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Tissue-specific expression of Le^Y antigen in high endothelial venules of human lymphoid tissues

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In this study, we demonstrated that the anti-Le^Y antibody (BM-1) especially reacted with high endothelial venules (HEVs) in peripheral lymph nodes of blood group O individuals. The Le^Y expression on HEVs showed a unique tissue-specific pattern, i.e., a large amount of the Le^Y expression in peripheral lymph nodes and no or small amounts in mesenteric lymph node. Statistical analysis showed that there was the significant difference between the percentage of Le^Y-positive HEVs in peripheral lymph nodes and mesenteric lymph nodes. No expression of Le^Y was observed in vessels of Payer's patch, thymus, spleen and other non-lymphoid organs. In blood group A or B individuals, the reactivity between HEVs and anti-Le^Y antibody increased after enzyme digestion with α-N-acetylgalactosaminidase or α-galactosidase. These findings show that the expression of difucosylated blood group ABH antigens are especially expressed on HEVs in peripheral lymph nodes. Furthermore, the tissue-specific pattern suggests that these antigens may be related to intercellular adhesion between lymphocytes and HEVs.

Keywords: **Le^Y antigen, high endothelial venules, fucosyltransferase, lymphocyte homing**

Introduction

Le^Y antigen expression seems to be correlated with apoptosis of some kinds of cancer cells or gastro-intestinal mucosal epithelia [1]. The Le^Y determinant has also been identified phenotypically as a marker of specific types of cells and possibly specific stages of murine embryogenesis [2], and it appears to function in cell-cell adhesion via blood group H antigen–Le^Y antigen interaction [3]. Furthermore, there is a report that Le^Y expression in breast cancer is thought to be related to tumor procoagulant and prognosis of cancer [4]. Taking these reports into consideration, the Le^Y determinant is thought to be related to intercellular adhesion. The presence of Le^Y determinants on the surface of mature human granulocytes has been reported by several groups of workers [5–8], and it has been suggested that the level of Le^Y expression in myeloid cells may be regulated during differentiation. Symington et al. [5] have reported that Le^Y antigen expression increases when the promyelocytic cell line HL-60 is induced to differentiate with retinoic acid. However, the functional significance of Le^Y determinant in these cells is uncertain. Most studies of Le^Y expression in human tissues have demonstrated that this antigen is not found in normal cells or that it is expressed in a low level, but is highly expressed in human tumors [4,9–12]. In this study, we demonstrate that Le^Y antigen was peculiarly and constantly expressed on HEVs of normal human lymphoid tissues and there was the tissue-specificity of Le^Y expression.

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Materials and methods

Tissue samples

Human lymphoid tissue materials were obtained from cadavers during autopsy in the Osaka Medical Examiner's Office, the Medical Examiner's Office of Hyogo Prefecture, the Department of Forensic Medicine and Sciences, Mie University School of Medicine, and the Department of Legal Medicine, Shiga University of Medical Science. All tissue samples from donors were used within 20 h of postmortem period. The tissue samples examined in this study were thymus, spleen, appendix, palatal tonsil, small intestine, cervical lymph node (CLN), axillary lymph node (AxLN), tracheal lymph node (TrLN), inguinal lymph node (InLN) and mesenteric lymph node (MLN). Tissues were fixed in 10% phosphate buffered

formalin, embedded in paraffin and serial sections were cut at a thickness of 3 μm. The ABO and Lewis blood groups were determined by a routine hemagglutination method, and the Lewis blood group status of each individual was decided by expression of Le^a and Le^b in intestinal mucosa and salivary glands [13] using an immunohistochemical technique.

Reagents

Anti-Le^Y mouse monoclonal antibody BM-1 was purchased from JIMRO (Gumma, Japan) and is a subclone of AH6 hybridoma producing anti-Le^Y antibody, with high-specificity for Le^Y antigen [14]. α-galactosidase (from coffee beans) was purchased from Boehringer Mannheim (Indianapolis, IU, USA) and α-N-acetylgalactosaminidase (from chicken liver) was purchased from SIGMA CHEMICAL CO. (St. Louis, MO, USA). Anti-A, anti-B, anti-Le^a and anti-Le^b mouse monoclonal antibodies were purchased from Ortho Diagnostic System (Raritan, NJ, USA). Anti-H antibody 92FRA2 (H type 2 blood group antigen-specific) was purchased from DAKO A/S (Glostrup, Denmark).

Histochemical procedure

Deparaffinized sections were treated with methanol containing 0.3% H_2O_2 to block endogenous peroxidase, hydrated and treated with PBS (0.1 M phosphate buffered saline) three times. They were then incubated in PBS containing 1% bovine serum albumin (BSA) for 30 min. After being blotted dry with paper filter, the tissue sections were incubated with monoclonal antibody (1 : 50) at room temperature for 2 h with or without prior enzyme digestion. Tissue sites reactive with monoclonal antibody were visualized with the streptavidin-biotin-peroxidase complex method, using the Histofine SAB-PO kit (Nichirei; Tokyo, Japan). Finally, the sections were counterstained with hematoxylin.

The percentage of Le^Y positive HEVs was calculated as follows: the number of Le^Y-positive HEVs and total HEVs were counted for 10 random microscopic fields (magnification $\times 400$) in the paracortical area of lymph nodes on each tissue slide. HEVs stained over 50% of its inner circumference was counted as Le^Y-positive. The percentage of Le^Y-positive HEVs was calculated from the equation

% Le^Y-positive HEVs = the number of Le^Y-positive HEVs/the number of total HEVs $\times$ 100.

Enzyme digestion

α-galactosidase digestion of tissue sections was carried out at concentration of 1 U/ml in 50 mM of citrate buffer (pH 6.5) at 37°C for 24 hr. α-N-acetylgalactosaminidase digestion of tissue sections was carried out at concentration of 2 U/ml in 0.2 M of citrate phosphate buffer (pH 3.85) at 37°C for 6 hr, 4 times (total 24 hr).

