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SPECIFIC EXPRESSION OF A COMPLEX SIALYL LEWIS X ANTIGEN ON HIGH ENDOTHELIAL VENULES OF HUMAN LYMPH NODES: Possible Candidate for L-Selectin Ligand

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SUMMARY: L-selectin is a cell adhesion molecule that mediates homing of lymphocytes to the peripheral lymph nodes and is speculated to bind to the carbohydrate determinant which is specifically expressed by the endothelial cells of high endothelial venules (HEVs) in the peripheral lymph nodes. One of the murine monoclonal antibodies (2H5, IgM, κ), which was raised against sialyl Le^x-active glycolipids which have long and complicated carbohydrate structures, was found to react strongly to the HEV endothelial cells of the human peripheral lymph nodes, while the typical anti-sialyl Le^x antibodies SNH-3, FH-6 and CSLEX-1 failed to detect the antigen on HEV under the same condition. This new antibody was reactive to essentially all endothelial cells of HEV in the peripheral lymph nodes, and moderately to some endothelial cells in Payer's patches and appendices, but never reacted with the endothelial cells of post capillary venules in the spleen, thymus, or other organs. The 2H5 antibody detected three major glycoproteins of 90, 110 and 250 kDa, the most abundant molecular species being 110 kDa, in the stroma of human lymph nodes. In Stamper-Woodruff assays employing cryostat sections of human lymph nodes, the 2H5 antibody significantly inhibited the adhesion of peripheral lymphocytes to the endothelial cells of HEV. These results indicate that the carbohydrate antigens defined by the 2H5 antibody, most probably sialyl Le^x determinants having complex carbohydrate structures, serve as the ligand for L-selectin on HEV endothelial cells. © 1993 Academic Press, Inc.

L-selectin (LECAM-1, LAM-1, Mel-14), a member of the selectin family, is thought to mediate homing of lymphocytes to the peripheral lymph nodes in both human and murine systems (1-3). The endothelial cells of high endothelial venules (HEVs) in lymph nodes are thought to express a carbohydrate ligand which binds to the L-selectin.

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Abbreviations used: HEV, high endothelial venule; Le^x, Lewis X; Le^a, Lewis A.

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The ligand carbohydrates for members of the selectin family are considered to be very similar, if not the same, because of high homology in their carbohydrate recognition domains. Ever since the ligands for E-selectin were first shown to be sialyl Le^x and sialyl Le^a (4-8), these carbohydrate ligands have been tested for the possibility of serving also as ligands for P- and L-selectins, mainly by *in vitro* experiments employing the pure carbohydrate ligand and the recombinant selectin molecules or cells transfected with selectin cDNAs (9-11). It was clearly shown from these experiments that P- and L-selectins are also capable of binding to the sialyl Le^x and sialyl Le^a determinants.

However, a problem concerning L-selectin ligands has been that the human HEV endothelial cells do not appear to appreciably express the sialyl Le^x antigen, if typical anti-sialyl Le^x antibodies, such as SNH-3, FH-6 and CSLEX-1, are used for detection. Both SNH-3 and FH-6 failed to stain the endothelial cells of HEV in human lymph nodes in our preliminary experiments. Some investigators reported that CSLEX-1 also failed to detect reactive antigens in human HEV (11,12), while others could detect the antigen using a higher concentration of the antibody (13). With regard to the other ligand candidate, sialyl Le^a, some investigators reported its presence on HEV (13), while others could not (10). These findings led some invetigators to propose other ligand candidates, like sulfated Le^x and other sulfated carbohydrate determinants, as specific ligands for L-selectin (13,14).

