# Differentiation-Dependent Expression of Sialyl Stage-Specific Embryonic Antigen-1 and I-Antigens on Human Lymphoid Cells and Its Implications for Carbohydrate-Mediated Adhesion to Vascular Endothelium

By Katsuyuki Ohmori, Akiko Takada, Tomoya Yoneda, Yumiko Buma, Kunimi Hirashima, Kiyotaka Tsuyuoka, Akira Hasegawa, and Reiji Kannagi

**Expression of two developmentally regulated carbohydrate antigens, the sialyl stage-specific embryonic antigen-1 (SSEA-1) and I-antigens, in human lymphocytes and lymphocytic leukemia cells was investigated using specific monoclonal antibodies. Sialyl SSEA-1 was expressed only on natural killer (NK) cells, and was essentially absent on resting mature T and B cells among normal peripheral lymphocytes. On the other hand, the I-antigen was strongly expressed on virtually all mature B cells, moderately expressed on most mature T cells, but not expressed on NK cells in normal donors. Expression of the two antigens on normal T and B cells was reversible; in vitro stimulation of normal lymphocytes with concanavalin A (Con A) resulted in the loss of I-antigen and appearance of sialyl SSEA-1 on $CD3^+$ T blasts, whereas stimulation with pokeweed mitogen led to loss of I-antigen expression and appearance of sialyl SSEA-1 antigen on $CD19^+$ B blasts. Among lymphocytic leukemia cells, sialyl SSEA-1 was detected primarily on leukemia cells having immature properties such as most common acute lymphocytic leukemia (cALL) blasts, while the I-antigen was frequently expressed on malignant cells having relatively mature properties, such as those found in adult T-cell leukemia or chronic lymphocytic leukemia, and only occasionally on cALL blasts. Among normal peripheral lymphocytes, the sialyl $SSEA\text{-}1^+I\text{-}antigen^-$ NK cells selectively underwent E-selectin (ELAM-1, endothelial-leukocyte adhesion molecule-1)-dependent adhesion to endothelial cells, while the $I\text{-}antigen^+sialyl\ SSEA\text{-}1^-$ mature T and B cells did not, in line with the recent finding that sialyl SSEA-1 serves as a specific ligand for E-selectin. Con A blasts, which are sialyl $SSEA\text{-}1^+I\text{-}antigen^-$, also exhibited significant E-selectin–dependent adhesion to endothelial cells. These results indicate that expression of the sialyl SSEA-1 and I-antigens varies alternately depending on the differentiation/activation status of the lymphocytes, and that this at least partly regulates the behavior of lymphocytes at the vessel wall.**



RECENTLY CELL surface carbohydrate antigens have been shown to be involved in the process of cell adhesion.[1,2] A cell adhesion molecule called E-selectin (endothelial-leukocyte adhesion molecule-1 [ELAM-1]), which appears at the surface of human endothelial cells after appropriate stimulation with cytokines such as interleukin-1$\beta$ (IL-1$\beta$) or tumor necrosis factor $\alpha$ (TNF$\alpha$), was shown to specifically recognize the sialyl $Le^x$ hapten.[3-7] The ligand carbohydrate for E-selectin is structurally related to a series of developmental carbohydrate antigens, known as SSEA-1 (stage-specific embryonic antigen-1). The SSEA-1 antigen, first described by Solter and Knowles[8] as the cognate antigen for the monoclonal antibody (MoAb) raised against murine teratocarcinoma F9 cells, was later shown to be a series of carbohydrate antigens carrying the $Le^x$ terminus.[9,10] The sialylated forms of SSEA-1 antigens, hence sometimes referred as sialyl SSEA-1,[11] have the terminal sialyl $Le^x$ structure and serve as the ligand for E-selectin.

We have previously investigated the distribution of sialyl SSEA-1 antigen in human lymphoid cells, and showed that this antigen is strongly expressed in lymphocytic leukemia cells having immature properties, whereas normal mature T and B lymphocytes lack the antigen.[11] The expression of this antigen is limited to a distinct subpopulation of natural killer (NK) cells among normal human peripheral lymphocytes.[11] In this report, we describe the distribution of another carbohydrate antigen, the I-antigen, which has a backbone carbohydrate structure very similar to that of sialyl SSEA-1.[10,12] We show that the I-antigen is expressed, in contrast to sialyl SSEA-1, preferentially on mature T and B cells but not on NK cells among normal peripheral lymphocytes, and that its expression is remarkably reduced in common acute lymphocytic leukemia (cALL) blasts. We further suggest that the expression status of these carbohydrate antigens affects the adhesion of lymphocytes to vascular endothelial cells.

## MATERIALS AND METHODS

*MoAbs used for flow cytometric analysis.* MoAbs FH-6[13,14] (specific to sialyl SSEA-1, supplied by Dr Sen-itiroh Hakomori, Biomembrane Institute, Seattle, WA) and C6[15] (specific to I-antigen, supplied by Dr Bruce A. Fenderson, Washington University, Seattle) are both murine IgM and were purified from ascitic fluids as described previously.[11,13-15] The carbohydrate structures of the sialyl SSEA-1 and I-antigens and the specificities of the MoAbs are summarized in Table 1. The FH-6 antibody reacts specifically to the carbohydrate sequence carrying sialyl $Le^x$ terminus that resides on the i-antigenic structure; hence, the antigenic epitope is called sialyl $Le^x$-i.[14]

Among the antibodies used for the cell surface marker analysis, Leu 4 (CD3) for T cells, Leu 12 (CD19), Leu 16 (CD20) for B cells, Leu 11 (CD16), Leu 19 (CD56) for NK cells, and the antibodies against IL-2 receptor, HLA-DR, and HPCA-1 were obtained from Becton Dickinson Immunocytometry Systems (Mountain View, CA). The antibody J5 was supplied from the Coulter Immunology Division (Hialeah, FL), and the anti-$\mu$, $\gamma$, $\kappa$, $\lambda$ antibodies were from Tago Immunochemicals (Burlingame, CA).