Statistical Analysis

Data are expressed as means ± SD. The Mann-Whitney's U test was used to determine the significance of differences in sample population means.

Results

A total of 11 blood group O donors, 4 blood group A donors, 4 blood group B donors and 1 blood group AB donor were examined in this study. Their age, sex and cause of death are summarized in Table 1.

Blood group O individuals

Anti-Le^Y antibody (BM-1) reacted unusually with HEVs in peripheral lymph nodes of blood group O individuals. In particular, Le^Y positive HEVs were likely to be distributed mostly in the paracortical area neighboring the follicles. In contrast, blood group H type 2 antigen detected with anti-H MoAB (92FRA2) was expressed equally on all vessels in all tissues (Figures 1 and 2 left columns). In adults, the Le^Y expression on HEVs showed a unique tissue-specific pattern (Figure 1 right columns) as a large amount of Le^Y expression was observed in HEVs of peripheral lymph nodes (CLN, AxLN, InLN and TrLN) and in contrast, low amount of Le^Y

Table 1. Blood group, age, sex, and cause of death of each donor in this study

Case	*Blood group*	*Age*	*Sex*	*Cause of death*
1	O	25 y.o.	F	Sudden death§
2	O	54 y.o.	F	Drowning
3	O	36 y.o.	F	Alcoholic liver cirrhosis
4	O	77 y.o.	F	Ischemic heart disease
5	O	59 y.o.	F	Dissecting aneurysm
6	O	15 y.o.	F	Sudden death§
7	O	49 y.o.	M	Hypovolemic shock, trauma
8	O	32 y.o.	M	Hypovolemic shock, trauma
9	O	12 d.o.	M	Asphyxia
10	O	2 y.10 m.o.	M	Subdural hemorrhege, trauma
11	O	8 m.o.	M	SIDS
12	A	39 y.o.	F	Hypovolemic shock, trauma
13	A	2 m.o.	F	SIDS
14	A	57 y.o.	M	Hypovolemic shock, trauma
15*	A	1 y.11 m.o.	F	Subdural hemorrhege, trauma
16	B	69 y.o.	M	Asphyxia
17	B	55 y.o.	M	Alcoholic cardiomyopathy
18	B	51 y.o.	M	Myocardial infarction
19**	B	56 y.o.	M	Asphyxia
20*	AB	49 y.o.	M	Sudden death, Plummer's disease

* Le (a − b −) individual. ** Le (a + b −) individual.
§ intrinsic sudden death, cause of death could not be determined by autopsy.

expression in HEVs of MLN and no Le^Y expression in HEVs of appendix and Payer's patch were observed. In MLN, The Le^Y antigen was expressed more strongly in part of the marginal sinus and trabecula than in HEV (Figures 1F, 3D). In contrast, Le^Y antigen could be barely detected in the marginal sinus and trabecula of peripheral lymph nodes. In HEVs of the tonsils, there was no regular pattern of the quantity of Le^Y expression in each individual.

In children (Figure 3), the tissue-specific pattern of Le^Y expression was similar to that in adult cases, however, it was likely that the difference in quantity of Le^Y expression in HEVs between peripheral lymph nodes and MLN was less than in adults. Moreover, a small amount of Le^Y expression in appendix (Figure 3E) and Payer's patch was observed in children.

The percentage of Le^Y-positive HEVs in each lymphoid tissue was calculated and shown in Table 2. As shown in Figure 2, the statistical analysis of the data showed a significant difference in quantity of Le^Y expression between the HEVs of MLN and of peripheral lymph nodes in adult cases ($P < 0.05$).

Blood group A and B individuals

As shown in Figures 4 and 5, the tissue sections obtained from blood group A individuals were stained with anti-A, anti-H and anti-Le^Y antibodies with or without α-N-acetylgalactosaminidase digestion. After enzyme digestion, reactivity with anti-A antibody to the vessels almost completely disappeared (Figures 4B and 5B), and reactivity with anti-H antibody was revealed in the corresponding sites (Figures 4D and 5D). This appearance showed the terminal α-N-galactosamine of blood group A antigen was almost completely removed in the vessels by α-N-acetylgalactosaminidase digestion. Le^Y expression in HEVs of peripheral lymph nodes was markedly increased after α-N-acetylgalactosaminidase digestion (Figure 4F). In MLN, Le^Y antigen was present in part of marginal sinus after α-N-acetylgalactosaminidase digestion (Figure 5F). The tissue-specific pattern of Le^Y expression with α-N-acetylgalactosaminidase digestion was also observed in blood group A individuals the same as in blood group O individuals, i.e.,

