymphocyte migration from the blood into most secondary lymphoid organs is initiated by a highly selective adhesive interaction with the endothelium of specialized blood vessels known as high endothelial venules (HEV). The propensity of lymphocytes to migrate to particular lymphoid organs is known as lymphocyte homing, and the receptors on lymphocytes that dictate interactions with HEV at particular anatomical sites are designated "homing receptors". Based upon antibody blockade experiments and cell-type distribution studies, a prominent candidate for the peripheral lymph node homing receptor in mouse is the 90-kD cell surface glycoprotein (gp90<sup>MEL</sup>) recognized by the monoclonal antibody MEL-14. Previous work, including sequencing of a cDNA encoding for this molecule, supports the possibility that gp90<sup>MEL</sup> is a calcium-dependent lectin-like receptor. Here, we show that immunoaffinity-purified gp90<sup>MEL</sup> interacts in a sugar-inhibitable manner with sites on peripheral lymph node HEV and prevents attachment of lymphocytes. Lymphocyte attachment to HEV in Peyer's patches, a gut-associated lymphoid organ, is not affected by gp90<sup>MEL</sup>. The results demonstrate that gp90<sup>MEL</sup>, as a lectin-like receptor, directly bridges lymphocytes to the endothelium.

VUPHOCVTES move extensively throughout the body, migrating from the blood into lymphoid organs and back to the blood again via lymphatics. The overall process, known as lymphocyte recirculation, is an essential component of immune surveillance as it brings potentially responsive lymphocytes into contact with antigens sequestered within secondary lymphoid organs (reviewed in Butcher, 1986; Duijvestijn and Hamann, 1989; Ford, 1975; Stoolman, 1989; Woodruff et al., 1987; Yednock and Rosen, 1989). Entry of blood-borne lymphocytes into all secondary lymphoid organs, except the spleen, is initiated by a highly specific cell–cell recognition event between lymphocytes and the endothelial cells of specialized postcapillary venules found within these organs (Gowans, 1959; Gowans and Knight, 1964). These venules are characterized by having a cuboidal or high endothelial lining and are referred to as high endothelial venules (HEV). The adhesive interactions between lymphocytes and HEV have been studied in vivo with short term recirculation studies (reviewed in Butcher et al., 1980) and in vitro with the highly validated Stamper-Woodruff adherence assay (Stamper and Woodruff, 1976), in which viable lymphocytes, overlaid onto cryostat-cut sections of lymphoid organs, selectively adhere to sectioned profiles of HEV. Three distinct adhesive specificities have been shown to exist, one governing lymphocyte attachment to HEV in peripheral lymph nodes (PN), another for gut-associated Peyer's patches (PP), and a recently identified specificity for lung-associated lymph nodes (Butcher et al., 1980; Chin et al., 1983; Chin et al., 1984; Geoffroy et al., 1988). It has been postulated that the endothelial cells of HEV bear cell surface ligands that are distinctive for particular lymphoid organs or anatomically related collections of lymphoid organs (Butcher and Weissman, 1984). The ligands are the heterophilic adhesion partners for a set of complementary lymphocyte receptors. Depending on a lymphocyte's developmental stage, class, subclass, and history of antigenic stimulation, the particular array of receptors expressed by the cell determines its ability to bind to and hence initiate extravasation across HEV in different anatomical sites (for review, see Butcher, 1986). "Homing receptor" is the designation applied to this set of lymphocyte adhesion receptors, reflecting their primary role in controlling the distinctive migratory or "homing" propensities of lymphocyte and lymphoma populations. The activity of these specific adhesion molecules appears to be supplemented by accessory molecules such as LFA-1, which act to strengthen the lymphocyte–HEV interaction without contributing to target organ specificity (Hamann et al., 1988; Pals et al., 1988). The use of this system of highly refined

1. Abbreviations used in this paper: HEV, high endothelial venules; PN, peripheral lymph node; PP, Peyer's patch.

2. Additional lymphocyte-endothelial adhesive specificities may regulate lymphocyte extravasation at sites of chronic inflammation such as inflamed joints (Jalkanen et al., 1986) and skin (Sackstein et al., 1988).
Materials and Methods