*From the Department of Laboratory Medicine, Kyoto University, School of Medicine, Kyoto; the Laboratory of Experimental Pathology, Aichi Cancer Center, Research Institute, Nagoya; and the Department of Applied Bio-organic Chemistry, Gifu University, School of Agriculture, Gifu, Japan.*

*Submitted January 28, 1992; accepted August 27, 1992.*

*Supported in part by Grants-in-aid for Scientific Research from the Ministry of Education, Science and Culture, Japan (03557114 and 03258218).*

*Address reprint requests to Reiji Kannagi, MD, PhD, Laboratory of Experimental Pathology, Research Institute, Aichi Cancer Center, 1-1 Kanokoden, Chikusaku, Nagoya, 464, Japan.*

*The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked* "advertisement" *in accordance with 18 U.S.C. section 1734 solely to indicate this fact.*



*0006-4971/93/8101-0005$3.00/0*

**Table 1. Carbohydrate Structures of Immunodominant Epitopes of the Sialyl SSEA-1 and I-Antigens, and Specific MoAbs Used in This Study**

| Antigen | Antibody | Structure of Immunodominant Epitope |
|---|---|---|
| Sialyl | FH-6 | NeuAc$\alpha$2 → 3Gal$\beta$1 → 4GlcNAc$\beta$1 → 3Gal$\beta$1 → 4GlcNAc$\beta$1 → 3R |
| SSEA-1 | (IgM) | Fuc$\alpha$1 ↗ 3 ±(Fuc$\alpha$1 ↗ 3)* |
| I-antigen | C6 | Gal$\beta$1 → 4GlcNAc$\beta$1 → 3Gal$\beta$1 → 4GlcNAc$\beta$1 → 3R |
| | (IgM) | Gal$\beta$1 → 4GlcNAc$\beta$1 ↗ 6 |

* The symbol ± indicates that the second fucose residue is not strictly required for the antibody to react with the antigen. The antibody FH-6 was initially raised against the sialyl Le$^x$ carried by polylactosamine with $\alpha$1 → 3 internal fucosyl substitution,[13] but later turned out to react also with sialyl Le$^x$ carried by a polylactosamine with at least two repeating N-acetyllactosamine units.[14]

*Preparation of cells and fluorescence-activated flow cytometry.* Peripheral blood samples were obtained from healthy donors and patients with nonhematologic disorders or patients with lymphoid malignancies at Kyoto University Hospital. Lymphocytes were isolated from Ficoll/Paque (Pharmacia, Uppsala, Sweden) by standard methods.[16] For stimulation of peripheral lymphocytes, lymphocytes were cultured at $1 \times 10^6$/mL in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) containing 5 $\mu$g/mL of concanavalin A (Con A; Pharmacia) for 96 hours, or containing 1% of the pokeweed mitogen (PWM) solution supplied by Life Technologies, Inc (Grand Island, NY) for 96 hours.

Cultured human lymphoid cell lines (Raji, FL-318, NALM-6, YT, MOLT-3, and ATL-2) were obtained from the First Division of Department of Internal Medicine, Kyoto University, and MOLT-15 and P12/Ichikawa were kindly supplied by Dr Jun Minowada (Fujisaki Cell Center, Hayashibara Biology Research Institute, Okayama, Japan). These cells were cultured in RPMI 1640 medium supplemented with 10% FCS.[17-19]

Flow cytometric analysis of lymphocytes was performed using FACScan (Becton Dickinson). The indirect immunofluorescence method was applied for staining of lymphocytes with the antibodies C6 and FH-6, using a fluorescein isothiocyanate (FITC)-labeled rabbit antimurine IgM ($\mu$-chain specific) antibody as the second antibody (Cappel Inc, Malvern, CA). Contamination of monocytes in the lymphocyte preparation in the flow cytometric analysis was checked for by staining with an anti-CD 14 antibody (Leu M3), and was less than 1% in all analyses described in this report.

*Monolayer cell adhesion assay using human umbilical vein endothelial cells (HUVECs).* HUVECs (2 to 6 passages after isolation; obtained from Kurabou Co Ltd, Osaka, Japan) were stimulated with 1.0 ng/mL of recombinant (r) IL-1$\beta$ for 4 hours in 24-well plates.[20-22] To these plates, lymphocytes prepared from peripheral blood ($5 \times 10^6$/0.5 mL/well) or Con A blasts ($8 \times 10^6$/0.5 mL/well) were added and incubated for 20 minutes at room temperature with rotation (100 rpm). Flat-bottomed 96-well plates were used in the case of leukemia cells ($1 \times 10^5$/60 $\mu$L/well) and incubation was performed for 30 minutes at room temperature with rotation (120 rpm). The number of attached cells was counted directly under a microscope.[22] The rIL-1$\beta$ was obtained from the Central Research Laboratory of Otsuka Pharmaceutical Co (Tokushima Japan), and the recombinant basic fibroblast growth factor used for the in vitro culture of HUVECs was from the Central Research Laboratory of Takeda Pharmaceutical Co (Juso, Japan). In some experiments, the adherent and nonadherent fractions of lymphocytes were recovered and subjected to flow cytometric analysis for surface markers.