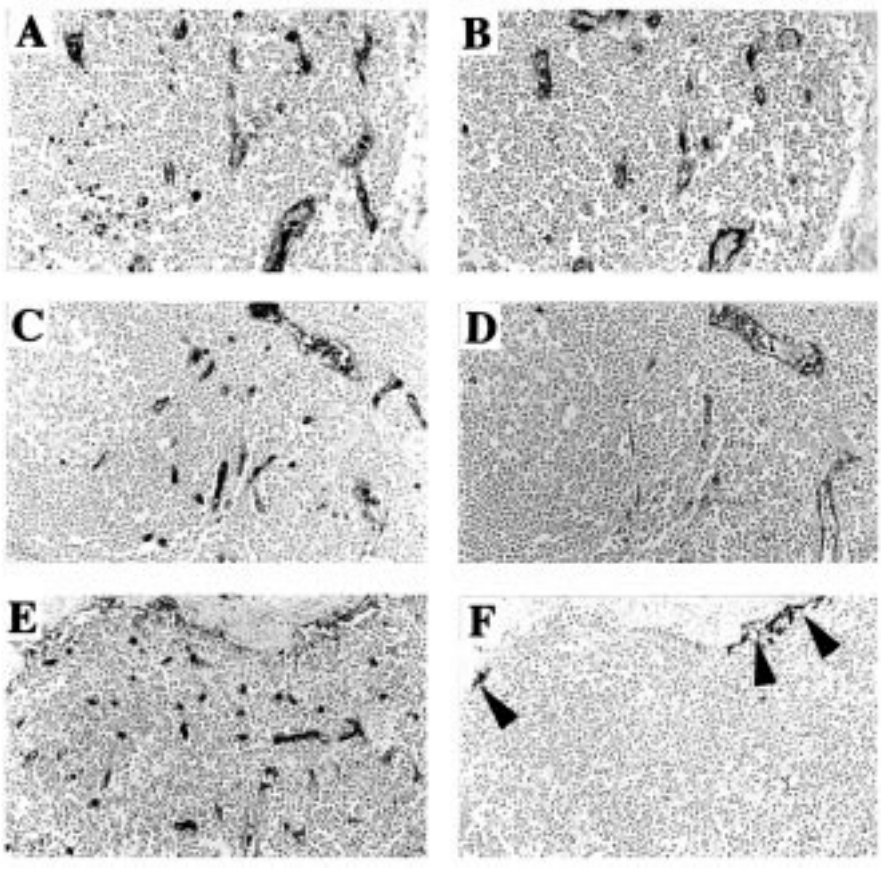

Figure 1. Immunohistochemical staining of the lymphoid tissue sections from 59-year-old blood group O donor (case 5 in Tables 1 and 2). Staining with anti-H antibody is shown in the left column (A, C, E, G, I and K), and staining with anti-Le^Y antibody is shown in the right coloumn (B, D, F, H and J). AxLN (A and B), CLN (C and D), MLN (E and F), appendix (G and H), spleen (I and J) and tonsil (K and L) are shown. In all tissues, vessels are equally stained with anti-H antibody (A, C, E, G, I and K). In AxLN, many HEVs are strongly Le^Y-positive (B). In CLN, the number of Le^Y-positive HEVs (D) are slightly below AxLN. In contrast, there are a few Le^Y-positive HEVs in MLN (F). In MLN, parts of the marginal sinus strongly reacted with anti-Le^Y antibody (arrowheads). There is no expression of Le^Y antigen in any vessels of the appendix (H) and spleen (J). In the tonsils, weak Le^Y-positive HEVs are observed (L). Magnification × 160.

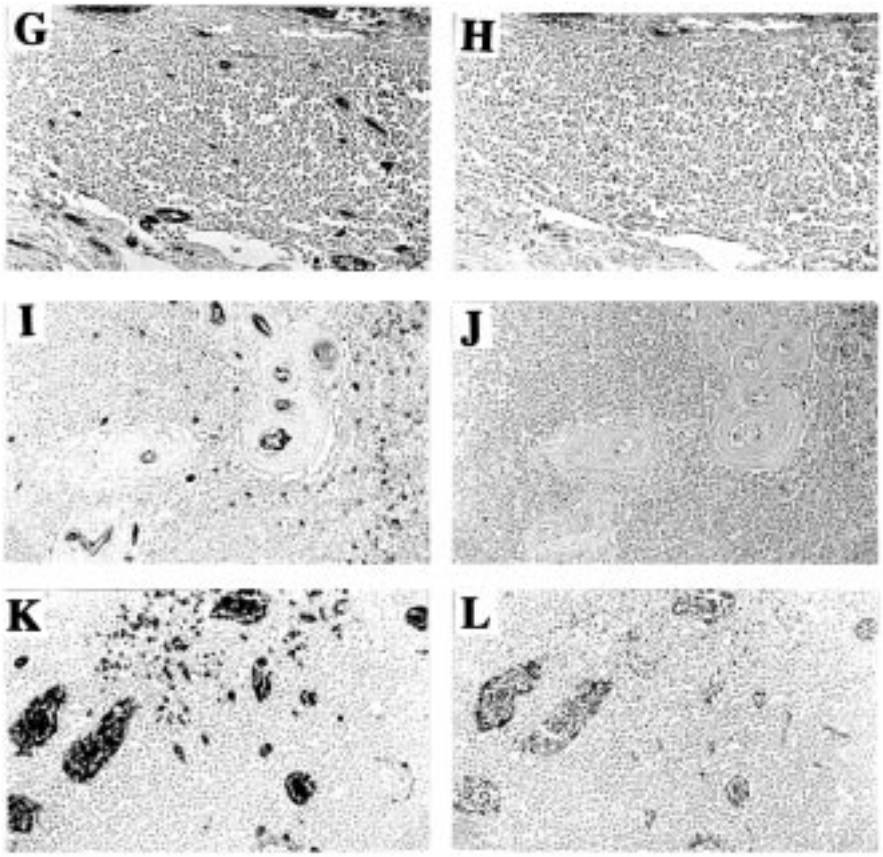

Figure 1. (*Continued*)