Materials

Frozen spleens from ICR and Swiss-Webster mice were obtained from Rockland, Inc. (Gilbertsville, PA) or Bioproducts for Science (Indianapolis, IN). Mesenteric lymph nodes and spleen were also dissected from ICR mice. Paraformaldehyde was purchased from EM Science (Cherry Hill, NJ). PPME was purified from Hansenula holstii core mannan, kindly provided by Dr. M. E. Slodki (U.S. Department of Agriculture, Northern Regional Center, Peoria, IL). The mnn 1 and mnn 2 mannans were generously gifts from Dr. C. E. Batlou (Department of Biochemistry, University of California, Berkeley, CA). The MEL-14 hybridoma was kindly provided by Dr. Eugene Butcher (Department of Pathology, Stanford University).

Frozen mouse spleens or mesenteric lymph nodes. Tissues were minced with a razor blade and then homogenized with a Potter-Elvehjem tissue grinder in 5 ml of lysis buffer (2% Triton-X 100 in Dulbecco's phosphate buffered saline [PBS], containing 1 mM PMSE, 1% aprotinin, and 0.02% NaN3 per gram [wet weight] tissue. Typical preparations used tissues from 200 animals. The lysates were prepared, clarified, and precleared with rat-Septarose 4B as previously described (Lasky et al., 1989). The flow through from the rat–serum column was applied to a 4-ml column of Sepharose 4B conjugated with MEL-14 mAb (1 mg mAb/ml Sepharose) which had been equilibrated in lysis buffer. The column was washed consecutively with 10 ml of lysis buffer followed by 30 ml of column buffer (10 mM CHAPS in PBS containing 1 mM PMSE, 1% aprotinin, and 0.02% NaN3). Antigen was released from the column in 15 ml of a low pH buffer (10 mM CHAPS, 100 mM glycine, 200 mM NaCl, 1% aprotinin, 1 mM PMSE, 0.02% NaN3, pH 3) and collected into 1.5 ml of 1 M Tris-HCl (pH 7.6) to neutralize the PH. After antigen release, the column was washed with 20 mM triethyamine, 200 mM NaCl (pH 11) and reequilibrated with column buffer. The antigen, which was diluted to 50 ml in column buffer, was reapplied to the column, and the elution procedure was repeated. The purified protein was concentrated in two steps by sequential use of Centriprep 30 and Centricon 30 concentrators. Purity was evaluated by SDS-PAGE with visualization by silver staining (Merrill et al., 1981). Based upon comparisons with alpha 1 acid glucoprotein standards, typical yields were 25–35 µg gp90MEL per 200 spleen and 20–30 µg from mesenteric lymph nodes from 200 animals. No difference could be detected in the biological activity of gp90MEL isolated from the two sources. The molecular weight distribution of antigen isolated from spleen was slightly broader than from mesenteric lymph node tissues.

Binding Assay

In a series of experiments, it was determined that the presence of 250 µM CHAPS in the assay medium did not interfere with the viability of lymphocytes (as assessed by Trypan blue exclusion) or their ability to bind to HEV in sections of PN and PP. Purified gp90MEL in column buffer was equilibrated with 500 µM CHAPS in RPMI 1640 (CHAPS/RPMI) by several exchanges in a Centricon 30 concentrator. BSA was added to antigen solutions to a final concentration of 1 mg/ml. Control buffer solutions were prepared in the same way. One part of antigen solution was mixed with one part of RPMI (2 mg/ml BSA) containing potential inhibitors (phosphorylated sugars, polysaccharides, or MEL-14 mAb), and the mixture was incubated for 1 h at 4°C before incubation with the tissue sections (see below). PN and PPs, dissected from ICR mice, were mounted on a cushion of gum tragacanth and snap-frozen in 2-methylbutane cooled in a liquid nitrogen bath. Cryostat sections (10 µm) were picked up on epoxy-coated well slides (Carlson Scientific, Inc., Peotone, IL). The slides were fixed in 1% paraformaldehyde in 0.1 M sodium cacodylate (pH 7.3) for 20 min at 4°C, and subsequently rinsed in PBS. Appropriate dilutions of purified antigen or control buffer were applied to the wells (75 µl per well), and the slides were placed on a metal pan supported by an ice pack and rotated on an orbital shaker for 30 min at 50 rpm. The slides were then washed in a 250 ml bath of PBS (4°C) and then subjected to the Stamper-Woodward in vitro adherence assay (Stamper and Woodruff, 1976) with the modifications described previously (Yednock et al., 1987b). Mesenteric lymph node lymphocytes (107 per ml in a volume of 100 µl per well) were used as test cells. At least four replicate sections were used for the control condition (buffer) and for each experimental condition. Values for lymphocytes bound per unit area HEV were determined by digital morphometry on a Bionscan image analyzer (R & M Biometrics, Nashville, TN). Samples were coded and evaluated blindly. At least 15 segments of HEV were counted for each PN and PP section. In the different experiments, control levels of binding ranged from 24 to 61 lymphocytes per 100 square micrometers HEV for PN and from 30 to 67 lymphocytes per 100 square micrometers HEV for PP.

