Human Vascular Adhesion Protein 1 (VAP-1) Is a Unique Sialoglycoprotein that Mediates Carbohydrate-dependent Binding of Lymphocytes to Endothelial Cells

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Summary

The regulated interactions of leukocytes with vascular endothelial cells are crucial in controlling leukocyte traffic between blood and tissues. Vascular adhesion protein-1 (VAP-1) is a novel, human endothelial cell molecule that mediates tissue-selective lymphocyte binding. Two species (90 and 170 kD) of VAP-1 exist in lymphoid tissues. Glycosidase digestions revealed that the mature 170-kD form of VAP-1 expressed on the lumenal surfaces of vessels is a heavily sialylated glycoprotein. The sialic acids are indispensable for the function of VAP-1, since the desialylated form of VAP-1 no longer mediates lymphocyte binding. We also show that L-selectin is not required for binding of activated lymphocytes to VAP-1 under conditions of shear stress. The 90-kD form of VAP-1 was only seen in an organ culture model, and may represent a monomeric or proteolytic form of the larger species. These data indicate that L-selectin negative lymphocytes can bind to tonsillar venules via the VAP-1–mediated pathway. Moreover, our findings extend the role of carbohydrate-mediated binding in lymphocyte–endothelial cell interactions beyond the known selectins. In conclusion, VAP-1 naturally exists as a 170-kD sialoglycoprotein that uses sialic acid residues to interact with its counter-receptors on lymphocytes under nonstatic conditions.

Efficient functioning of the immune system requires that leukocytes have the ability to undergo rapid changes from circulating cells to effector cells in lymphatic and other tissues (1, 2). Vascular endothelium is strategically positioned for regulating the extravasation of leukocytes. At the blood–endothelial cell interface, the blood-borne leukocyte makes an initial adhesive contact with the endothelial cell. Through a series of steps this interaction may then ultimately lead to the transmigration of the leukocyte between the endothelial cells into the tissue. At the molecular level, multiple sequential contacts between several endothelial adhesion molecules and their leukocyte ligands are required for a successful emigration cascade (for reviews see references 1–7). Vascular adhesion protein 1 (VAP-1)[1] is one of the endothelial cell molecules that mediates binding of lymphocytes to endothelial cells (8). VAP-1 is preferentially expressed in high endothelial venules (HEV) through which most lymphocyte trafficking takes place. VAP-1 displays a certain tissue specificity, since it is abundantly present in peripheral lymph node HEV, but considerably less is seen in HEV in normal muscosal lymphatic tissues. However, in the setting of chronic inflammation, functional VAP-1 is induced/upregulated in the vessels of tonsil, gut, skin, and synovium (9). mAb 1B2 against VAP-1 inhibits lymphocyte binding to HEV, to inflamed vessels, and to affinity-purified VAP-1. Thus, VAP-1 supports leukocyte–endothelial cell interactions both in normal lymphatic tissues and at sites of inflammation.

For functional analysis of VAP-1, a detailed knowledge of the biochemical structure of this molecule is required. In particular, elucidation of the posttranslational modifications of VAP-1 is important since such determinants are critical in conferring the recognition specificities of several adhesion molecules. For example, mAb MECA-79 peripheral lymph node–specific vascular addressins (PNAd) (10) absolutely require sialic acid decorations and sulphate for their function (11, 12). The 50-kD species of these addressins (glycosylation-dependent cell adhesion molecule-1 [GlyCAM-1]) is expressed as a sialylated, sulphated mucinlike molecule (13) that binds to lymphocyte L-selectin (11). It is striking that the protein core of this molecule with the

[1] *Abbreviations used in this paper:* ddNANA, 2,3,-dehydro, 2-deoxy-N-acetylneuraminic acid; HEV, high endothelial venule; PNAd, mAb MECA-79 defined peripheral lymph node addressin; VAP-1, vascular adhesion protein 1.

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same primary amino acid sequence is also present in lactating mammary epithelial cells and in milk, but the lack of sulphate modifications renders it incapable of binding to L-selectin (14). It has also been shown that removal of sialic acids or N-linked glycans from the specific ligands of P- and E-selectin on neutrophils abolishes binding to their respective selectin (15).

In the present study, we elucidate the structural modifications of VAP-1 that are involved in lymphocyte binding and examine the relationship between the 170- and 90-kD forms of VAP-1. We show that the mature 170-kD form of VAP-1 is a heavily sialylated glycoprotein. The sialic residues are crucial for the adhesive function of VAP-1. We also show that L-selectin is not required for lymphocyte binding to VAP-1. Together, these data indicate that VAP-1 is a novel endothelial sialoglycoprotein that mediates carbohydrate-dependent, selectin-independent lymphocyte binding under shear stress.

Materials and Methods

Cells, Tissues, and Antibodies. Human tonsils were obtained from surgical operations. PBL were isolated from healthy adult volunteers using Ficoll gradient centrifugation. IL-2–dependent T cell lines from peripheral lymph nodes were established and maintained as described (16).

mAbs 1B2, an inhibitory antibody against VAP-1 (8), Leu-8 (from Becton Dickinson & Co., Mountain View, CA), and Dreg-56 (a kind gift from Professor E. Butcher, Stanford University, Stanford, CA) against L-selectin (17, 18), 3G6, a nonbinding negative control against chicken T cells (8), and HB116, a binding control against HLA ABC (19), were used as purified Igs or serum-free hybridoma supernatants (all mouse IgG1). FITC-conjugated sheep anti–mouse Ig was from Sigma Chemical Co. (St. Louis, MO) and rabbit anti–mouse Ig and peroxidase-conjugated sheep anti–mouse Ig were from Dako (Glostrup, Denmark).

Immunoblotting. Proteins from NP-40 lysates of tonsil stromal elements (150 mM NaCl, 10 mM Tris-base, pH 7.2, 1.5 mM $MgCl_2$, 1% NP-40, 1% Aprotinin, and 1 mM PMSF) were used as the source of VAP-1 antigen. The lysate supernatants were depleted of Igs by rocking with protein G–Sepharose beads (Pharmacia LKB Biotechnology, Piscataway, NJ) for 2 h at room temperature and then discarding the beads. Aliquots of the lysate were mixed with equal volumes of Laemmli's sample buffer with or without reduction (5% 2-ME). Samples were then subjected to gentle heating (20 min at 37°C) or boiling (5 min at 100°C) and loaded on 5–12.5% SDS-PAGE gels. The resolved proteins were transferred onto nitrocellulose sheets (Hybond-ECL; Amersham International, Amersham, Bucks, UK) by Hoefer electroblotter. Nitrocellulose strips were then developed using an enhanced chemiluminescence detection kit for Western blotting (Amersham International) according to the manufacturer's recommendations. Briefly, blocking was done with PBS containing 10% nonfat milk powder and 0.3% Tween 20 for 1 h, and the primary antibodies were used at 2 μg/ml.

Metabolic Labeling. Small tonsil cubes were subjected to metabolic labeling in an in vitro organ culture as described (20). Briefly, ~100 mg tissue/well was starved and labeled with 0.5 mCi/ml [^{35}S]methionine/[^{35}S]cysteine (Translabel; ICN Biomedicals, Inc., Costa Mesa, CA) for 4 h. After lysis and four rounds of preclearing, the lysates were immunoprecipitated for 4 h at 4°C with 15 μl aliquots of protein A beads armed with mAb 1B2 or 3G6. The beads were then washed, and the antigens were eluted with Laemmli's sample buffer containing 5% 2-ME, and resolved in 5–12.5% SDS-PAGE. After electrophoresis, the gels were fixed, enhanced, dried, and subjected to autoradiography at −70°C.