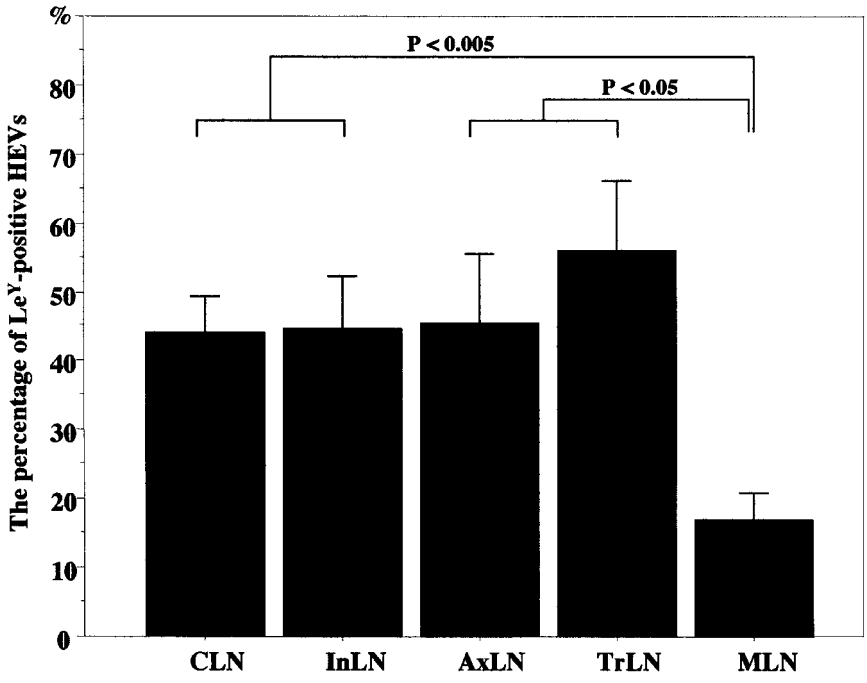

Figure 2. The percentage of Le^Y-positive HEVs in each lymph node from adult blood group O donors (see Table 2). Error bar shows the standard error (SE). The percentage of Le^Y-positive HEVs in MLN is less than those of the other lymph nodes with the significance of difference.

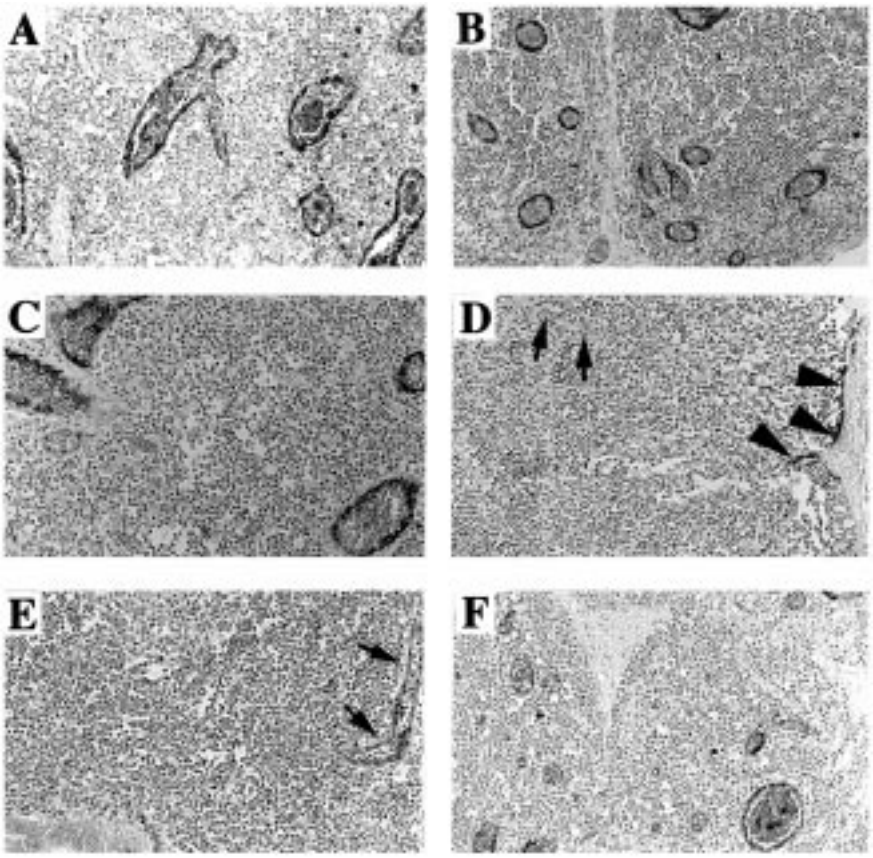

Figure 3. Le^Y expression in the lymphoid tissues from an 8-month-old blood group O donor (case 11 in Table 1). A: AxLN, B: InLN, C: CLN, D: MLN, E: appendix and D: tonsil. Many HEVs of AxLN (A), InLN (B) and CLN (C) strongly reacted with anti-Le^Y antibody, in contrast, those of MLN (D) and appendix (E) show no or mean reactivity with anti-Le^Y antibody (arrows). In MLN, parts of marginal sinus strongly reacted with anti-Le^Y antibody (arrowheads) the same as adult blood group O donors (see Fig. 1F). There are small numbers of HEVs expressing Le^Y antigen in the tonsils (F). Magnification $\times 160$.

Table 2. The rate of Le^Y positive HEVs in adult blood group O individuals

Case	*AxLN*	*CLN*	*InLN*	*BrLN*	*MLN*
1	–	0.47	0.74	0.59	0.31
2	–	0.52	0.33	–	–
3	–	0.57	0.51	0.56	0.23
4	–	0.22	0.33	0.30	0.17
5	0.58	0.46	–	–	0.21
6	0.67	0.66	0.54	0.79	0.06
7	0.25	0.31	–	–	0.00
8	0.32	0.32	0.23	0.23	0.21
Mean $\pm$ SD	0.46 ± 0.18	0.44 ± 0.14	0.45 ± 0.17	0.56 ± 0.17	0.17 ± 0.10

large amounts of Le^Y expression in HEVs of peripheral lymph nodes, small amounts of Le^Y expression in HEVs of MLN and no Le^Y expression in HEVs of the appendix and Payer's patch.