Results

Our overall approach was based upon one developed by Springer, Shaw, and their respective colleagues ( Dustin et al., 1989; Plunkett et al., 1987; Selvaraj et al., 1987) to study a receptor–ligand interaction that underlies a number

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cytotoxic T-cells to target cells and the attachment of thymocytes to thymic epithelial cells. In these studies, solubilized receptor from T-cells (CD2 glycoprotein) was shown to interact with ligand sites (i.e., LFA-3 glycoprotein) on partner cells and block intercellular adhesion. In reciprocal experiments, isolated LFA-3 was demonstrated to interact with CD2 on intact T-cells and block adhesion. A key to these studies was that the solubility and biological activity of the purified transmembrane glycoproteins could be maintained in a low detergent concentration, which was compatible with the viability of the test cells. Guided by the strategy of these experiments, we wished to determine whether purified gp90MEL could bind to HEV in cryostat-cut sections of PN and prevent lymphocyte attachment in the Stamper-Woodruff in vitro adhesion assay. With mouse spleen and mesenteric lymph nodes as the sources of antigen, purification was achieved by immunoaffinity chromatography on a MEL-14 mAb column (see Materials and Methods). In a typical preparation, the purified material was resolved by SDS-PAGE as a major diffuse band at 90 kD and variable amounts of a minor (<10%) 180-kD band (Fig. 1). The larger component appears to be a dimer of the smaller, since upon electroelution and reelectrophoresis it is substantially converted to the 90-kD form (Brian Williams and S. Rosen, unpublished observations).

We chose CHAPS as the detergent for the purified antigen, because it is relatively non-denaturing and can be readily removed by membrane filtration. We determined (data not shown) that the maximum concentration of CHAPS compatible with the Stamper-Woodruff assay was 250 μM. When the assay was performed at this concentration or lower, the density of lymphocytes bound per unit area of HEV was equivalent to that in detergent-free buffer.

Cryostat-cut sections of PN were pretreated for 30 min with gp90MEL in 250 μM CHAPS or with this concentration of detergent alone. The sections were washed in detergent-free medium and tested for their ability to support lymphocyte attachment to HEV. As shown in Fig. 2, lymphocyte binding to PN HEV was almost completely blocked after exposure of the sections to 1 μg of gp90MEL (~100 nM). In contrast, pre-exposure of lymphocytes to the same antigen concentration followed by washing of the cells resulted in no decrement in lymphocyte attachment to HEV. Thus, the isolated antigen exerted its inhibitory effect on the HEV and not the lymphocyte, consistent with the suspected role of gp90MEL as a lymphocyte-associated adhesive receptor.

Although the antigen preparations were >90% pure (Fig. 1), it was essential to demonstrate that the inhibitory activity of the purified antigen was, in fact, due to gp90MEL and not to a contaminant. To address this issue, PN sections were pretreated with purified gp90MEL in the presence and absence of MEL-14 mAb. After the preincubation step, the sections were washed extensively so that antibody would not be available to react with the test lymphocytes. As shown in Fig. 3, the antibody completely prevented the inhibitory activity of the purified antigen. Thus, the active constituent in the immunoisolated material must be the antigen itself.