Glycosidase Digestions and Lectin Blotting. For glycosidase treatments, tonsil stroma were lysed in different buffers without protease inhibitors: (*a*) 50 mM sodium acetate, pH 5.5, containing 1% NP-40 (acetate lysate); (*b*) PBS containing 1% NP-40 (PBS lysate); and (*c*) 10 mM Tris-base, pH 7.2, 150 mM NaCl, 1.5 mM $MgCl_2$, and 1% NP-40 (Tris lysate). Insoluble material was removed by centrifugation and supernatants were depleted of Igs with protein G beads. The size of VAP-1 antigen was identical in all three lysates when analyzed in parallel lanes in the same gel. For sialidase treatments, 0.2–25 mU *Vibrio cholerae* neuraminidase (Behringwerke AG, Marburg, Germany), 5 mU *Clostridium perfringens*, and 5 mU *Arthrobacter ureafaciens* (Boehringer Mannheim, Mannheim, Germany) were added to 50 μl acetate lysate for 2 h at 37°C and 10 mU Newcastle disease virus neuraminidase (Genzyme Corp., Cambridge, MA) for 1 and 4 h. To digest glycoproteins with *O*-glycanase, 50 μl PBS lysate was first treated with *V. cholerae* sialidase (this enzyme caused a similar change in the size of VAP-1 when used to digest acetate or PBS lysates) and then incubated with 16 mU recombinant endo-α-*N*-acetylgalactosaminidase (*O*-glycanase; Genzyme Corp.) overnight at 37°C. *N*-glycanase treatment was performed overnight at 37°C using Tris-lysate supplemented with 0.5% SDS and 5 mM EDTA or PBS lysate to which 1.2 U peptide/N-glycosidase F (Genzyme Corp.) was added (with similar results). Alternatively, PBS lysate was first subjected to neuraminidase treatment and then to overnight digestion with *N*-glycanase. For sequential digestion of a single sample with both *N*- and *O*-glycanase, 50 μl PBS lysate was first treated overnight with *N*-glycanase, then for 1 h with sialidase, and and finally overnight with *O*-glycanase. For *Pasteurella hemolytica* *O*-sialoglycoprotein endopeptidase (Cedarlane Laboratories Ltd., Hornby, ON, Canada) treatment, 2 or 20 μl enzyme was added to PBS lysate for 1–4 h at 37°C. Almond meal α-fucosidase (Genzyme Corp.) and α1,3/4-L-fucosidase from *Streptomyces sp.* 142 (Takara Biomedicals, Shiga, Japan) were used to digest acetate lysate and acetate lysate preincubated with sialidase (10 μU fucosidase, overnight at 37°C).

To study the specificity of the enzymatic treatments, 5 mU *V. cholerae* sialidase and 10 μl *O*-sialoglycoprotease were preincubated with different amounts of a sialidase inhibitor 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (ddNANA; also known as 2,3-dehydro-2,3-dideoxy-*N*-acetylneruaminic acid; Boehringer Mannheim) for 30 min at 37°C before adding the Ig-depleted tonsil lysate into the reaction mixture. Boiled (5 min at 95°C) sialidase and *O*-sialoglycoprotease, and an irrelevant enzyme, β-galactosidase (from *Diplococcus pneumoniae*, reported to lack all detectable sialidase and protease activity; Boehringer Mannheim; 2.5 mU enzyme for 25 μl lysate, 3 h at 37°C), were used to digest tonsillar lysate.

[^{35}S]Methionine/[^{35}S]cysteine–labeled VAP-1 immunocomplexes were treated with 0.1 U neuraminidase from *V. cholerae* (Calbiochem-Novabiochem Corp., San Diego, CA) at 37°C for 4 h. The enzyme was removed by washing twice in PBS, 4 mU *O*-glycanase was added, and the incubation was continued overnight at 37°C. Samples were then subjected to gel electrophoresis and enhancement as described above.

For lectin blotting, tonsil lysates (Tris-lysate supplemented with 2 mM $CaCl_2$ and 2 mM $MnCl_2$) were depleted of Ig with protein G. Lectins (specificity, as stated by the manufacturers, is

shown in brackets) immobilized on agarose beads (Con A [α-D-mannose, α-D-Glc], *Lens culinaris* [α-D-mannose], *Helix pomatia* [D-GalNAc], *Ulex europaeus* I [L-fucose], wheat germ agglutinin [D-GlcNA$_2$, NeuNAc], *Bandeiraea simplicifolia* II [D-GlcNAc], *Artocarpus integrifolia* [O-linked glycans]) were purchased from Sigma Chemical Co. Succinylated wheat germ agglutinin (D-GlcNAc) and elderberry bark lectin (αNeuNAc [2-6/2-3]D-Gal/GalNAc) were from Vector Laboratories, Inc. (Burlingame, CA). Agarose beads on which human IgG had been immobilized (Sigma Chemical Co.) were used as controls. Beads were washed three times with lysis buffer; 50 μl aliquots of tonsil lysate were then mixed with 50 μl lectin beads and rocked for 3 h at room temperature. Thereafter, the supernatants were subjected to a second round of preclearing with fresh lectin beads. Finally, supernatants were mixed with Laemmli's sample buffer and analyzed by immunoblotting.

Immunostainings and FACS® Analyses. Immunoperoxidase stainings of acetone-fixed frozen sections were done exactly as described earlier (8). Lymphoid cells were stained for immunofluorescence and analyzed using a FACScan® cytometer (Becton Dickinson & Co.) as described earlier (16).

HEV-binding Assay. The in vitro frozen section assay was conducted as described earlier (21). In brief, 8-μm frozen sections from tonsil were preincubated with mAbs (100 μg/ml diluted in RPMI 1640 containing 10% FCS and 10 mM Hepes) for 30 min at 7°C under constant rotation on an orbital shaker (60 rpm). Thereafter, 3 × 10^6 PBL in the same medium were added to tissue sections and incubation continued for another 30 min under rotation. The nonadherent cells were gently tilted off, and the adherent cells were fixed in 1% glutaraldehyde. PBL binding to HEV was counted from coded samples under dark-field illumination. At least 120 HEV per sample were counted. The number of cells adherent to HEV in the presence of the negative control defines 100% adherence. IL-2–driven T cells were preincubated with mAbs Dreg-56 and 3G6, and thereafter their binding to HEV was analyzed similarly.

For evaluating the role of sialic acids of VAP-1 in lymphocyte binding to HEV, the target tissue was pretreated with glycosidases. Neuraminidase treatment was performed by incubating the frozen sections with 5 mU *V. cholerae* neuraminidase (Behringwerke) in 50 mM sodium acetate buffer, pH 5.5, with 100 mM NaCl and 5 mM $CaCl_2$ or in PBS with 2 mM $CaCl_2$ (with similar results) for 30 min in a humidified chamber at 37°C. For O-sialoglycoprotease digestion, the sections were incubated with PBS containing 5 μl enzyme for 30 min at 37°C in a humidified chamber. After digestion, the enzymes were washed away and the sections used for immunohistochemical stainings or binding assays. In HEV assays, the sections were preincubated with mAbs, and finally, PBL were added as in standard HEV assays. Control sections were incubated with the appropriate buffers (lacking only the enzymes) at the first step.