As shown in Figures 6 and 7, the tissue sections obtained from blood group B individuals were stained with anti-B, anti-H and anti-Le^Y antibodies with or without α-galactosidase digestion. The results from blood group B individuals were the same as for blood group A individuals. The tissue sections obtained from blood group AB individual were stained with anti-A, anti-B, anti-H and anti-Le^Y antibodies with or without sequential digestion of α-N-acetylgalactosaminidase and α-galactosidase (data not shown). The results from blood group AB individuals were the same as for blood group A and B individuals.

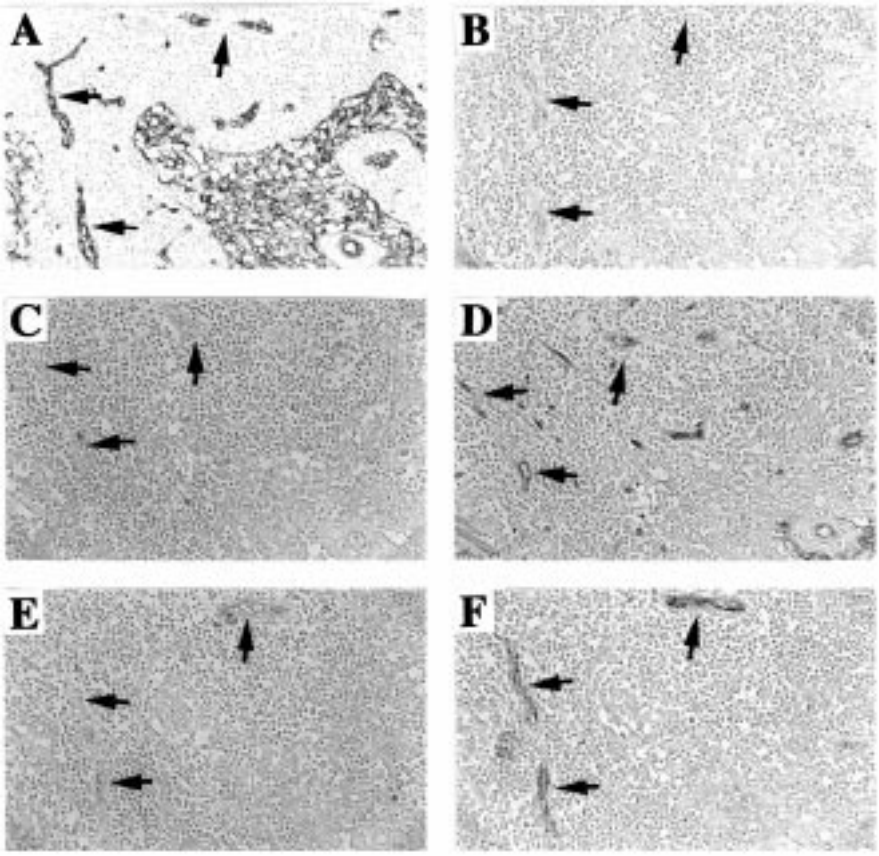

Figure 4. Sections of CLN from a 57-year-old blood group A donor (case 14 in Table 1). The left column (A, C and E) shows the sections with no enzyme pretreatment, and the right column (B, D and F) shows the sections after enzyme digestion with α-N-acetylgalactosaminidase. The sequential sections were stained with anti-A antibody (A and B), anti-H antibody (C and D) or anti-Le^Y antibody (E and F). After α-N-acetylgalactosaminidase digestion, the reactivity between anti-A antibody and the vessels is almost completely eliminated (B), and the reactivity of anti-H antibody in most vessels is revealed (D). Le^Y expression in some HEVs was markedly increased after α-N-acetylgalactosaminidase digestion (F). Arrows show Le^Y-positive HEVs. Magnification × 160.

The rate of Le^Y positive HEVs from A, B and AB donors are shown in Table 3.

Discussion

There have been no reports that Le^Y antigen is expressed especially in HEVs of human lymphoid tissues, and the only previous report showed that the Le^Y determinant was expressed in pig lymph node HEVs [15]. In this study, we demonstrated that the Le^Y antigen was expressed in the HEVs of human lymphoid tissues with tissue specificity. In blood group O individuals, Le^Y antigen detected with anti-Le^Y monoclonal antibody BM-1 was markedly expressed in many HEVs of peripheral lymph nodes (AxLN, InLN, TrLN and CLN), but in contrast, was poorly expressed in gut-associated lymphoid tissues (GALT) (appendix and Payer's patch) and MLN. Le^Y antigen could not be detected in any vessels of the thymus, spleen or non-lymphoid organs. Therefore, it is presumed that Le^Y antigen should be one of the peripheral lymph node-type high endothelial cell markers. In the blood group A or B phenotypes, Le^Y antigen in HEVs could be detectable as for blood group O phenotypes after α-N-acetylgalactosaminidase or α-galactosidase digestion. This observation shows that HEVs in blood group A or B phenotypes should express difucosyl A (ALe^Y) or difucosyl B (BLe^Y) antigens (Figure 8).

To synthesize the Le^Y determinant, there are two kinds of fucosyltransferase, i.e., α1,2-fucosyltransferase (α1,2-Fuc-T) and α1,3-fucosyltransferase (α1,3-Fuc-T) (Figure 8). α1,2-Fuc-T(s) which is responsible for forming the H antigen, an essential precursor of the A and B antigens, plays a regulatory role in the tissue expression of the ABH antigens. There are at least two α1,2-Fuc-Ts expressed in human tissues [16,17]. One is the H gene-encoded α1,2-Fuc-T (H enzyme) [18], which regulates mainly the expression of H type-2 antigen in tissues from ectoderm and mesoderm. The other is the secretor (*Se*) gene-encoded α1,2-Fuc-T (Se enzyme) [19,20], which regulates mainly the expression of H type-1 antigen in tissues from endoderm. Generally, it is thought that the type 2 blood group antigens in vascular endothelium are regulated by the H