As reviewed above, gp90MEL is believed to be involved in organ-specific lymphocyte adhesion to HEV. It was therefore of interest to determine whether isolated gp90MEL would interfere with lymphocyte attachment to PP HEV as it did with attachment to PN. When sections of PP were pretreated with up to 2 μg of purified antigen, there was no inhibition of subsequent lymphocyte attachment (Fig. 4). With the same antigen preparation tested in parallel, 0.1 μg of antigen produced ~50% inhibition of lymphocyte attachment to PN HEV, and
phosphate (B), all previously shown to be relatively inactive in adhesion-blocking activity of gp90 M~L. As shown in Fig. 6, 100 nM of either M6P (A) or F1P (B) largely prevented the blockade of lymphocyte attachment to PN HEV, whereas two control mannans (mnn 1 and mnn 2) were inactive (Fig. 7).

Discussion

Previous work has established that gp90 MEL is essential for PN homing, but heretofore, there has been no direct information bearing on whether this receptor could, itself, bind to HEV-ligands. The earlier results, for example, are compatible with the possibility that gp90 MEL interacts with other lymphocyte surface molecules, either integral or peripheral membrane components, to form a homing receptor complex. Thus an element of the complex, other than gp90 MEL, could form the actual bridge to the endothelium. The demonstration above that isolated gp90 MEL can react with HEV and block the lymphocyte attachment activity of the HEV leads to the unequivocal conclusion that gp90 MEL is capable of a direct binding interaction with HEV ligands. Moreover, this interaction is organ specific as established by the selective inactivation of PN sites as compared to PP sites. Thus, the information required for the selective interaction of lymphocytes with the endothelium of PN HEV resides, at least in part, within the gp90 MEL molecule. Determining whether gp90 MEL, by itself, is capable of mediating the lymphocyte-HEV adhesive interaction will require reconstitution experiments with the purified glycoprotein or alternatively, transfection experiments with its cDNA. Although gp90 MEL may be sufficient for the interaction, it seems probable, in light of results from this system (Hamann et al., 1988; Pals et al., 1988) and from related systems (Goverman et al., 1986; Springer et al., 1987), that general adhesion molecules (e.g., LFA-1) may be necessary to supplement the molecules conferring target organ-specificity (e.g., gp90 MEL).

Our finding that isolated gp90 MEL blocks the adhesive ligands of PN HEV is highly reminiscent of the activity of HEBFLN in the rat system (Chin et al., 1983; Chin et al., 1984; Rasmussen et al., 1985). This factor was originally detected as a soluble factor in lymph fluid. It is apparently derived by a shedding process from integral membrane components of the lymphocyte plasma membrane. In this regard, it is noteworthy that brief incubation of mouse lymphocytes

Figure 3. The effect of MEL-14 mAb on the adhesion-blocking activity of purified gp90 MEL. Sections of peripheral node were preincubated with control buffer, or 2 μg of MEL-14 mAb, 1 μg of gp90 MEL, or 1 μg of gp90 MEL + 2 μg of MEL-14 mAb. Sections were then washed and subjected to the lymphocyte binding assay. Values of lymphocytes bound per unit area HEV were computed as a percent of control binding, referenced to the level of binding in the buffer control. The error bars represent SEMs based upon four independent replicates. The MEL-14 mAb alone treatment was included to establish that the section washing procedure was adequate to reduce the antibody concentration to a level that did not interfere with lymphocyte binding.

at 0.25 μg and above, inhibition was >80% (Fig. 4). Thus, gp90 MEL was at least 20-fold more active on the PN HEV sites than the PP HEV sites. Representative micrographs illustrating the differential effects of antigen on lymphocyte attachment activity of the two kinds of HEV are shown in Fig. 5.