In another set of control HEV-binding assays, the enzymes were (*a*) treated with ddNANA (5 mU sialidase plus 50 μg inhibitor, 10 μl O-sialoglycoprotease plus 50 μg inhibitor); or (*b*) boiled, adjusted to 100 μl with PBS, and used to predigest the target tissue. Other control sections were preincubated with 2.5 mU β-galactosidase.

Results

The 170-kD Form Is the Major Immunoreactive Species of VAP-1 in Tonsil. Immunoblotting of tonsil lysate was used to determine which form of VAP-1 (~90/180 kD) is the mature, immunoreactive molecule. A specific band in the range of 170 kD was seen (Fig. 1 *A*, lane *4*), when the sample was not reduced and only mildly heated. VAP-1 reactivity was destroyed by reduction or boiling (Fig. 1 *A*). Thus, in tonsil, the 1B2 immunoreactive epitope is in the 170-kD form of the molecule when the analysis is done under gentle conditions.

To analyze further the synthesis of VAP-1 in tonsil, slices of human tonsil tissue were metabolically labeled and NP-40 soluble proteins were used for immunoprecipitations. From this material, two mAb 1B2 specific bands were detected (Fig. 1 *B*). The size of the more prominent band at 170 kD was similar to the mAb 1B2 reactive band seen in

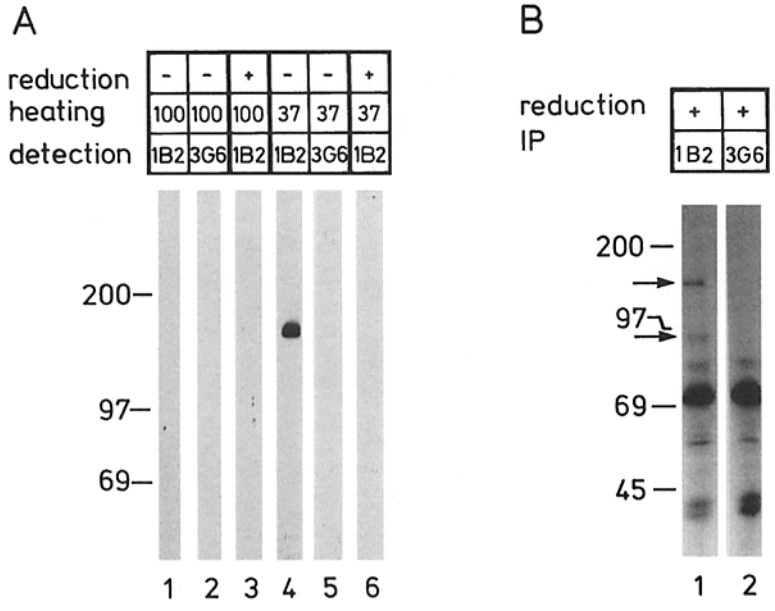

Figure 1. The 170-kD form is the major immunoreactive species of VAP-1. (*A*) Ig-depleted NP-40 lysate of tonsil stroma were analyzed without (*reduction* −) or with (*reduction* +) 2-ME after boiling for 5 min at 100°C (*100*) or heating for 20 min at 37°C (*37*) using SDS-PAGE and immunoblotting. mAb 1B2 only reacted with the 170-kD molecule under nonreducing conditions when boiling was omitted (lane *4*). 3G6 is a negative control mAb. (*B*) in [^{35}S]methionine/[^{35}S]cysteine–labeled tonsil organ culture, mAb 1B2 specifically precipitates a major 170-kD and a less prominent 90-kD form of VAP-1 (*arrows*). (*IP*) Immunoprecipitation. Mol. wt. standards are indicated on the left.

immunoblotting, and represents the intact VAP-1 molecule. The small difference between the apparent size of the 170-kD form and the 180–200-kD form seen earlier by surface iodination (8) is due to the differences in the gel systems (linear vs. gradient) and in the sample treatment. A less abundant 90-kD species was also specifically immunoprecipitated with mAb 1B2 (Fig. 1 *B*). These data indicate that both the 170- and 90-kD forms of VAP-1 are found in human lymphatic tissues.

VAP-1 Is a Sialoglycoprotein in Tonsil Vessels. To assess the potential oligosaccharide modifications of VAP-1, tonsil lysates were subjected to different glycosidase digestions before gel electrophoresis and immunoblotting (Fig. 2 *A*). Neuraminidase (from *V. cholerae*) treatment, which removes sialic acids from oligosaccharide side chains (22), increased the apparent molecular weight of VAP-1 antigen (Fig. 2 *A*, lane *2*). This paradoxical change in the electrophoretic mobility after sialidase treatment is consistent with a reduction in the net charge of the molecule due to the removal of negatively charged sialic acid residues. Sialidases from *C. perfringens* and *A. ureafaciens* (22) produced a similar increase in the apparent molecular weight of VAP-1 (data not shown). A less pronounced upward shift of VAP-1 was detected after digestion with Newcastle disease virus neuraminidase that specifically cleaves α2.3 (and α2.8) linked sialic acids (Fig. 2 *A*, lane *3*). Since the substrate specificity of Newcastle disease virus neuraminidase is less stringent during prolonged incubations (23), it is important to note that 1- and 4-h digestions with this enzyme gave identical results. Together, the results from digestions with four different sialidases indicate that VAP-1 contains abundant sialic acid residues at least some of which are α2,3 linked to underlying oligosaccharides.

VAP-1 was also susceptible to cleavage by the enzyme *O*-sialoglycoprotease (Fig. 2 *A*, lane *4*) that only cleaves glycoproteins carrying sialylated O-linked oligosaccharides (23, 24). After *O*-sialoglycoprotease digestion, VAP-1 was still detectable with mAb 1B2 and the increase in apparent molecular weight was slightly less than that obtained with *V. cholerae* sialidase. Enzymatic digestion of sialidase-treated samples with *O*-glycanase had only a very minor effect on the size of VAP-1 when compared to samples treated with sialidase alone (Fig. 2 *A*, lane *6*). In contrast, neither *N*-glycanase nor fucosidase (with or without prior desialylation) treatments affected the mobility of VAP-1 antigen in these assays. Also, when VAP-1 was digested sequentially with *N*-glycanase, sialidase, and *O*-glycanase, the effect was similar to that of sialidase alone, suggesting that *O*-glycanase did not work under these conditions (Fig. 2 *A*). The enzymatic activities of *O*- and *N*-glycanase were confirmed in