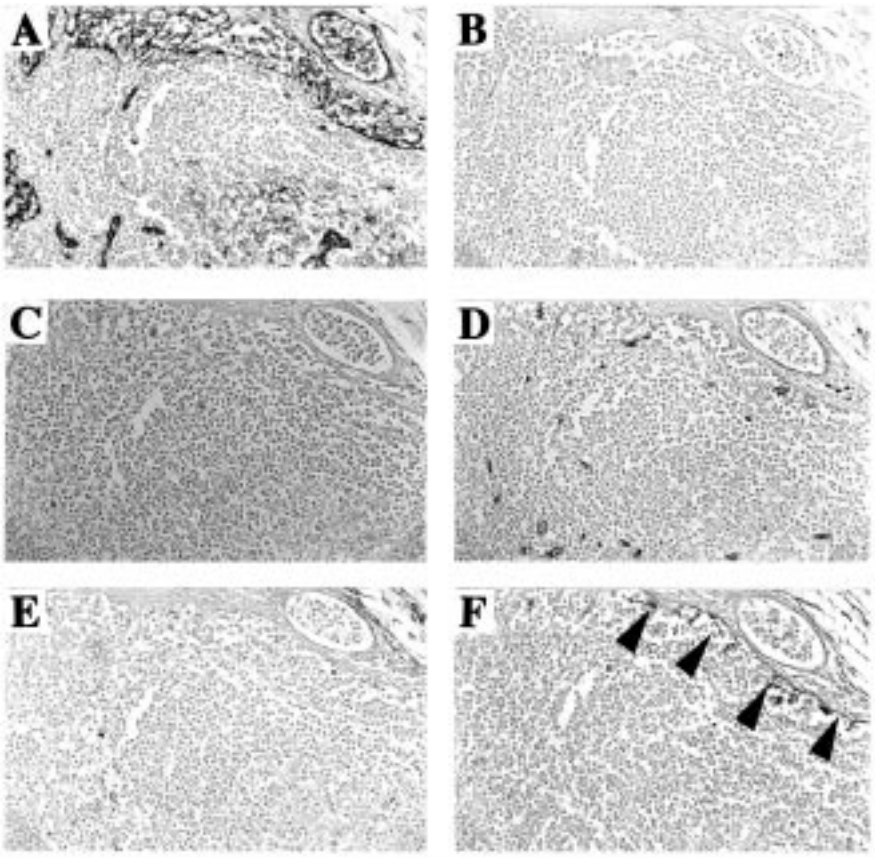

Figure 5. Sections of MLN from the same donor as shown in Fig. 4. The left column (A, C and E) shows the sections with no enzyme pretreatment, and the right column (B, D and F) shows the sections after enzyme digestion with α-N-acetylgalactosaminidase. The sequential sections were stained with anti-A antibody (A and B), anti-H antibody (C and D) or anti-Le^Y antibody (E and F). In MLN, Le^Y antigen was expressed more strongly in part of marginal sinus than in HEV after α-N-acetylgalactosaminidase digestion (F, arrowheads). Magnification × 160.

enzyme. In fact, the expression of Le^Y antigen in human HEVs of peripheral lymph node was independent of the secretor status of the donor in this study (Case 19 in Table 1).

To date, five α1,3-Fuc-Ts (Fuc-TIII, Fuc-TIV, Fuc-TV, Fuc-TVI and Fuc-TVII) have been cloned and characterized in humans [21–29], the *Fuc-TIII (Le gene)* product is an α1,3/4-Fuc-T which catalyses the transfer of L-fucose from GDP-fucose to the N-acetylglucosamine of various type 1 and type 2 acceptor substrates. The others, Fuc-TIV-VII, have α1,3-Fuc-T activity for type 2 acceptors substrates, but no or low α1,4-Fuc-T activity [26–29]. *Fuc-TV* and *Fuc-TVI* have a high level of sequence homology with *Fuc-TIII*, and like *Fuc-TIII* are located on chromosome 19 [30,31]. These three α1,3-Fuc-Ts can be virtually excluded as determinants of leukocytic selectin ligand expression, as they are not expressed in leukocytic cells. Similarly, there have been no reports of these three α1,3-Fuc-Ts in HEVs and it is difficult to conceive that they can play a role in Le^Y synthesis in HEVs. In fact, for *Fuc-TIII*, the Le^Y expression on HEVs of the peripheral lymph nodes was detected in the Le(a-b-) phenotypes (*Fuc-TIII*-deficiency) similar to the Le (a + b −) or Le (a − b +) phenotypes (*Fuc-TIII*-wild type) in this study (Case 15 and 20 in Table 1).

Fuc-TIV and *Fuc-TVII* are located on chromosomes 11 and 9, respectively [32,28,29]. These two α1,3-Fuc-Ts are expressed mainly in myeloid cell lineages from mesoderm. The Fuc-TVII locus is transcribed in leukocytic cells and, when expressed in COS or CHO cell lines [28,29,33], consistently yields sialyl Le^X expression, E-selectin ligand activity [27], and P-selectin ligand activity when coexpressed with PSGL-1 [34,35]. In the study of the Fuc-TVII mutant mice, Maly et al. [36] reported that Fuc-TVII is essential to the expression of not only E- and P-selectin ligand activity but also L-selectin ligand activity in mice, however, the role of Fuc-TIV in selectin ligand synthesis is not directly addressed and remains uncertain. Goelz et al. [23] showed that ELAM-1 ligand fucosyltransferase, identical to Fuc-TIV, was expressed in a myeloid lineage cell line HL-60 and conferred E-selectin binding activity to transfected CHO cells. However, the capability of Fuc-TIV of generating the selectin ligands in