All these preceding observations, which partly conflict, imply that the situation is rather complicated in the case of the L-selectin ligand expressed on HEV, when compared to the E-selectin ligand carried by granulocytes and monocytes which is readily detectable with typical anti-sialyl Le^x antibodies. It is well known that the Le^x or sialyl Le^x determinant is carried by multiple molecular species of glycolipids and glycoproteins, and that their antigenicity is considerably affected by the linkage, length, branching or other modification in the core carbohydrate structure which carries the antigenic determinant (15-17). Kojima et al. more recently proposed that these factors may also affect binding specificities of the carbohydrate determinants towards selectins (18). In this study, we generated a series of monoclonal antibodies against long-chain sialyl Le^x glycolipids having complicated carbohydrate structures, and tested the reactivity of these antibodies against the HEV endothelial cells of human peripheral lymph nodes.

Materials and Methods

Preparation of sialyl Le^x-active complex glycolipids: Human colon carcinoma cells LS174T were cultured in Dulbecco's modified MEM supplemented with 10% fetal calf serum. The tumors xenografted to nude mice served as starting

materials for the preparation of glycolipids from these cells (19). Briefly, total lipids were extracted from these tissues with isopropanol/ hexane/ water (55:20:25, v/v/v). The glycolipids in the upper layer fraction of the Folch's partition were subjected to DEAE-Sephadex A-25 (Pharmacia, Uppsala, Sweden) column chromatography. The neutral glycolipids were eluted with chloroform/ methanol/ water (30:60:8, v/v/v, fraction A), and gangliosides were then eluted with chloroform/ methanol/ 0.8N sodium acetate (30:60:8, v/v/v, fraction B). The gangliosides in the fraction B were further separated by TLC using the solvent system; chloroform/ methanol/ 2% $CaCl_2$ (60:35:8, v/v/v), and immunostained with the antibody FH6. TLC-immunostaining was performed using an HPTLC plate (Si-HPF plate 7011-3, J. I. Baker Chemical Co., Phillipsburg, NJ) and ^{125}I-protein A (Dupont, Boston, MA) as described (20). The several glycolipids having more than 10 sugar residues that were cross-reactive to the FH-6 antibody were pooled and used for the immunization of BALB/c mice for the generation of monoclonal antibodies. These glycolipids are the sialyl Le^x-active glycolipids which contain branched core structures as ascertained by the reactivity with an anti-I antibody (IgM-Ma), and their carbohydrate structures are much longer and more complicated than the well-known short-chain sialyl Le^x active glycolipid, which has only 6 sugar residues and has been widely used for *in vitro* experiments involving selectins in preceding papers.

Generation of the 2H5 monoclonal antibody: Hybridoma cells which secrete monoclonal antibodies directed to these sialyl Le^x-active complex glycolipids were generated according to the method described by Köhler and Milstein (21), and subsequently modified for anti-carbohydrate antibodies (20). Briefly, the sialyl Le^x-active glycolipids prepared as described above were absorbed to *Salmonella minnesota R595 strain* and used for repeated intraperitoneal immunizations of BALB/c mice on day 0 (7μg glycolipid), day 7 (15μg), day 14 (23μg) and day 28 (23 μg). Three days after the final immunization, the spleen cells were harvested and fused with mouse myeloma P3/X63-Ag8U1 (P3U1). The sialyl Le^x-active complex glycolipids were used as the antigen in solid phase enzymo-immunoassays of hybridoma culture supernatants for the cloning procedures. Six positive hybridoma clones, 2H5, 4F9, 5A12, 9B1 and 10A4 (all IgM) were obtained, with only the 2H5 antibody showing a strong reactivity to HEV.

Immunohistochemical methods: Monoclonal antibodies SNH-3 (IgM) and FH-6 (IgM) were supplied by Dr. Sen-itiroh Hakomori, Biomembrane Institute, Seattle, WA) and were purified from ascitic fluids as described previously (2,21). The 2D3 antibody (IgM) specific to the sialyl Le^a antigen was prepared in our laboratory as described previously (7,22). Human peripheral lymph nodes and other tissue samples were obtained at therapeutic operation or autopsy at Aichi Cancer Center Hospital. Consecutive sections of 4 μm thickness were prepared from formalin-fixed and paraffin-embedded tissue samples. The avidin-biotin complex technique for the immunohistochemical study was performed as described in the instructions for the kits (Vectastain) provided by Vector Inc. (Burlingame, CA)(23).