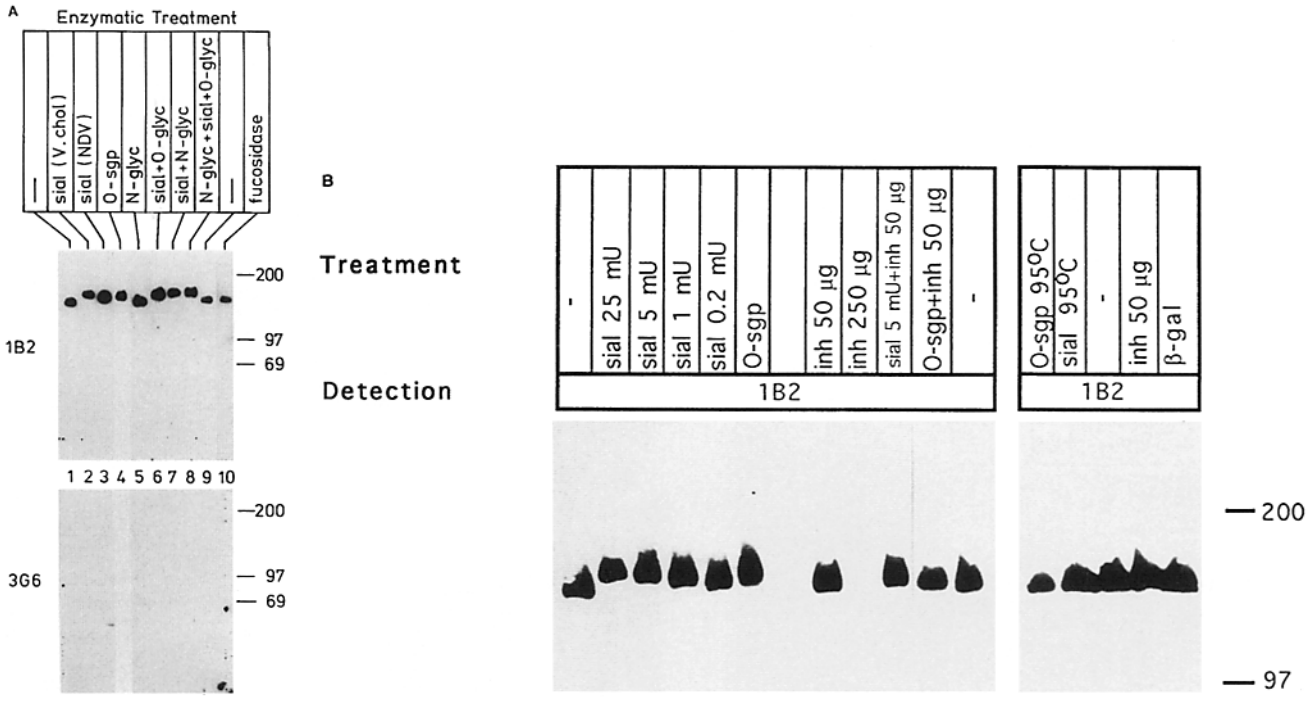

Figure 2. Tonsillar VAP-1 is a sialoglycoprotein. (*A*) Tonsil lysates were subjected to different enzymatic treatments before SDS-PAGE and immunoblotting with mAb 1B2 and negative control (mAb 3G6). Lane *1*: no treatment (acetate lysate); lane *2*: *V. cholerae* neuraminidase; lane *3*: Newcastle disease virus neuraminidase; lane *4*: *O*-sialoglycoprotease; lane *5*: *N*-glycanase; lane *6*: sialidase treatment followed by *O*-glycanase; lane *7*: sialidase treatment followed by *N*-glycanase; lane *8*: *N*-glycanase treatment followed by sialidase digestion and then *O*-glycanase; lane *9*: mock treatment (as in lane 8, but PBS was added instead of enzymes), and lane *10*: fucosidase. (*B*) Specificity of the sialidase and *O*-sialoglycoprotease treatments. Same amount of tonsil lysate was digested with different concentrations of *V. cholerae* sialidase (*sial*, 25–0.2 mU), with sialidase (5 mU) or *O*-sialoglycoprotease (*O-sgp*, 10 µl) preincubated with varying amounts of a sialidase inhibitor ddNANA (*inh*), with the same enzymes alone, with the inhibitor alone, with the same amounts of boiled enzymes (95°C), and with 2.5 mU β-galactosidase (*β-gal*). (−) No pretreatment. For details of enzymatic treatments, see Materials and Methods.

parallel immunoblottings in which the expected reduction in mass was observed with CD44 (data not shown) which is known to be modified with these sugars (25).

The dose dependence of sialidase digestions was shown by adding increasing amounts of sialidase to a constant amount of lysate. 0.2 mU enzyme caused only a minor increase in the apparent molecular weight of VAP-1, whereas increasing amounts of sialidase (up to 25 mU) resulted in a gradual upward shift in the mobility (Fig. 2 *B*). The specificity of sialidase treatment was further corroborated by using a sialidase inhibitor ddNANA. 7 mM of inhibitor effectively reversed the effect of sialidase, but had no effect on the electrophoretic mobility of VAP-1 when used alone (Fig. 2 *B*). Use of higher concentrations of the inhibitor was unfeasible since it denatured VAP-1 protein (due to the low pH), rendering it undetectable in immunoblotting. Finally, boiled sialidase or an irrelevant enzyme β-galactosidase caused no shift in the molecular mass of VAP-1 when compared to nontreated samples (Fig. 2 *B*). Thus, the effect of sialidase on VAP-1 is highly specific.

O-sialoglycoprotease had an unusual effect on the electrophoretic mobility of VAP-1, since only minimal degradation of the VAP-1 protein core rather than complete fragmentation or marked reduction in molecular weight typical of most mucinlike molecules was seen. The results would be compatible with the possibility that the commercial enzyme preparation might be contaminated with small amounts of sialidases. Therefore, O-sialoglycoprotease was preincubated with the sialidase inhibitor ddNANA and used thereafter to digest tonsil lysate. After this pretreatment O-sialoglycoprotease had no effect on the molecular mass of VAP-1 (Fig. 2 *B*). However, the inclusion of the sialidase inhibitor also had variable effects on the ability of O-sialoglycoprotease to degrade known molecules that contain mucinlike domains (CD34 and GlyCAM-1 reactivities were not affected, but CD44 was only partially degraded, data not shown; see also Discussion). As expected, boiled O-sialoglycoprotease had lost all its activity (Fig. 2 *B*).

The composition of the VAP-1 oligosaccharide side chains was further analyzed using lectins. In particular, the possibility of the presence of N-linked glycans in VAP-1 was assessed since the reaction conditions for *N*-glycanase had to be compromised because of the requirements of immunoblotting. To that end, tonsil lysates were precleared with lectins immobilized on agarose beads and the supernatants (containing the glycoproteins that were not recognized by the lectins) were then assayed for VAP-1 reactivity (Fig. 3). Lectins specific for mannose (*lens culinaris* agglutinin [LCA] and Con A) as well as those specific for GlcNAc (*Bandeiraca simplicifolia* [BS-II] and succinylated wheat germ agglutinin [WGA]) and for GlcNAc or NeuNAc (WGA) effectively absorbed all VAP-1 reactivity from tonsil lysates. Since mannose and GlcNAc residues often exist in N-linked glycans in humans, these data suggest that VAP-1 may also contain N-linked oligosaccharides. Consistent with the existence of O-linked oligosaccharides, VAP-1 reactivity also disappeared by preclearing with *Helix pomatia* (HP) and Jacalin *Artocarpus integrifolia* (AI) lectins that