*MoAbs used for inhibition of cell adhesion.* Monoclonal anti-E-selectin and anti-ICAM-1 antibodies (BBA2 and BBA4, both murine $IgG_1$) were obtained from British Biotechnology Ltd (Abington, Oxon, UK), and these antibodies, when used, were preincubated with HUVECs at 50 $\mu$g/mL for 30 minutes at room temperature before the adhesion experiments with lymphocytes.[22] Monoclonal anti-lymphocyte function-associated antigen 1$\beta$ (anti-LFA1$\beta$) (CBL5, $IgG_1$) and very late antigen 4 (anti-VLA4) (HP2/1, $IgG_1$) were obtained from Immunotechs S.A. (Marseille, France), and these antibodies were preincubated with lymphocytes at 50 $\mu$g/mL for 30 minutes before application to the monolayer of HUVECs.

*Inhibition of cell adhesion with liposomes containing the ligand glycolipid.* The pure synthetic sialyl Le$^x$ glycolipid used for the inhibition experiment had the structure: NeuAc$\alpha$2 → 3Gal$\beta$1 → 4(Fuc$\alpha$1 → 3)GlcNAc$\beta$1 → 3Gal$\beta$1 → 4Glc$\beta$1 → 1Cer.[22,23]

The anomeric structures and linkages of the terminal four-sugar residues of this synthetic glycolipid are identical to those in the sialyl SSEA-1 epitope as shown in Table 1. The sialyl Le$^x$ liposome contained 40 $\mu$g of the glycolipid, 20 $\mu$g of cholesterol, and 40 $\mu$g of phosphatidylcholine/well. The control liposome contained the same amount of cholesterol and phosphatidylcholine, but no glycolipid. The liposome suspensions were preincubated with HUVECs for 30 minutes at room temperature before the addition of lymphocytes.

## RESULTS

*Expression of the sialyl SSEA-1 and I-antigens among peripheral lymphocytes in normal individuals.* The carbohydrate antigen sialyl SSEA-1 was expressed only in a very minor population (7.6% ± 4.6% [n = 60]) of peripheral lymphocytes of healthy individuals, when the FH-6 antibody was used for the flow cytometry. These sialyl SSEA-1$^+$ cells were confirmed to be essentially NK cells (Fig 1a), as reported by us earlier.[11] The antigen was absent on normal resting T cells (both CD4$^+$ and CD8$^+$ cells) and B cells.

On the other hand, a majority of peripheral blood lymphocytes in healthy individuals expressed the I-antigen as detected using the antibody, C6. The mean ± SD of the percentages of the I-antigen$^+$ cells in healthy donors were 67.9% ± 6.9% (n = 52). As shown in a typical cytofluorogram depicted in Fig 2a, the I-antigen$^+$ cells consist of two peaks; one small population, indicated by the arrow A, exhibited a very high antigen density, while the other population, as indicated by the arrow B, displayed a relatively low antigen density. In normal individuals, the I-antigen$^{high+}$ cells comprised 12.6% ± 3.5% and the I-antigen$^{dim+}$ cells comprised 56.2% ± 8.2% on average (n = 52).

Two-color analysis indicated that the I-antigen$^{high+}$ cells were B cells that were CD19$^+$ (Fig 2a). In fact, more than 95% of B cells in all of the tested healthy individuals strongly expressed the I-antigen. On the other hand, the I-antigen$^{dim+}$