Assessment of specificity of monoclonal antibodies against various glycolipids: ELISA was performed using glycolipid antigens which were immobilized at the bottom of 96-well culture plates, by a standard method described previously (19). Peroxidase-conjugated goat anti-mouse IgM (μ-chain specific) was obtained from Cappel Inc., (Malvern, PA). The pure synthetic sialyl Le^x glycolipid used as the antigen in ELISA had the structure, NeuAcα2→3Galβ1→4(Fucα1→3)GlcNAcβ1→3Galβ1→4Glcβ1→1Cer (16). The pure sialyl Le^a glycolipid having the structure, NeuAcα2→3Galβ1→3(Fucα1→4)GlcNAcβ1→3Galβ1→4Glcβ1→1Cer was prepared from human cultured colon cancer cells Colo201 as described previously (24).

Western blotting analysis of lymph node stromal glycoproteins: SDS-polyacrylamide gel electrophoresis of stromal proteins of human peripheral lymphocytes was performed using 10% resolving gels and 3% stacking gels according to the procedure of Laemmli (25) under reducing conditions. Proteins were transferred to a nitrocellulose membrane (Schleicher & Schnell, FRG) for immunoblotting (26). The membrane was first incubated with murine monoclonal antibodies, followed by the addition of peroxidase-conjugated goat anti-murine IgM

(μ-chain specific). The membrane was developed using 3,3'-diamino-benzidine.

Cell adhesion experiments using cryostat sections of human lymph nodes: Cryostat sections of 10 μm thickness were prepared from the human lymph nodes freshly obtained at therapeutic surgical operation at Aichi Cancer Center Hospital and were fixed with 1.0% paraformaldehyde at 4 °C for 20 min. The sections were pretreated with the 2H5 antibody solution or the class-matched control antibody (MK1-8) solution (100 μg/ml) for 30 min at room temperature. To this, human lymphocytes prepared from peripheral blood of healthy individuals by Ficoll/Hypaque method as described below, were over-laid at the concentration of $5x10^7$/ml, and incubated for 30 min at 4°C with continuous rotation (60 rpm)(27). The lymphocytes were freed of thrombocytes by repeated centrifugation at 100xg, and treated with neuraminidase from *Arthrobacter ureafaciens* (0.05 unit/ml, Nakarai Co. Ltd., Kyoto, Japan) according to the method of Stoolman et al. (28). The sections were then fixed with 3% glutaraldehyde at 4 °C for 20 min, followed by staining with 0.5% Toluidine blue in 20% ethanol. The number of lymphocytes attached to endothelial cells of HEV was counted under a microscope, and was expressed as the number of attached lymphocytes per unit area of the HEV.

Results

Specific expression of the complex sialyl Le^x-like antigen defined by the 2H5 antibody in endothelial cells of HEV in human peripheral lymph nodes: By immunohistochemical examinations of the human lymph nodes including cervical, lung hilar, parastomach and mesenteric lymph nodes, essentially all endothelial cells in HEV were strongly stained by the 2H5 antibody, with a typical example shown in panel *A* of Fig. 1. Staining of the HEV endothelial cells with 2H5 was completely abolished by pretreatment of the sections with neuraminidase, indicating that the detected carbohydrate antigen carried a sialic acid terminus. The 2H5 antibody also stained granulocytes and monocyte-like cells present in the sections.

On the other hand, the hitherto known typical anti-sialyl Le^x antibodies SNH-3 and FH-6 did not stain any of the endothelial cells of HEV in peripheral lymph nodes, with typical examples shown in panels *B* and *C* of Fig. 1. The only cells positively stained with these antibodies were granulocytes and monocyte-like cells. The CSLEX-1 antibody did not stain HEV when used at concentrations up to 10 μg/ml, and only a faint staining of HEV was observed at concentrations above 20 μg/ml. In contrast, a strong staining was readily obtained with 2H5 at concentrations of 0.1 μg/ml or even below. No positive staining was observed with the anti-sialyl Le^a antibody 2D3 in any of the lymph nodes studied (Panel *D*, Fig. 1).