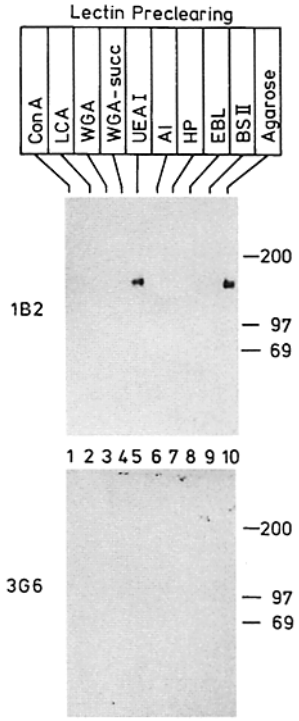

Figure 3. Lectin blotting of VAP-1. Tonsil lysates were precleared with lectins immobilized on agarose beads, the supernatants collected and analyzed by SDS-PAGE and immunoblotting. Lectin preclearing was as follows: lane *1*: Con A; lane *2*: lens culinaris agglutinin; lane *3*: wheat germ agglutinin; lane *4*: succinylated wheat germ agglutinin; lane *5*: ulex europaeus agglutinin I; lane *6*: artocarpus integrifolia; lane *7*: helix pomatia agglutinin; lane *8*: elderberry bark lectin; lane *9*: bandeiraea simplicifolia II; lane *10*: agarose control beads. Strips were probed with mAbs 1B2 and 3G6 (a negative control). For details of preclearing protocol and specificity of lectins, see Materials and Methods.

bind O-linked carbohydrates. Elderberry bark lectin (EBL), which recognizes sialic acids linked to Gal or GalNac, also interacted with VAP-1 in tonsil lysates. In contrast, *Ulex europacus aggiutinin* I (UEAI) (against fucose) or control agarose beads coupled with human Ig did not bind VAP-1 from tonsil lysates. Together, the glycosidase digestions and lectin blottings indicate that VAP-1 contains abundant sialic acid decorations and that mAb 1B2 detects a protein rather than a sugar epitope.

To confirm the immunoblotting results by an independent method, VAP-1 was also affinity isolated from metabolically labeled tonsil fragments and treated with glycosidases. Because of technical difficulties (weak signal), we had to limit the enzymes used in these experiments to sialidase and *O*-glycanase. Again, this treatment increased the size of VAP-1 (Fig. 4). However, only a fraction of the 170-kD VAP-1 displayed altered electrophoretic mobility, indicating that during a 4-h pulse, sialylation of all labeled VAP-1 molecules was not complete. It is interesting to note that the 90-kD form of VAP-1 also contained sialic acids/O-linked carbohydrates, since its size was reduced after neuraminidase and O-glycanase treatment.

Sialic Acid Modifications of VAP-1 Are Required for Lymphocyte Binding. To analyze the role of sialic acid decorations of VAP-1 in lymphocyte binding, an in vitro frozen section assay was employed. Staining of neuraminidase (from *V. cholerae*) or O-sialoglycoprotease treated sections revealed that the 1B2-epitope remained intact after these enzymatic digestions. Enzymatic treatments were effective and specific since all mAb CSLEX-1 (against sialyl Lewis^x) reactivity disappeared after the neuraminidase digestion, and all CD44 reactivity after O-sialoglycoprotease digestion, whereas CSLEX-1 reactivity remained intact after

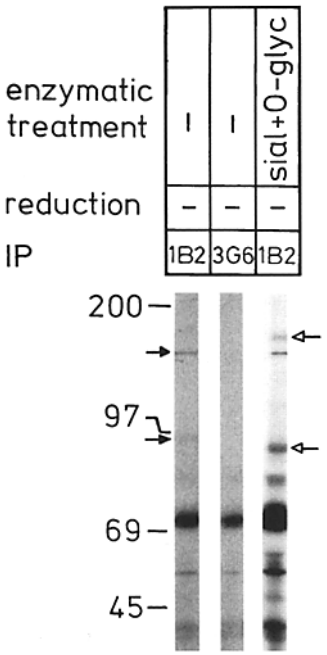

Figure 4. Both the 170- and 90-kD forms of VAP-1 are glycoproteins. VAP-1 isolated from [^{35}S]methionine/[^{35}S]cysteine–labeled tonsil pieces was either treated with sialidase and O-glycanase or left untreated. Black arrows point to the original 170- and 90-kD forms, and white arrows to the altered forms detected after the enzymatic treatment. Note that three times more digested material than untreated material was loaded, and that all the nonspecific bands are also visible in the controls, albeit weakly.

O-sialoglycoprotease and CD44 after sialidase treatment, and buffer controls did not affect the staining pattern with either antibody (Fig. 5). The sialidase inhibitor ddNANA completely reversed the effect of sialidase on CSLEX-1 epitope (data not shown).

For HEV-binding assays, tonsil sections were treated with neuraminidase or acetate buffer only, washed, and incubated with mAb 1B2 or a negative control mAb; finally, lymphocytes were applied onto the sections (Fig. 6 *A*). mAb 1B2 abrogated >50% of lymphocyte binding to tonsil HEV in the buffer-treated control samples. In this context it should be noted that mAb 1B2 stains 50–70% of tonsil HEV, depending on the tissue donor (8). When the target tissue was pretreated with neuraminidase, lymphocyte binding was reduced by ~50% when compared to buffer controls. It is striking that after neuraminidase treatment, mAb 1B2 had no further inhibitory effect on lymphocyte binding to tonsil HEV, i.e., lymphocytes bound equally well to neuraminidase-treated sections incubated with a negative control mAb and mAb 1B2 (Fig. 6 *A*). This binding is therefore totally attributable to the sialidase-insensitive, VAP-1 negative vessels.

The effect of sialidase on abrogating VAP-1–dependent lymphocyte binding to tonsil HEV was specific, since pretreatment of the target sections with boiled enzyme or enzyme preincubated with a sialidase inhibitor completely reversed the observed changes. In the presence of a control mAb, HB116 lymphocytes bound practically as well to HEV in tonsil sections pretreated with boiled sialidase or with sialidase plus sialidase inhibitor as to control sections preincubated with PBS only, whereas ~60% fewer lymphocytes bound to HEV in sialidase-treated sections (Fig. 6 *B*). mAb 1B2 did not inhibit PBL adherence to sialidase-treated HEV, but it did cause ~60% reduction in binding to sections treated with sialidase inhibitor plus sialidase or

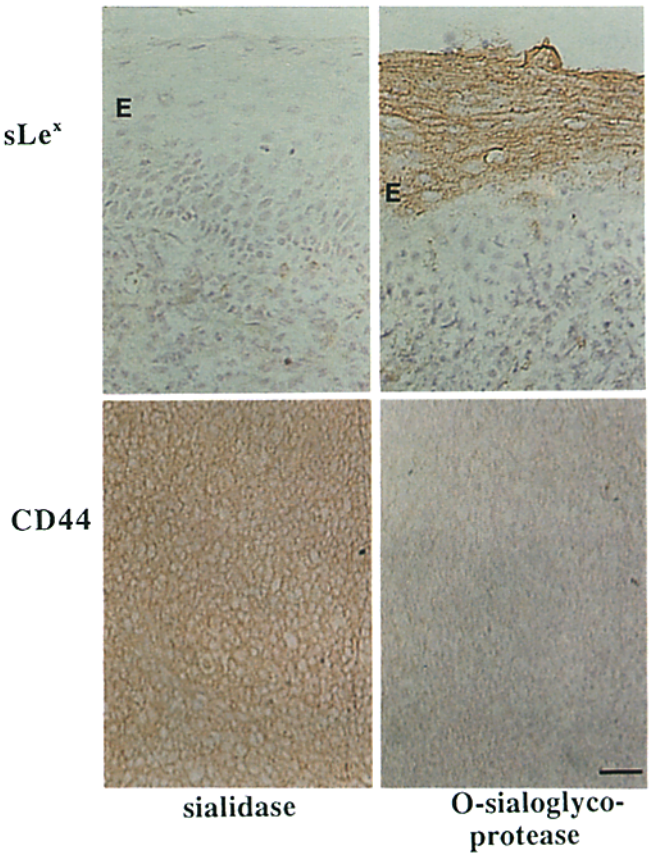

Figure 5. Enzymatic digestion of tonsil sections. Frozen sections were treated with sialidase and O-sialoglycoprotease and stained with CSLEX (against sialyl Lewis x-antigen, sLe^x) and Hermes-1 (against CD44). (*E*) Epithelium. Bar, 20 μm.

with boiled sialidase. β-galactosidase had no effect on PBL binding to tonsil HEV or on the capacity of mAb 1B2 to inhibit the adherence (Fig. 6 *B*). These data also demonstrated that control sections treated with a nonbinding (mAb 3G6) or a binding (anti-HLA ABC mAb HB116, which stains cells extremely brightly) antibody supported lymphocyte adherence to a similar extent.