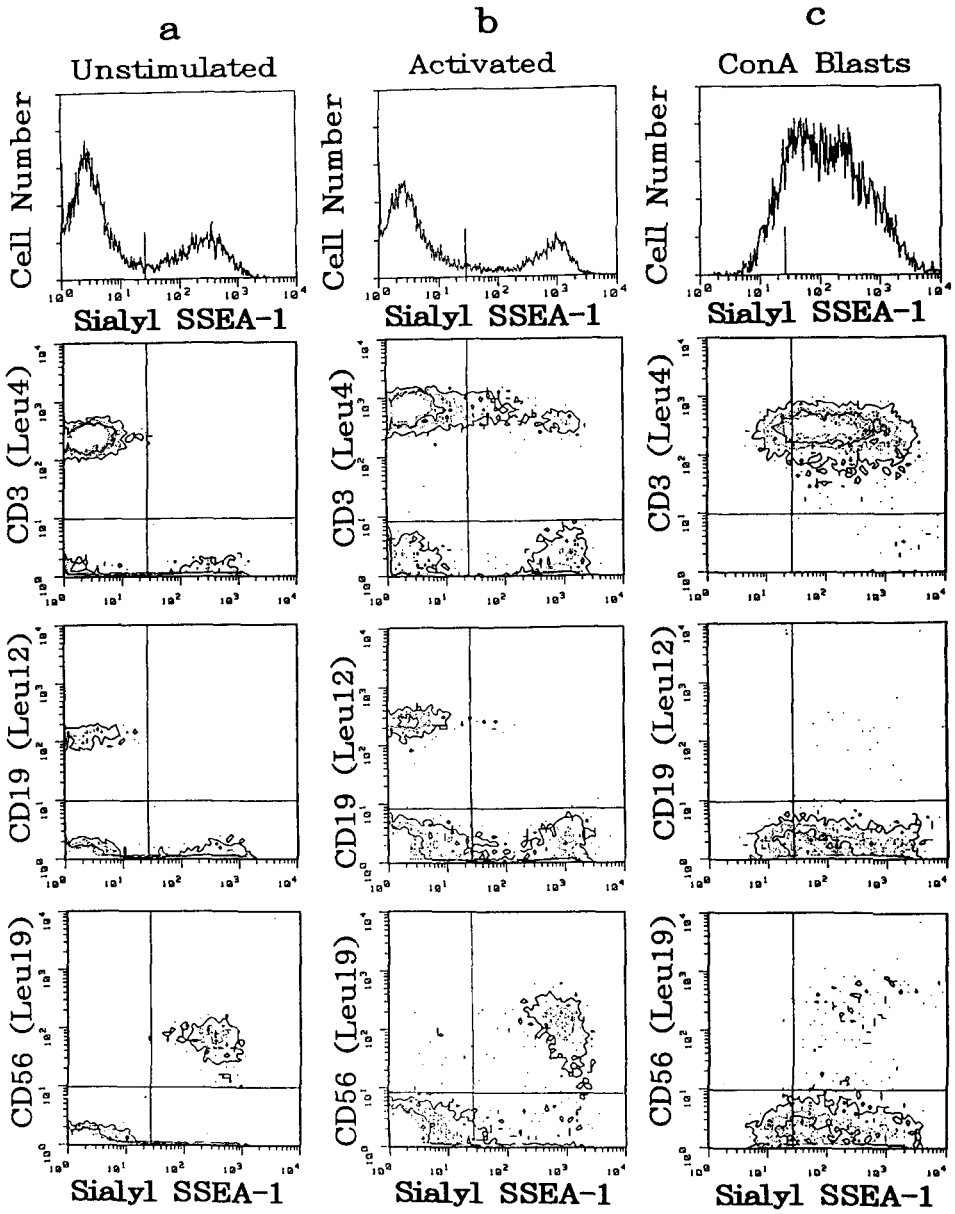


**Fig 1. Expression of sialyl SSEA-1 in normal peripheral lymphocytes (a), peripheral lymphocytes containing activated T cells (b), and Con A-stimulated lymphocytes (c). In each panel, the uppermost panel shows a cytofluorogram of the sialyl SSEA-1$^+$ cells, and the lower three panels show the results of two-color analysis. Lymphocytes were prepared from the peripheral blood of a healthy donor in panels a and c, and from a patient with a nonhematologic disorder in panel b. Sialyl SSEA-1 was detected with the FH-6 antibody, which is specific to sialyl Le$^x$-i hapten, by an indirect immunofluorescence method with FITC-labeled rabbit antimurine IgM ($\mu$-chain specific) antibody. In two-color analysis, lymphocytes were double-stained with phycoerythrin (PE)-labeled MoAbs against Leu 4 (CD3), Leu 12 (CD19), or Leu 19 (CD56) after staining with FH-6.**

cells were mostly CD3$^+$ T cells (Fig 2a). On average, 72.6% ± 6.8% of CD3$^+$ T cells were I-antigen$^+$ (n = 30).

Expression of the I-antigen was not specific to any particular subset of T cells; 85.6% ± 6.7% of CD4$^+$ cells and 50.7% ± 14.1% of CD8$^+$ cells expressed the antigen (n = 30). The NK cells in healthy individuals, as far as monitored with CD16 and CD56, were entirely I-antigen negative. In healthy individuals, the percentages of I-antigen$^{high+}$ and I-antigen$^{dim+}$ cells were essentially dependent on the percentages of mature B and T cells; the percentages of I-antigen$^-$ cells was a function of the percentage of NK cells.

*Expression of the sialyl SSEA-1 and I-antigens among peripheral lymphocytes in patients with nonhematologic disorders.* When the peripheral lymphocytes from patients with various nonhematologic disorders were tested for expression of the sialyl SSEA-1 and I-antigens ad libitum, some patients with chronic infection or malignancies of organs other than the hematopoietic system were occasionally found to have a relatively low frequency of I-antigen$^{dim+}$ cells. A typical example is shown in Fig 2b. The results of detailed surface marker analysis indicated that these patients had a relatively high percentage of activated T cells, which was reflected by the increased number of HLA-DR$^{dim+}$ T cells. In patients with nonhematologic disorders, a good negative correlation ($r = -.704$) was observed between the percentage of the I-antigen$^+$CD3$^+$ cells and the percentage of the HLA-DR$^{dim+}$CD3$^+$ cells among CD3$^+$ cells (Fig 3a). The expression of I-antigen on T cells is concluded to be highly dependent on their activation status.