Staining patterns of the endothelial cells in post-capillary venules of various lymphoid organs with these antibodies are summarized in Table 1. The 2H5 antibody stained the endothelial cells of most HEV in the tonsils strongly (Fig. 1, panel *E*), and the endothelial cells of some HEVs in the Payer's patches (Fig. 1, panel *F*) and appendices (photograph not shown) moderately. These endothelial cells were not stained with the antibodies SNH-3, FH-6 or

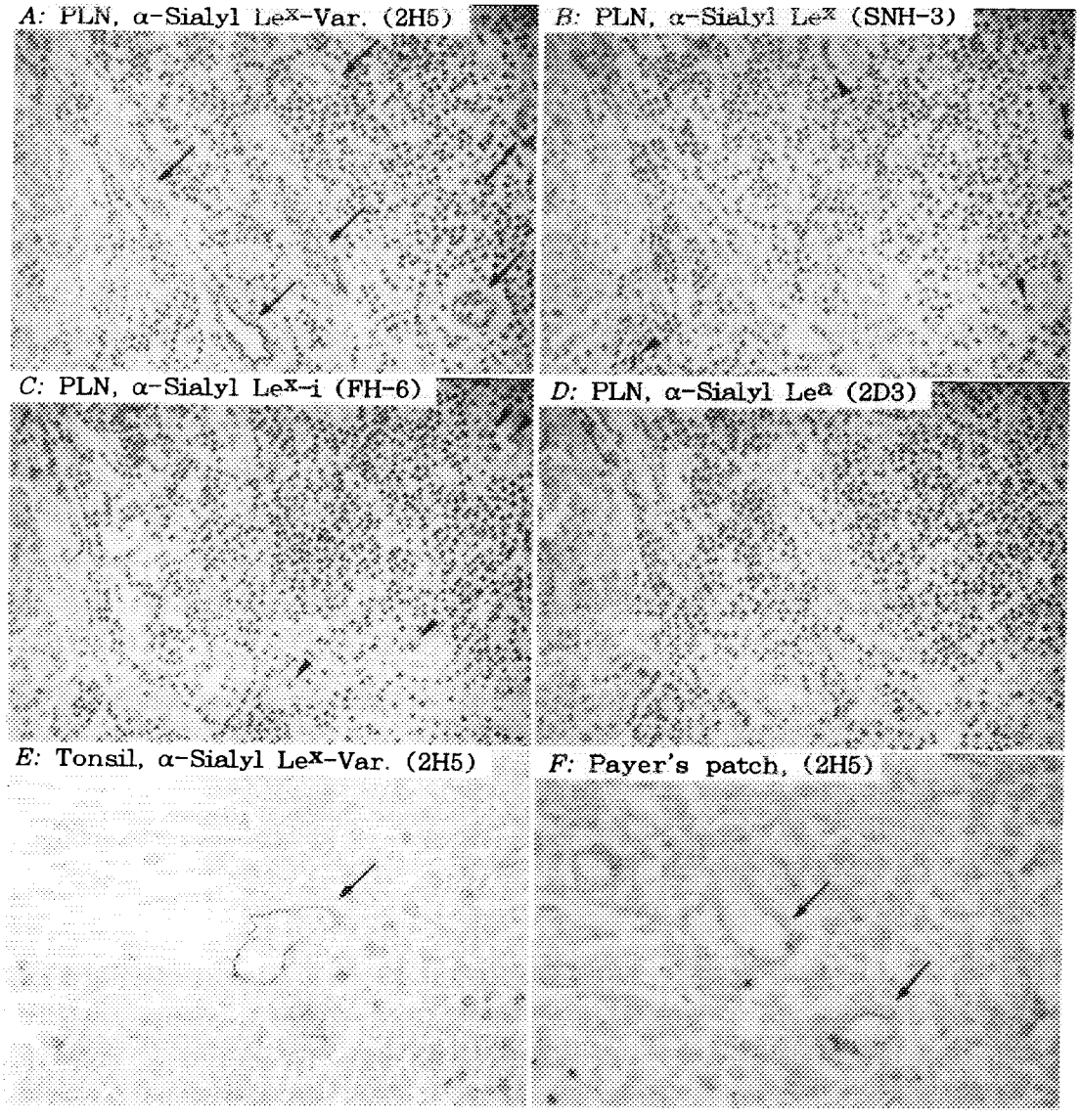

Fig. 1. Immunohistochemical detection of the 2H5-defined carbohydrate antigen in HEV of lymphoid organs. Panels *A–D*; immunohistochemical detection of sialyl Le^x-like antigen defined by the 2H5 (panel *A*), SNH-3 (panel *B*), and FH-6 antibodies (panel *C*), or detection of sialyl Le^a antigen defined by the 2D3 antibody (panel *D*), in a section of a human peripheral lymph node. Arrows indicate HEV; arrowhead, granulocytes. Panels *E* and *F*; Immunohistochemical detection of sialyl Le^x antigen defined by the antibody in a section of tonsil (panel *E*) or Payer's patch (panel *F*).

2D3. The antibody CSLEX-1 failed to stain HEVs in the Payer's patches or appendices even at concentrations above 20 μg/ml. The endothelial cells in the spleen and thymus were not stained with the 2H5 antibody, or the SNH-3, FH-6 and 2D3 antibodies (Table 1).

Table 1. Expression of sialyl Le^x, sialyl Le^a and related antigens in endothelial cells of venules in various organs