O-sialoglycoprotease digestion also reduced lymphocyte adherence to tonsil HEV by >50%. mAb 1B2 did not exert any additional inhibitory effect on PBL binding after this enzymatic treatment (Fig. 6 *C*). When O-sialoglycoprotease was preincubated with the sialidase inhibitor, 65% of maximal lymphocyte binding was observed to control mAb-treated HEV (Fig. 6 *D*). Furthermore, mAb 1B2 prevented all of the restored lymphocyte binding, indicating that PBL adherence had again become VAP-1 dependent. The reason for incomplete recovery of lymphocyte recognition of HEV treated with O-sialoglycoprotease plus sialidase inhibitor is probably the fact that mucin-type addressins such as PNAd remain nonfunctional (due to the specific action of the enzyme) and VAP-1 alone cannot support adherence of all tonsil-binding PBL. As expected, boiled O-sialoglycoprotease was completely inactive in these assays (Fig. 6 *D*).

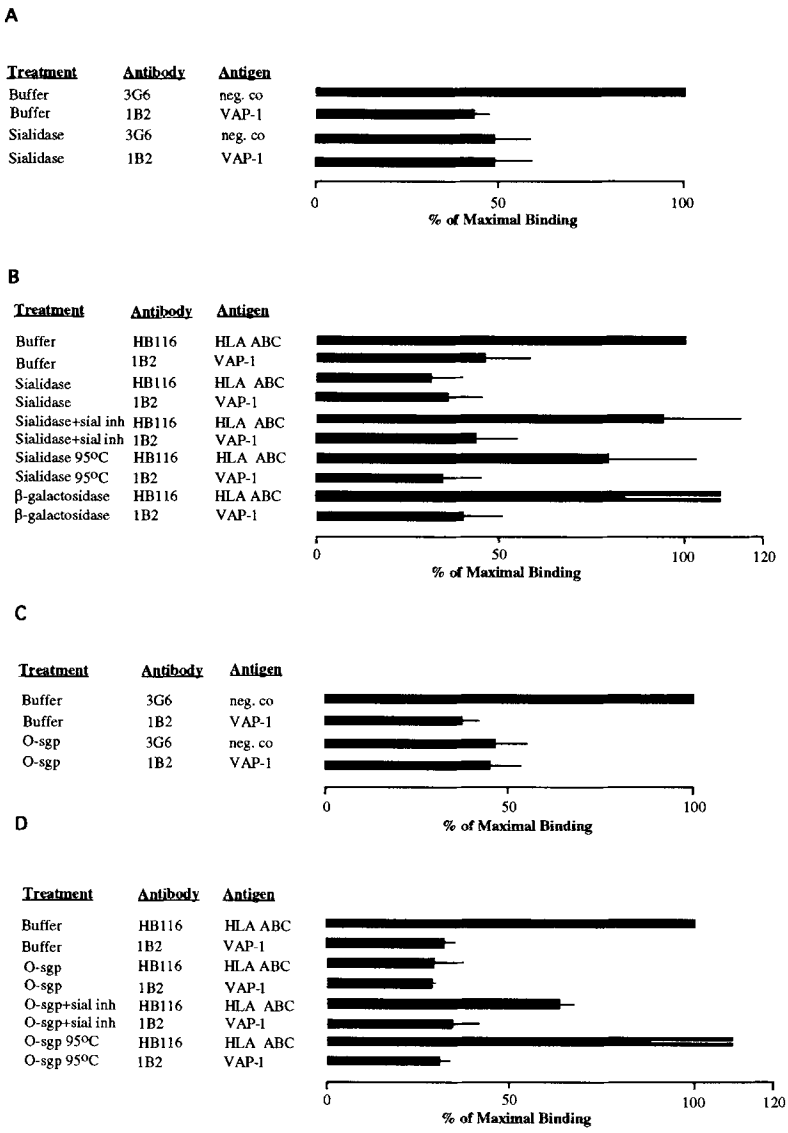

Figure 6. Sialic acids of VAP-1 are a prerequisite for lymphocyte binding. Tonsil sections were treated with enzymes (or with the appropriate buffers alone), washed and preincubated with the indicated mAbs, and used for HEV assays. Results are the mean ± SEM of four (*A* and *C*) or two (*B* and *D*) independent experiments (different tonsils and PBL each time). Results are expressed as the percentage of maximal binding (binding to the buffer-treated sections in the presence of the control mAb [*3G6* or *HB 116*] defines 100% binding). See Materials and Methods for the details of pretreatments of the target tissue. (*sial inh*) ddNANA, (*O-sgp*) O-sialoglycoprotease.

Thus, removal of sialic acids dramatically alters the capacity of tonsil venules to bind lymphocytes. Specifically, the desialylated form of VAP-1 can no longer mediate lymphocyte binding, although it is still recognized by mAb 1B2. These results suggest that both sialic acids and 1B2-epitope contribute to the ligand binding site of VAP-1.

The Lymphocyte Ligand of VAP-1 Is a Non-L-Selectin Molecule. Sialic acid–dependent binding of PNAd to lymphocytes has been shown to be mediated by L-selectin on lymphocytes (26). Therefore, we next wished to determine whether L-selectin is also the counter–receptor of VAP-1. To that end we took advantage of an IL-2–propagated T cell line that retains its capacity to bind to peripheral lymph node type HEV (16). This cell line expresses no L-selectin (Fig. 7 *A*). However, it binds to HEV 1.4 times better than L-selectin positive PBL under nonstatic conditions (Fig. 7 *B*). Pretreatment of the tonsil sections with mAb 1B2 inhibited 47% of HEV binding of this L-selectin negative cell line when compared to the noninhibitory control (Fig. 7 *C*). As expected, a function-blocking anti-L-selectin mAb had no effect on the binding of these cells to tonsil HEV. Similar results were obtained when a binding control mAb against HLA ABC was used (Fig. 7 *D*). The binding of the IL-2–driven T cell line to VAP-1 was also sialic acid dependent (data not shown). These data directly demonstrate that VAP-1 defines a novel non-L-selectin–dependent adhesion pathway that mediates lymphocyte binding to HEV under shear stress.