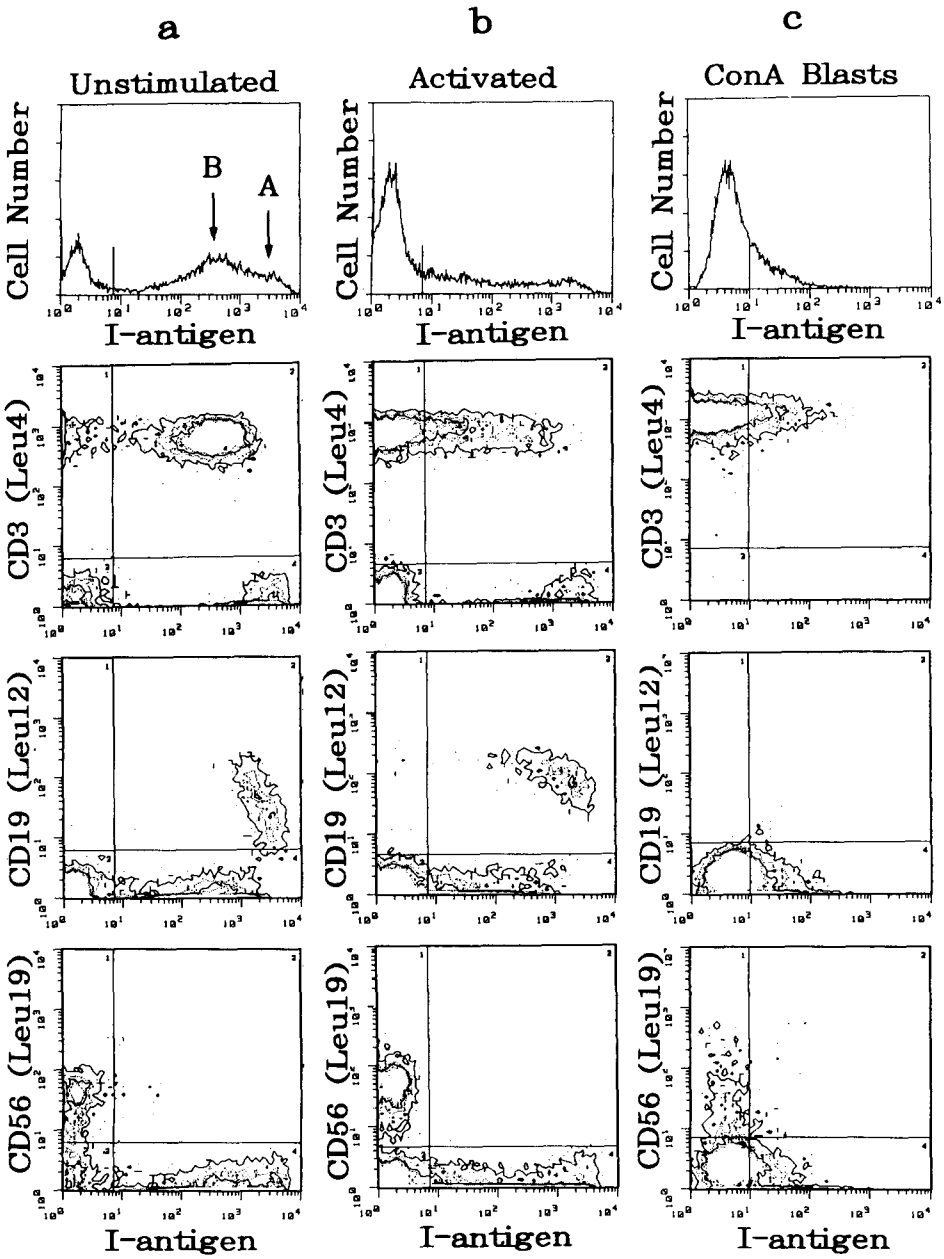


**Fig 2. Expression of the I-antigen in normal peripheral lymphocytes (a), peripheral lymphocytes containing activated T cells (b), and Con A-stimulated lymphocytes (c). In each panel, the uppermost panel shows a cytofluorogram of the I-antigen$^+$ cells, and the lower three panels show the results of two-color analysis. Lymphocytes were prepared from the peripheral blood of a healthy donor in panels a and c, and from a patient with a nonhematologic disorder in panel b. The I-antigen was detected with the monoclonal C6 antibody by an indirect immunofluorescence method with FITC-labeled rabbit anti-murine IgM ($\mu$-chain specific) antibody. In two-color analysis, lymphocytes were double-stained with PE-labeled MoAbs against Leu 4 (CD3), Leu 12 (CD19), or Leu 19 (CD56), after staining with C6. Arrow A indicates I-antigen$^{high+}$ cells, and arrow B indicates I-antigen$^{dim+}$ cells.**

A considerable proportion of these I-antigen–negative activated T cells expressed sialyl SSEA-1. A typical example of such a patient is shown in Fig 1b, indicating that an activated population of CD3$^+$ T cells expresses sialyl SSEA-1. Again a significant negative correlation ($r$ = .528) was observed between the proportion of the I-antigen$^+$CD3$^+$ cells and the proportion of the sialyl SSEA-1$^+$CD3$^+$ cells in the CD3$^+$ cells of these patients (Fig 3b). The relative percentages of I-antigen$^{dim+}$ cells and sialyl SSEA-1$^+$ cells among peripheral T cells seemed to reflect the activation status of T cells in the respective patient.

*Expression of the sialyl SSEA-1 and I-antigens in lymphocytes undergoing blastogenesis.* To further test the hypothesis that activation of T cells results in a decrease of I-antigen$^+$ cells and increase of sialyl SSEA-1$^+$ cells, peripheral lymphocytes from normal individuals were cultured and stimulated with Con A. This resulted in the rapid loss of I-antigen expression (Fig 2c), and appearance of sialyl SSEA-1 (Fig 1c). After 4 days of culture, the I-antigen$^+$ cells almost entirely disappeared, as shown in Fig 2c. More than 85% of the Con A blasts strongly expressed sialyl SSEA-1, which were identified as CD3$^+$ T cells undergoing blastogenesis (Fig 1c).