Organs		Staining with the antibody[a] (No. of Positive Cases / No. of Tested Cases)					
		2H5	SNH-3	FH-6	CSLEX-1 1μg/ml	CSLEX-1 20μg/ml	2D3
Lymphoid Tissues	Lymph Node	7/7	0/7	0/7	0/5	2/3	0/5
	Tonsil	4/4	0/4	0/4	0/4	0/4	0/4
	Payer's Patch	3/3	0/3	0/3	0/3	0/3	0/3
	Appendix	2/2	0/2	0/2	0/2	0/2	0/2
	Spleen	0/6	0/6	0/6	0/5	n.t.[b]	0/5
	Thymus	0/1	0/1	0/1	n.t.	n.t.	0/1
Other Organs	Esophagus	0/1	0/1	0/1	n.t.	n.t.	0/1
	Stomach	0/3	0/3	0/3	0/1	0/1	0/3
	Intestine	0/6	0/6	0/6	0/4	0/4	0/5
	Liver	0/6[c]	0/6	0/6	0/5	0/5	0/6
	Pancreas	0/3	0/3	0/3	0/1	0/1	0/2
	Lung	0/6[d]	0/6	0/6	0/5	0/5	0/5
	Kidney	0/2[e]	0/2	0/2	0/2	0/1	0/1
	Skin	0/5	0/5	0/5	0/5	0/5	0/5

a The results when used at the concentration of 1 μg/ml are shown for the 2H5, SNH-3, FH-6 and 2D3 antibodies. For the CSLEX-1 antibody, the results obtained at the concentrations of both 1 μg/ml and 20 μg/ml are shown.

b n.t., Not tested.

c The liver parenchymal cells and Kupffer cells sometimes showed significant staining, but essentially no staining was observed on the endothelial cells of blood vessels including sinusoids.

d The bronchial gland cells sometimes showed positive staining, but no staining was observed on the endothelial cells of blood vessels.

e The epithelial cells of the proximal convoluted tubules showed positive staining, but essentially no staining was observed on the endothelial cells of blood vessels including the glomerulus.