Discussion

In the present study we have shown that intact VAP-1 is a 170-kD sialoglycoprotein in vivo. The sialic acid residues are indispensable for the function of VAP-1, since desialylated VAP-1 does not support lymphocyte binding. Moreover, VAP-1 does not use L-selectin as its counter–receptor

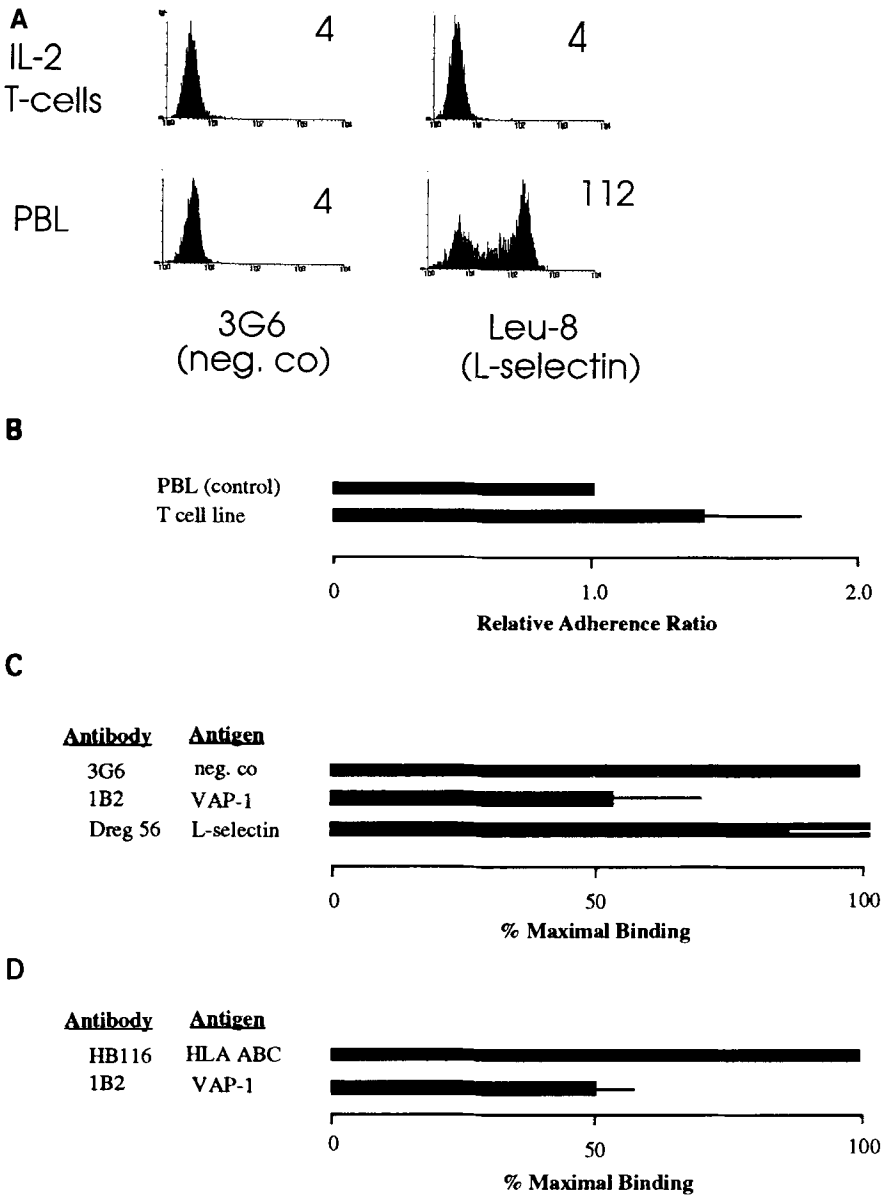

Figure 7. VAP-1 mediates L-selectin independent binding of lymphocytes to HEV. (*A*) The IL-2–driven T cell line was stained with Leu-8 (against L-*selectin*), and with a negative control (*3G6*). Note that these cells completely lack all surface L-selectin. L-selectin expression on freshly isolated PBL is shown for comparison. Mean fluorescence intensities are shown in the upper right corners. (*B*) Relative HEV-binding efficiency of the IL-2–driven T cell line and PBL. (*C*) Tonsil sections were pretreated with mAb 1B2, Dreg-56 (a functional anti-L-selectin mAb), or 3G6, and standard HEV assays with the T cell line were performed. (*D*) Similar frozen section assays as in (*C*) were performed but a binding control antibody (*HB 116* against *HLA ABC*) was used. Results in *B–D* are the mean ± SEM of two independent experiments and are expressed as a percentage of maximal binding (binding in the presence of a control mAb).

on lymphocytes. These findings extend the role of carbohydrate-mediated lymphocyte–endothelial interactions beyond the known selectins. Thus, VAP-1 provides a novel, sialic acid–dependent pathway by which lymphocytes adhere to the endothelium under nonstatic conditions using a non-L-selectin ligand.

The 170-kD form of VAP-1 is a heavily sialylated glycoprotein in tonsil. VAP-1 was susceptible to three different neuraminidases isolated from *V. cholerae*, *C. perfringens*, and *A. ureafaciens* which all have a broad substrate specificity against terminal sialic acids linked by one of the common glycosidic linkages (α2,3, α2,6, or α2,8) to the oligosaccharide core (22). Newcastle disease virus neuraminidase which specifically cleaves sialic acids linked by α2,3 (and α2,8) linkages to the underlying oligosaccharide core (27), also cleaved sialic acids from VAP-1. However, Newcastle disease virus neuraminidase caused less pronounced increase in the apparent molecular weight of VAP-1 than the other neuraminidases. Thus, some, but not all, sialic acids in VAP-1 are α2,3 linked, and the rest are most likely in an α2,6 linkage since the α2,8 linkage is unusual in naturally occurring glycoproteins (28). The effects of sialidase digestions were specific since they were dose dependent, readily reversed by a preincubation of the sialidase with a sialidase inhibitor, and not detected when using boiled enzyme. The sialylation capacity of tonsil high endothelial cells is strikingly different from those of cultured endothelial cells, since VAP-1 found at low levels in certain cultured endothelial cell lines completely lacks all sialic acids and is solely intracytoplasmic (20). Thus, the endothelial cell type-specific differences in posttranslational modification capacity critically affect proper surface location and function of adhesion molecules.

O-sialoglycoprotease, a novel metalloprotease from *P. hemolytica*, cleaves only proteins containing at least two adjacent (or closely spaced) O-linked sialylated oligosaccha-