The demonstration above that purified gp90 MEL has biological activity allowed us to test directly the predicted lectin function of the molecule. Our previous work had shown that millimolar levels of mannose-6-phosphate (M6P) or of the structurally related fructose-1-phosphate (FIP) block lymphocyte attachment to PN HEV by reacting with gp90 MEL or a closely associated molecule on the lymphocyte surface (Yednock et al., 1987a,b). If these effects were, in fact, through a direct interaction of the sugars with gp90 MEL, then biological activity of isolated gp90 MEL should be competed by the same sugars. As shown in Fig. 6, 100 nM of either M6P (A) or FIP (B) largely prevented the blockade of PN HEV sites by gp90 MEL. Three control sugars, fructose-6-phosphate (A), galactose-6-phosphate, and glucose-6-phosphate (B), all previously shown to be relatively inactive in blocking lymphocyte attachment to PN HEV, did not antagonize the inhibitory activity of the isolated antigen. Competition experiments were also conducted with the M6P-rich mannan called PPME, a highly specific ligand for the active site of the PN homing receptor (Stoolman et al., 1984; Yednock et al., 1987b). As expected, this polysaccharide prevented the adhesion-blocking activity of gp90 MEL, whereas two control mannans (mnn 1 and mnn 2) were inactive (Fig. 7).
Figure 5. Lymphocyte binding to PN and PP HEV after pretreatment of sections with gp90<sup>MEL</sup>. PN (A and B) and PP (C and D) sections were preincubated with control buffer (A and C) or with 2 μg/section of gp90<sup>MEL</sup> (B and D) for 30 min before the binding assay. Micrographs show representative fields. The dark round cells are the exogenously added lymphocytes which have attached to the sections during the adhesion assay. Dashed lines have been drawn to outline the basement membranes of the HEV. Bar, 100 μm.

at 37°C results in the shedding of substantial amounts of soluble gp90<sup>MEL</sup> with a slightly decreased (4–5 kD) molecular mass (Yednock, T. A., J. S. Geoffroy, and S. D. Rosen, unpublished). Both the soluble and membrane forms of HEBF<sub>LN</sub> are capable of blocking the activity of PN HEV ligands in an organ selective manner (Chin et al., 1984; Rasmussen et al., 1985). The molecular masses of the membrane form (135, 63, and 40 kD) of HEBF<sub>LN</sub> (rat), as defined by immunopre-
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Figure 6. Effects of phosphorylated monosaccharides on the adhesion-blocking activity of gp90MEL. Purified gp90MEL was mixed with control buffer or with sugars (100 nM final concentration) for 60 min before the mixtures were applied (1 μg of gp90MEL per section) to sections of PN. The sections were pretreated for 30 min, and washed before the binding assay. Data are expressed as a percent of control binding (no gp90MEL) as above. Error bars denote SEMs for four replicate sections. (A) Mannose-6-phosphate (Man-6-P) or fructose-6-phosphate (Fruc-6-P). (B) Fructose-1-phosphate (Fruc-1-P), galactose-6-phosphate (Gal-6-P), or glucose-6-phosphate (Glc-6-P).

Figure 7. Effects of polysaccharides on the adhesion-blocking activity of gp90MEL. Purified gp90MEL was incubated with control buffer or 100 μg/ml of PPME, man1 mannan, or man2 mannan for 60 min before applying mixtures (1 μg of gp90MEL per section) to sections of peripheral node. Sections were pretreated for 30 min and then rinsed thoroughly before the binding assay. Data are expressed as a percent of control binding (no gp90MEL). Error bars denote SEMs for four replicate sections.

The demonstration presented herein, that gp90MEL can interact directly with sites on PN HEV focuses attention on the nature of the HEV-associated ligands for this receptor. In view of the mounting evidence that this receptor functions as a lectin, it is strongly suspected that the recognition determinant of its cognate HEV-ligand will be carbohydrate in nature. Evidence is already available that sialic acid residues are essential for the function of the PN HEV ligands, although it is not yet known whether this sugar contributes directly to the actual recognition determinant or instead has a modulatory role (Rosen et al., 1989; Rosen et al., 1985). One prominent candidate for the PN HEV ligand is the 92-kD HEV-associated antigen detected by the MECA-79 antibody (Streeter et al., 1988b; Berg et al., 1989). This antibody selectively stains HEV of PN and blocks lymphocyte attachment. Future work should address whether this antigen or a physically associated molecule expresses a carbohydrate determinant that is recognized by gp90MEL. The possibility exists that the EGF-like and complement regulatory domains of gp90MEL might also contribute to the interaction with ligand molecules. The capability to produce recombinant forms of gp90MEL in substantial quantities should provide tools that expedite the identification and biochemical dissection of the relevant endothelial molecules of PN HEV. Eventually, the analysis will undoubtedly progress to the distinct,
References