Table 1 also summarizes reactivity of the 2H5 and other antibodies with endothelial cells of the various organs other than lymphoid organs, such as the liver, stomach, large and small intestine, kidney, lung and skin. None of the endothelial cells of small vessels in these organs were stained with any of these antibodies.

Specificity of the 2H5 antibody against glycolipid and glycoprotein sialyl Le^x antigens: The 2H5 antibody was strongly reactive to the sialyl Le^x-active complex glycolipids which were used to raise this antibody. The antibody was also significantly cross-reactive to the short-chain sialyl Le^x glycolipid, and was not reactive to sialyl Le^a glycolipid as shown in Fig. 2, panels *A* and *B*. Reactivity of 2H5 against the short chain sialyl Le^x glycolipid was essentially the same as that of the antibody SNH-3. On the other hand, the typical anti-sialyl Le^a antibody 2D3, which served as a control antibody in these experiments, was reactive only to sialyl Le^a and was not reactive to sialyl Le^x Fig. 2).

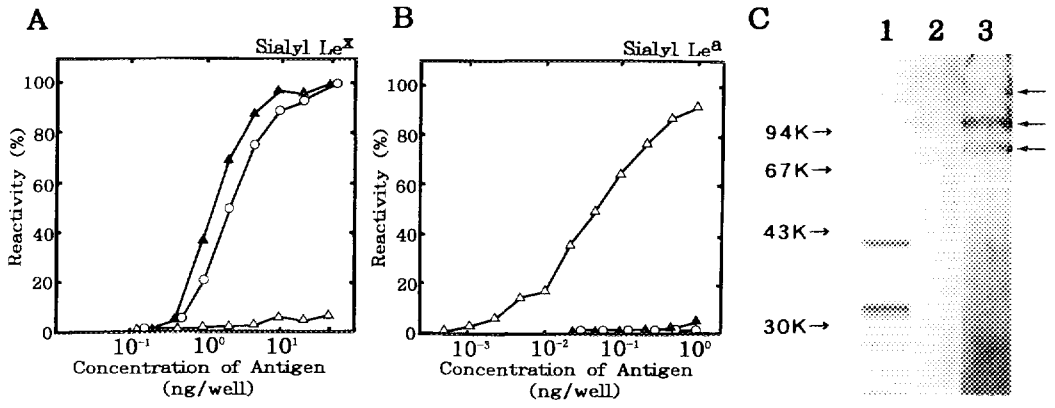

Fig. 2. Reactivity of the 2H5 antibody against glycolipids and glycoproteins. Panels *A* and *B*; Comparison of the reactivity of the 2H5 antibody with that of the SNH-3 (typical anti-sialyl Le^x) and 2D3 (typical anti-sialyl Le^a) antibodies towards a short-chain sialyl Le^x glycolipid (panel *A*) or sialyl Le^a glycolipid (panel *B*) as ascertained by ELISA. The structures of the glycolipid used for the immobilized antigen were sialyl Le^x glycolipid (synthetic), NeuAcα2→3Galβ1→4(Fucα1→3)GlcNAcβ1→3Galβ1→4Glcβ1→1Cer; sialyl Le^a glycolipid (purified from Colo201 cells), NeuAcα2→3Galβ1→3(Fucα1→4)GlcNAcβ1→3Galβ1→4Glcβ1→1Cer.
Panel *C*; Western blotting analysis of human peripheral lymph node stroma proteins with the SNH-3 and 2H5 antibodies. Lane 1, protein staining; lane 2, staining with SNH-3; lane 3, staining with 2H5.

When the reactivity of glycoproteins in the human peripheral lymph node stroma were tested by Western blotting technique with these antibodies, only the 2H5 antibody detected a set of the glycoproteins as indicated by arrows in lane 3 of panel *C* of Fig. 2. The molecular masses of the major three reactive glycoproteins were estimated to be 250, 110 and 90 kDa, respectively. The most abundant molecular species was 110 kDa. No positive bands were observed with the SNH-3 antibody tested under the same condition (lane 2 of panel *C*, Fig. 2), or with the FH-6, CSLEX-1, or 2D3 antibodies.