Similarly, when normal peripheral lymphocytes were stimulated with PWM, B cells, as well as T cells, gradually lost the I-antigen from the cell surface (Fig 4, right panel), whereas CD19$^+$ B blasts acquired sialyl SSEA-1 (Fig 4, left panel).

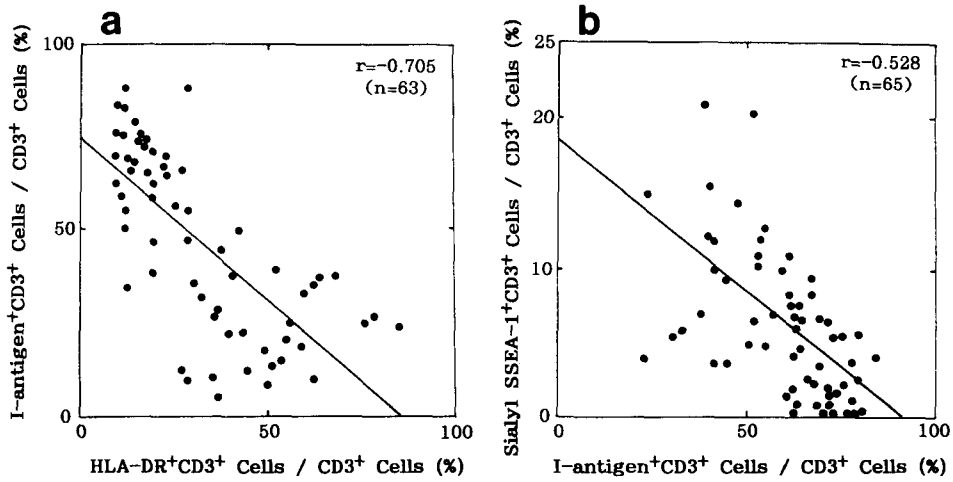


**Fig 3. Expression of the sialyl SSEA-1 and I-antigens on peripheral T cells in patients with nonhematologic disorders. (a) The correlation of the percentage of I-antigen$^+$ cells in total T cells versus the percentage of HLA-DR$^+$ cells in total T cells. (b) The correlation of the percentage of sialyl SSEA-1$^+$ cells in total T cells versus the percentage of I-antigen$^+$ cells in T cells. Sialyl SSEA-1 was stained with the FH-6 antibody, which is specific to the sialyl Le$^x$-i hapten, and the I-antigen was stained with the C6 antibody.**

*Expression of the sialyl SSEA-1 and I-antigens in malignant cells of various types of lymphocytic leukemia.* Expression of the sialyl SSEA-1 and I-antigens appears to be differentiation dependent in lymphocytic leukemia cells. Figure 5a shows the frequencies of sialyl SSEA-1$^+$ cells in leukemic cells of various human lymphoid malignancies. The antigen is preferentially expressed on blasts having immature characteristics such as cALL blasts, and is essentially absent on leukemia cells in patients with chronic lymphocytic leukemia (CLL) and adult T-cell leukemia (ATL) (see also ref 8). This distribution pattern contrasts with that of the I-antigen (Fig 5b). The leukemia cells in patients with CLL and ATL, which have relatively well-differentiated properties such as surface expression of IgM or CD4, exhibited a very high frequency of I-antigen, whereas the antigen was less frequently positive on blasts in cALL that have immature characters.

When the ratio of the two antigens, ie, I-antigen$^+$ leukemia cells (%)/sialyl SSEA-1$^+$ leukemia cells (%), was plotted for these patients, it was usually over 10 in patients with CLL and ATL, and mostly below 1.0 in patients with cALL. The ratio showed a wide range of values, from 0.04 to 2.1, in patients with cALL.

From the results described above, the I-antigen was concluded to be a marker for well-differentiated leukemia cells, and sialyl SSEA-1 a marker for leukemia cells having relatively undifferentiated properties. Table 2 displays the results on the cultured human lymphocytic leukemia cells, which also generally support this conclusion. These carbohydrate markers were distinct and did not parallel other B-cell markers