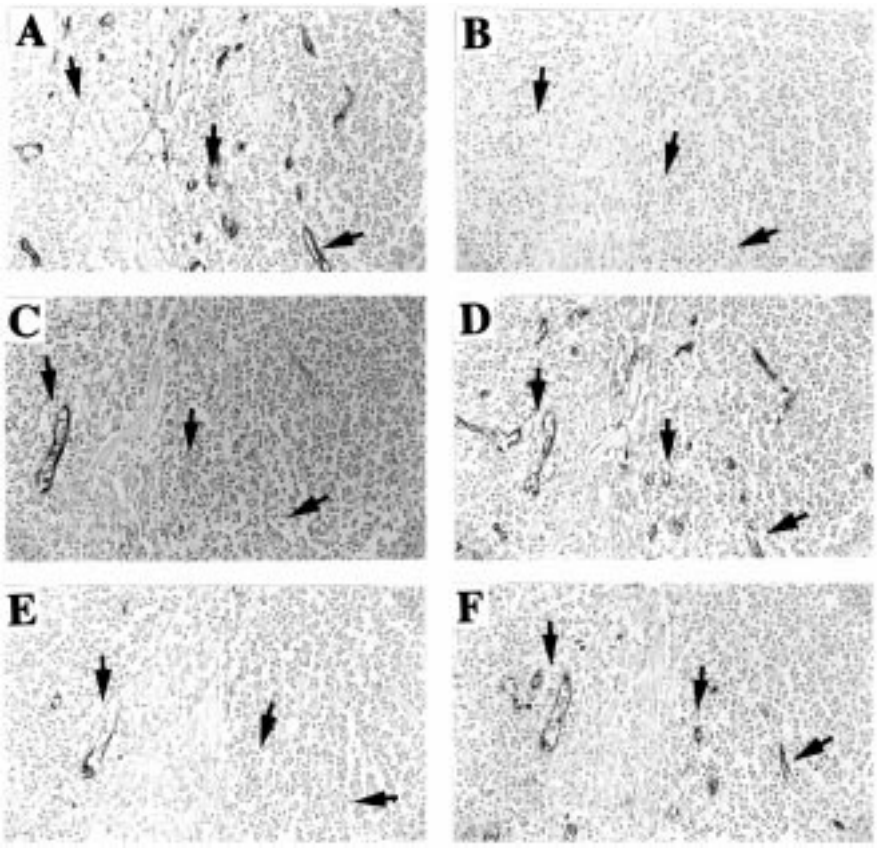

Figure 6. Sections of CLN from 51-year-old blood group B donor (case 18 in Table 1). The left column (A, C and E) shows the sections with no enzyme pretreatment, and the right column (B, D and F) shows the sections after enzyme digestion with α-galactosidase. The sequential sections were stained with anti-B antibody (A and B), anti-H antibody (C and D) or anti-Le^Y antibody (E and F). After α-galactosidase digestion, the reactivity between anti-B antibody and the vessels is almost completely eliminated (B), and the reactivity of anti-H antibody in most vessels are revealed (D). Le^Y expression in some HEVs was markedly increased after α-galactosidase digestion (F). Arrows show Le^Y-positive HEVs. Magnification × 160.

either transfected CHO and COS cells has not been confirmed by other studies [24,25]. In our preliminary experiments (data not shown), Le^Y determinants were expressed on cultured human umbilical vein endothelial cells (HUVECs) after tumor necrosing factor-α (TNF-α) stimulation and peaked 1–2 days. Simultaneously, the elevation of the relative Fuc-TIV mRNA level was observed by the RT-PCR method in cultured HUVECs after stimulation. These findings suggest that Fuc-TIV may play a role in Le^Y synthesis. In fact, several groups [8,37,38] have reported that Fuc-TIV shows a marked preference for neutral type 2 substrates, especially H type 2 substrate. Clarke and Watkins [8] concluded that insofar as the developing myeloid cells are concerned, the rise in expression of Le^Y on the cell surface appears to correlate with an increase in the level of Fuc-TIV mRNA expressed in KG1 and HL-60 cells. They also presumed that *Fuc-TIV* and *Fuc-TVII* genes are independently regulated in developing myeloid cells.

Consequently, in HEVs of human lymphoid tissues, we suggest that the products of Fuc-TIV and Fuc-TVII may be distinct and one of the candidate of Fuc-TIV products may be Le^Y determinants. The functional or biological significance of Le^Y determinants *in vivo* is still unknown, but its peculiar distribution in human lymphoid tissues is so interesting in marked contrast to mucosal addressin cell adhesion molecule-1 (MAdCAM-1) [39,40] expressed mainly in HEVs of GALT. Therefore, we presumed that the Le^Y determinant may be related to lymphocytes homing to peripheral lymph nodes in humans.