Inhibition of lymphocyte adhesion to HEV by the 2H5 antibody: In order to know whether the carbohydrate determinants detected on HEV by the 2H5 antibody are involved in the L-selectin-mediated adhesion of human lymphocytes to HEV, cryostat sections of human peripheral lymph node were pretreated with 2H5 and tested in Stamper-Woodruff assays. As shown in Fig. 3, considerable inhibition of adhesion was observed in the sections pretreated with the 2H5 antibody, compared to the sections pretreated with class-matched control antibody MK1-8, which was directed against an unrelated carbohydrate antigen, G_{M2}. The HEVs in the frozen lymph node sections treated with the control antibody adhered as many as 22.6 ± 4.2 lymphocytes/kunit HEV area on average, while the HEVs in the frozen sections treated with the 2H5 antibody adhered only 5.5 ± 3.2 lymphocytes/kunit HEV area on average (these results

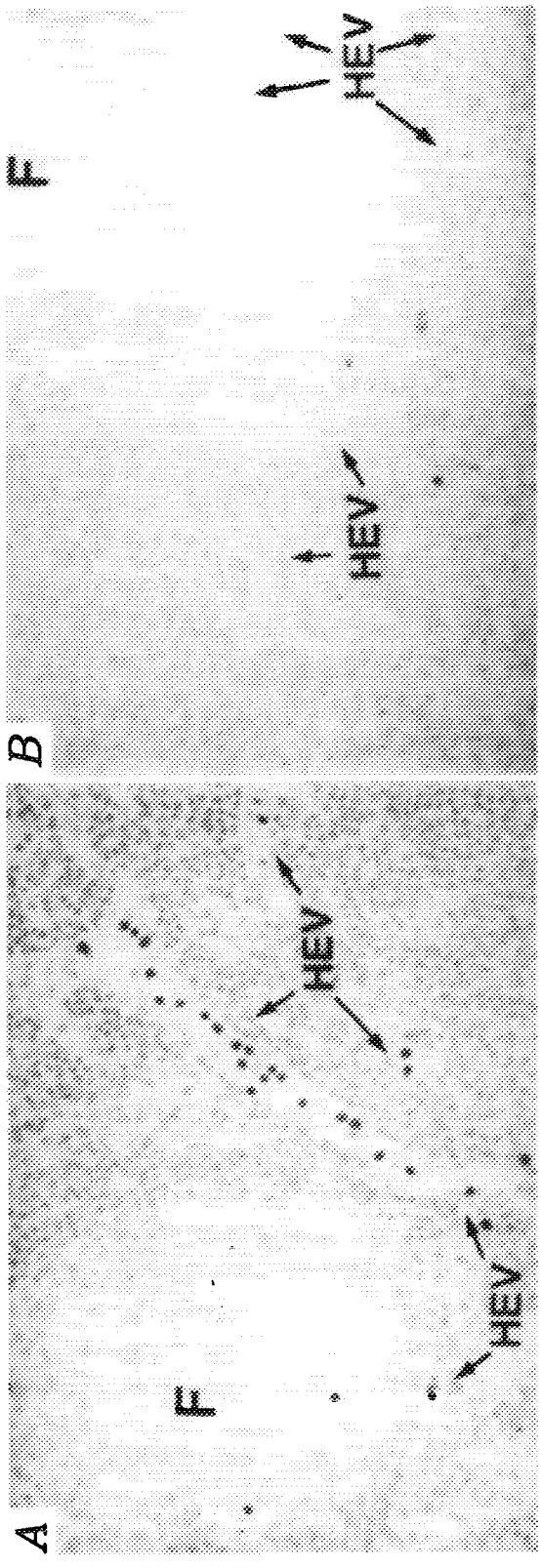

Fig. 3 Inhibition of lymphocyte adhesion to HEV of a human peripheral lymph node by the 2H5 antibody. Results of a typical experiment are shown. Panel *A*, the lymph node frozen section was pretreated with the control antibody MK1-8; panel *B*, the section was pretreated with the 2H5 antibody.

represent the mean of calculations on 6 independent microscopical fields). The reduction of the lymphocyte adhesion to HEV by the 2H5 antibody pretreatment was 75 %, and the difference was statistically significant at $P<0.001$ by the Student's *t*-test.