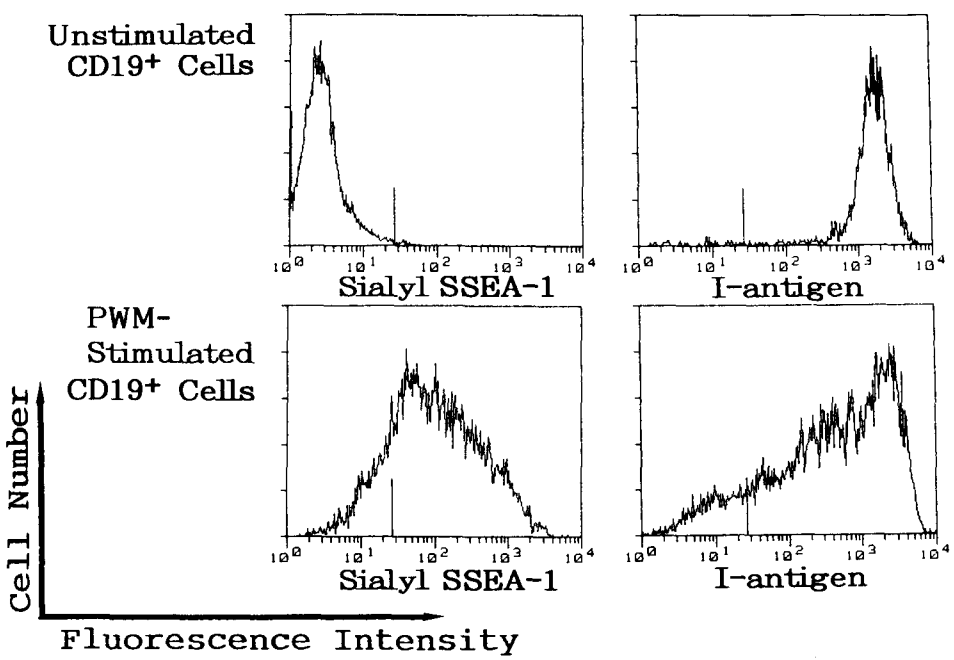


**Fig 4. Expression of the sialyl SSEA-1 and I-antigens in normal peripheral CD19$^+$ lymphocytes (upper panel) and PWM-stimulated CD19$^+$ lymphocytes (lower panel). Sialyl SSEA-1 was stained with the FH-6 antibody that is specific to the sialyl Le$^x$-i hapten, and the I-antigen was stained with the C6 antibody.**

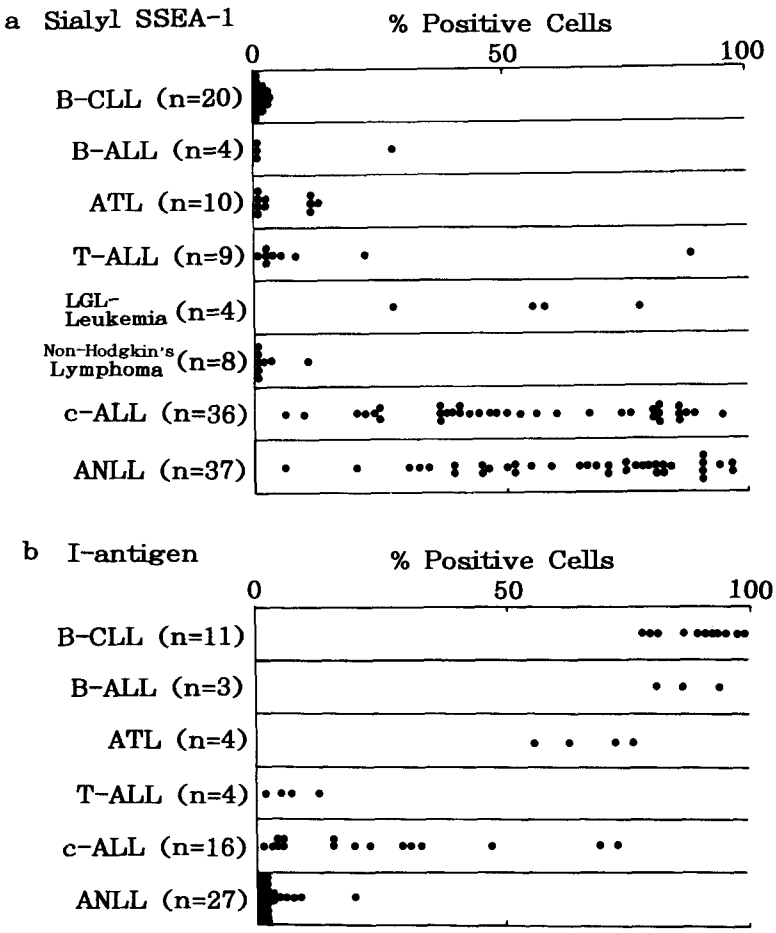


**Fig 5. Occurrence of the sialyl SSEA-1 (upper panel) and I-antigens (lower panel) in malignant cells in patients with various lymphocytic and nonlymphocytic leukemias.**

like CD10, CD19, CD20, and SmIg, or T-cell markers such as CD3, CD4, CD5, CD7, and CD8.

*Adhesion of normal peripheral lymphocytes to endothelial cells and its relation to the expression of carbohydrate antigens.* Because sialyl SSEA-1 has recently been reported to be the specific ligand for E-selectin, a cell adhesion molecule that is present on vascular endothelial cells,[4-7] we next investigated the role played by these carbohydrate antigens in the adhesion of lymphocytes to cultured HUVECs preactivated with rIL-1$\beta$.

Lymphocytes prepared from healthy individuals exhibited significant adhesion to the rIL-1$\beta$–activated HUVECs in a monolayer cell adhesion assay as indicated in Fig 6a. Peripheral lymphocytes of normal individuals contained variable percentages of the sialyl SSEA-1$^+$ NK cells. Case 1 in Fig 6a represents a healthy individual who had a high percentage of sialyl SSEA-1$^+$ NK cells (more than 30%). Case 2 is an example of a case with an average number of sialyl SSEA-1$^+$ NK cells. Adhesion of lymphocytes to the rIL-1$\beta$–activated HUVECs was strongly inhibited in case 1, and