ride side chains (i.e., mucinlike domains (23, 24)). Susceptibility of VAP-1 to *O*-sialoglycoprotease therefore initially suggested that sialic acids in VAP-1 would be attached to O-linked oligosaccharide chains and that VAP-1 would have certain mucinlike characteristics. The fact that after this endopeptidase treatment mAb 1B2 reactivity remains intact and that the apparent size of VAP-1 is only slightly smaller than with sialidase treatment is, however, atypical of proteins containing mucinlike domains (such as CD44, CD34, and GlyCAM-1) that are completely degraded by *O*-sialoglycoprotease. Nevertheless, this enzyme does not destroy the antibody reactivity of all mucins. CD45, for example, which is known to carry the sialylated O-linked glycans at the very NH_2-terminal end of the molecule, is still detectable after *O*-sialoglycoprotease treatment and its apparent molecular mass undergoes only a slight change (24, and our unpublished observations). After preincubation with ddNANA, which effectively inhibited the function of *V. cholerae* sialidase, *O*-sialoglycoprotease no longer altered the molecular weight of VAP-1. These data favor the idea that the observed effects of *O*-sialoglycoprotease on VAP-1 can be caused by sialidase contaminants. This interpretation is, however, complicated by two observations. (*a*) *O*-sialoglycoprotease did not destroy sialyl-Lewis^x on tissue sections, whereas the CSLEX-1 epitope was readily abolished by sialidase treatment. Therefore, *O*-sialoglycoprotease preparations are unlikely to contain major sialidase contaminants, and yet the effect of *O*-sialoglycoprotease on the electrophoretic mobility of VAP-1 was equal to that of 1 mU sialidase. (*b*) ddNANA is known to also inhibit *O*-sialoglycoprotease. 10 mM of inhibitor has little effect on the susceptibility of glycoprotein A (a model substrate for *O*-sialoglycoprotease) to this enzyme, but 20 mM causes 100% inhibition (Professor Alan Mellors, personal communication); 2 mM sialidase inhibitor already affected the sensitivity of CD44 to *O*-sialoglycoprotease. The relative sensitivity of different target molecules to sialidases and *O*-sialoglycoprotease is variable, making generalization of the results from sialidase inhibitor studies impossible. Thus, we cannot yet conclusively determine the cause of the observed mobility shifts of VAP-1 after O-sialoglycoprotease treatment. It is possible that: (*a*) VAP-1 contains only one or a few clusters of sialylated O-linked oligosaccharides that occur terminally in the protein core; or (*b*) all the effects of *O*-sialoglycoprotease on VAP-1 are merely due to the low-level sialidase contamination in the enzyme preparations.

Enzymatic incubation with *N*-glycanase resulted in no change in size of VAP-1. However, since the detection method was based on immunoblotting, we could not use ideal digestion conditions for *N*-glycanase. Therefore, several lectins recognizing sugars common in N-linked structures were analyzed for their ability to bind VAP-1. It was evident that all lectins that bound mannose or GlcNAc depleted VAP-1 from tonsil lysates, suggesting that VAP-1 also contains N-linked glycans. However, we cannot formally exclude the possibility that VAP-1 could be complexed to some other N-linked protein in tonsil lysate that would then indirectly result in the disappearance of VAP-1 after lectin preclearing. Moreover, it is also known that mannose and GlcNAc can exist in O-linked oligosaccharides, and that mannose is the linkage sugar of glycophosphoinositol lipid anchors. Taken together, the biochemical analyses and lectin blotting showed that VAP-1 is a large glycoprotein carrying abundant sialic acid decorations on O-linked oligosaccharides and, likely, also N-linked oligosaccharide side chains.

In the present study, the 90-kD form of VAP-1 was detected by metabolic labeling in a tonsil organ culture model, and in previous studies we have also seen it in surface iodination of tonsil tissue fragments (8). This species of VAP-1 also appears to be sialylated/*O*-glycosylated, since its molecular mass is reduced after sequential neuraminidase/*O*-glycanase treatments. The 90-kD form was not seen in immunoblotting. Thus, it may represent a proteolytic degradation product of the larger molecule that loses the mAb 1B2 epitope. Alternatively, if the larger form of VAP-1 is a dimer that is only partially reduced by 2-ME, the 90-kD could then be the monomeric form which lacks the conformation-sensitive mAb 1B2 epitope.

The function of VAP-1 is critically dependent on proper sialylation. In HEV assays removal of sialic acids from VAP-1 completely abolished its capacity to mediate lymphocyte binding to tonsil vessels. In mouse, sialidase treatment has been shown to reduce lymphocyte binding to peripheral lymph nodes by 90%, to mesenterial nodes by 50%, and to have no effect on binding to mucosal HEV (29). Our results extend these earlier observations on the critical importance of sialic acids in lymphocyte binding to a human system. Tonsil is structurally similar to mesenteric nodes in the sense that it is a mixed type lymphoid organ containing both peripheral lymph node type HEV and mucosal type HEV. Therefore, the ~50% inhibition in lymphocyte binding after sialidase treatment alone is consistent with the abrogation of cell adherence to desialylated peripheral lymph node type HEV but not to those of mucosal type. Since VAP-1 is expressed in peripheral type HEV but not significantly in normal mucosal HEV (8), it is obvious that mAb 1B2 against this molecule only inhibits lymphocyte binding to the VAP-1 positive subpopulation of tonsil HEV (i.e., gives a 50–70% inhibition). We show that both sialidase and *O*-sialoglycoprotease treatment completely destroy the adhesive pathway that can be inhibited by mAb 1B2. In other words, after desialylation no functionally intact VAP-1 remains in the tissue sections that could support lymphocyte adherence in the presence of a control mAb but not in the presence of mAb 1B2. The effects of sialidase were specific, since pretreatment of the target tissue with boiled sialidase, sialidase preincubated with a sialidase inhibitor, or a nonsialic acid modifying enzyme β-galactosidase did not destroy the VAP-1–dependent adhesion. Hence, VAP-1 (together with PNAd) is a principal neuraminidase-sensitive molecule that mediates lymphocyte binding to peripheral lymph node type venules under nonstatic conditions. Although we currently cannot unambiguously define the type of sialylated oligosaccharide in VAP-1, there are

antecedents for both O-linked (endothelial sialomucins GlyCAM-1, MAdCAM-1, and PSGL-1) and N-linked (ESL-1) α2,3-sialic acid–dependent adhesion during the initial step of leukocyte–endothelial cell recognition (30).

VAP-1 was initially judged to be different from all other known endothelial adhesion molecules mediating lymphocyte binding based on its expression pattern, molecular weight, function, and a unique NH_2-terminal sequence. Although the reported NH_2-terminal sequence (8) comes from a coprecipitating mouse cyclophilin-C–associated protein via a specific mimotypic interaction (the mouse protein binds to mAb 1B2 during antibody synthesis in hybridoma cells; Salmi M., D. Smith, P. Bono, and S. Jalkanen, manuscript submitted for publication), all other previous and present data unambiguously show that VAP-1 is a unique human endothelial molecule. Specifically, PNAd and VAP-1 are differentially expressed in tonsil HEV and are differentially sensitive to O-sialoglycoprotease digestion. In particular, we have ruled out the identity of VAP-1 with the 170–180-kD species of human PNAd (26) by enzyme immunoassay (EIA) which shows that the molecules detected by mAbs 1B2 and MECA-79 are distinct (8).

L-selectin negative IL-2–activated T cells can adhere to peripheral lymph node type HEV using VAP-1 under shear stress. In more general terms, when these experiments were performed, VAP-1 presented the first molecularly defined pathway that bypasses the selectin–carbohydrate interaction in the first step of lymphocyte–endothelial cell interactions. [During the revision of this paper, a couple of reports on an α4-dependent rolling of leukocytes on mucosa-associated endothelium have been published (31, 32). However, VAP-1 is not an α4-ligand (our unpublished results)]. Two alternative but not mutually exclusive possibilities exist to integrate VAP-1 into the multistep paradigm of lymphocyte–endothelial cell binding. (*a*) Certain lymphocytes can use VAP-1 and others PNAd to make the first contacts with the vascular lining in peripheral lymph nodes. (*b*) PNAd and VAP-1 can act sequentially during the cascade. In this case, initial contacts can be accounted for by lymphocyte L-selectin binding to PNAd which then could lead to progression to the stage where non-L-selectin ligands make additional contacts to endothelial VAP-1. Clearly, at least activated L-selectin negative cells can circumvent the PNAd-mediated phase of adhesion and already initially rely on VAP-1. In either case, VAP-1 increases the possibilities for regulating the diversity and specificity of lymphocyte–endothelial cell interactions.

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