Discussion

Our present results indicated that a sialyl Le^x-like carbohydrate determinant is expressed in HEV of the human peripheral lymph nodes, and this determinant is efficiently detected by the newly established antibody 2H5. This 2H5-defined sialyl Le^x-like determinant seems to serve, at least partly, as a ligand for L-selectin, as ascertained by the clear inhibition of adhesion by the addition of the antibody in the Stamper-Woodruff assay. This L-selectin ligand detected in peripheral lymph nodes seems to be significantly different from the conventional E-selectin ligand sialyl Le^x expressed at the surface of the granulocytes; only the 2H5 antibody effectively detects the former ligand, while the latter is quite readily detected with almost every typical anti-sialyl Le^x antibody ever reported.

Sialyl Le^a as well as sialyl Le^x is known to bind to L-selectin from *in vitro* experiments employing recombinant L-selectin and/or L-selectin transfected cells (9,10). However, we tentatively conclude that the 2H5-defined determinant is most probably complex carbohydrates which carry the sialyl Le^x-like determinant, and not the sialyl Le^a determinant, for the following reasons. First, the antibody 2H5 which efficiently detects this determinant was successfully obtained by immunization of the sialyl-Le^x-active complex glycolipid fraction; second, this antibody has a specificity to sialyl Le^x hapten and has no cross-reactivity to sialyl Le^a; and third, even the CSLEX-1 at very high concentrations could detect this determinant, while an anti-sialyl Le^a antibody 2D3 failed to detect any reactive antigen on HEV.

We could detect significant expression of the 2H5-defined sialyl Le^x-like determinant also in HEV of the Payer's patches and appendices, although less strongly than in HEV of the peripheral lymph nodes. It had been postulated that L-selectin is an adhesion molecule which specifically mediates homing of lymphocytes to the peripheral lymph nodes, and that homing of the gut-associated lymphocytes to the Payer's patches and appendices is mediated by an entirely different molecular mechanism (1-3). The ligand for L-selectin had been assumed to be present only in HEV of the peripheral lymph nodes but not in HEV of the Payer's patches or appendices. However, a recent study indicated that the L-selectin molecules are partly involved in homing of the gut-associated lymphocytes (27). The determinant detected by 2H5 in the Payer's patches and appendices is probably the carbohydrate ligand also

operating as a ligand for L-selectin, and this determinant is not detectable by the conventional anti-sialyl Le^x antibody CSLEX-1 even at higher concentrations.

The 2H5 antibody could detect sialyl Le^x-like determinants on stromal glycoproteins in human peripheral lymph nodes, while other antibodies fail to detect the antigen under the same condition. These proteins seem to carry the complex sialyl Le^x-active carbohydrate determinants which are most probably similar in their carbohydrate structure to the carbohydrate determinants carried by the complex glycolipids used as immunogens to raise the 2H5 antibody. A recombinant human L-selectin was shown to detect two major glycoproteins SGP50 (50 kDa) and SGP90 (90 kDa) on HEV of murine lymph nodes (28). SGP50 was named as GlyCAM-1, and cDNA for this molecule was cloned (28). On the other hand, Berg et al. identified 65, 90, 105, 150 and 200 kDa proteins in human lymph nodes as the L-selectin ligands using the MECA-79 antibody (29). Our 2H5 antibody detected glycoproteins of 250, 110 and 90 kDa in human peripheral lymph nodes. No explanation for these discrepancies in molecular mass is yet available.

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