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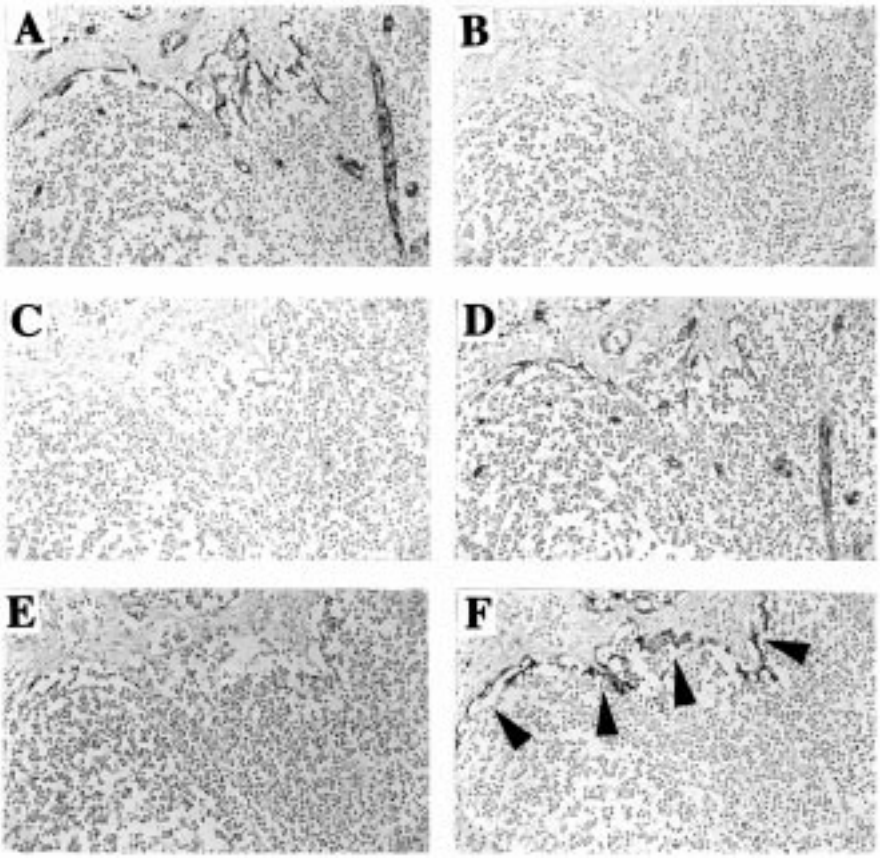

Figure 7. Sections of MLN from the same donor as shown in Fig. 6. The left column (A, C and E) shows sections with no enzyme pretreatment, and the right column (B, D and F) shows the sections after enzyme digestion with α-galactosidase. The sequential sections were stained with anti-B antibody (A and B), anti-H antibody (C and D) or anti-Le^Y antibody (E and F). In MLN, Le^Y antigen was expressed more strongly in part of marginal sinus than in HEV after α-galactosidase digestion (F, arrowheads). Magnification × 160.

Table 3. The rate of Le^Y positive HEVs in adult blood group A, B and AB individuals

	Case 12 (blood group A)		*Case 18 (blood group B)*		*Case 20 (blood group AB)*	
	*Enzyme digestion**					
	(−)	*(+)*	*(−)*	*(+)*	*(−)*	*(+)*
AxLN	0.03	0.56	0.23	0.76	0.02	0.56
CLN	0.03	0.45	0.30	0.69	0	0.14
InLN	0.08	0.17	0.08	0.49	0.03	0.33
BrLN	0.02	0.09	0.11	0.36	–	–
MLN	0	0	0	0.11	0	0

*The enzymes used in this study were α-N-acetylgalactosaminidase and α-galactosidase for blood group A and blood group B donors, respectively. For blood group AB donor, sequential digestion of α-N-acetylgalactosaminidase and α-galactosidase was performed.

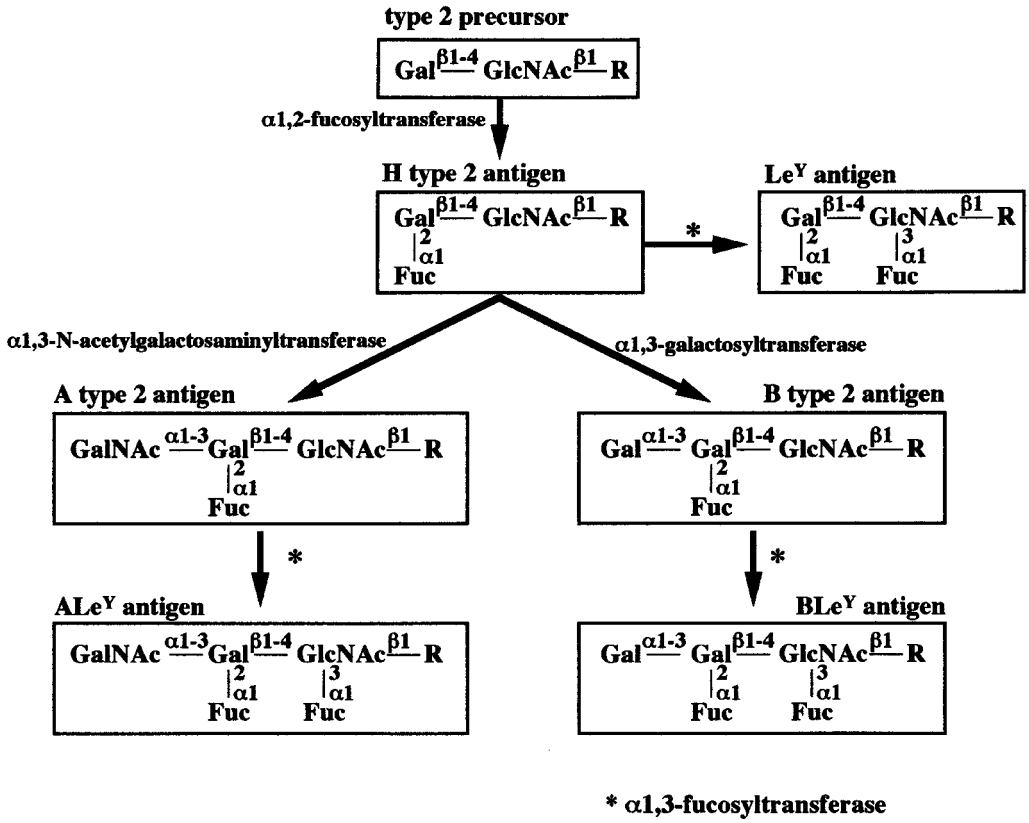

Figure 8. Schematic illustration of the enzymatic effect of α1,2Fuc-$T_{(s)}$ and α1,3Fuc-$T_{(s)}$ on the synthesis of difucosylated ABH antigens.

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