**Table 2. Expression of the Sialyl SSEA-1 and I-Antigens in Cultured Human Lymphocytic Leukemia and Lymphoma Cells (%)**

| Cells | Origin | Sialyl SSEA-1 | I-Antigen | CD3 Leu4 | CD4 Leu3a | CD8 Leu2a | CD5 Leu1 | CD7 Leu9 | CD10 J5 | CD19 Leu12 | CD20 Leu16 | SmIg | CD25 IL2R | HLA-DR | CD34 HPCA-1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| FL-318 | B-NHL | 0.1 | 64.3 | 1.3 | 0.3 | 0.5 | 0.3 | 0.4 | 99.1 | 99.9 | 99.9 | 98.2 | NT | 99.6 | 0.2 |
| Raji | Burkitt | 0.3 | 98.3 | 0.3 | 0.1 | 0.7 | 0.2 | 0.5 | 99.9 | 100.0 | 100.0 | 0.7 | 0.7 | 99.8 | 0.2 |
| ATL-2 | ATL | 1.1 | 89.1 | 2.0 | 99.7 | 2.0 | 98.9 | 1.4 | 2.0 | 1.4 | 0.9 | 0.9 | 99.9 | 99.9 | 0.7 |
| NALM-6 | ALL | 79.9 | 41.4 | 1.1 | 0.2 | 0.7 | 0.2 | 0.6 | 99.9 | 100.0 | 19.1 | 0.2 | 0.4 | 99.3 | 0.3 |
| P12/Ichikawa | ALL | 70.7 | 15.4 | 6.4 | 29.2 | 2.6 | 99.1 | 97.3 | 0.8 | 5.4 | 1.9 | 1.8 | 0.8 | 6.0 | 1.2 |
| MOLT-15 | ALL | 55.1 | 11.7 | 1.2 | 0.4 | 1.6 | 0.4 | 71.3 | 1.9 | 1.1 | 1.7 | 0.6 | 1.3 | 1.5 | 88.8 |
| MOLT-3 | ALL | 92.7 | 1.9 | 1.4 | 0.8 | 1.2 | 0.6 | 63.6 | 2.0 | 0.6 | 1.1 | 0.6 | 0.4 | 98.3 | 0.8 |
| YT | LGLL | 90.1 | 0.4 | 0.2 | 0.2 | 0.2 | 0.1 | N.T. | 1.6 | 0.1 | 1.8 | 0.1 | 0.1 | 0.2 | 0.2 |

Abbreviations: NT, not tested; B-NHL, B-cell type non-Hodgkin's lymphoma; LGLL, large granular lymphocytic leukemia; ALL, acute lymphocytic leukemia.

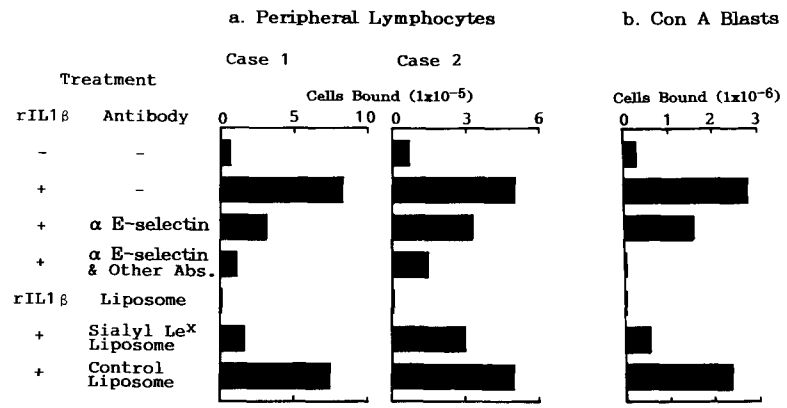


**Fig 6. Adhesion of normal peripheral lymphocytes (a) or Con A blasts (b) to rIL-1 β–activated HUVECs, and effect of pretreatment with MoAbs directed to cell adhesion molecules or liposomes containing sialyl Le$^x$ determinant. Anti–E-selectin antibody was used for the pretreatment of HUVECs at 50 μg/mL for 30 minutes at room temperature before the incubation with lymphocytes. Pretreatment with "Anti–E-selectin and other antibodies" indicates that HUVECs were pretreated with 50 μg/mL of anti–E-selectin and anti–ICAM-1 antibodies, while lymphocytes were pretreated with 50 μg/mL of anti-LFA1 and anti-VLA4 antibodies for 30 minutes at room temperature before the adhesion experiment. For liposome inhibition, HUVECs were pretreated with a liposome suspension containing 40 μg of synthetic sialyl Le$^x$ glycolipid for 30 minutes at room temperature before the incubation with lymphocytes. For cases 1 and 2, see text.**

moderately in case 2, by the preincubation of HUVECs with anti–E-selectin antibody. In these cases, liposomes containing pure sialyl Le$^x$ glycolipid carrying the same hapten structure as sialyl Le$^x$ also significantly inhibited the adhesion of lymphocytes to HUVECs.

Adhesion of lymphocytes to activated HUVECs is known to be mediated not only by E-selectin/sialyl SSEA-1, but also by intercellular adhesion molecule-1 (ICAM-1)/LFA1 and vascular cell adhesion molecule-1 (VCAM-1)/VLA4.[24,25] Therefore, the effect of the addition of the antibodies against cell adhesion molecules other than E-selectin, ie, ICAM-1, LFA1, and VLA4, was studied, to evaluate the participation of the other two cell adhesion systems. In case 1, the roles played by the two systems other than the E-selectin/sialyl SSEA-1 were less significant, as the addition of anti-ICAM, LFA1, and VLA4 antibodies produced an almost negligible increase in the inhibitory effect. In case 2, a significant participation of the two cell adhesion systems was observed, as the addition of the antibodies directed to the components of the ICAM-1/LFA1 and VCAM-1/VLA4 systems further inhibited the lymphocyte adhesion.

Next, monolayer cell adhesion experiments were performed on a large scale using peripheral lymphocytes from case 1, and the expression of sialyl SSEA-1 and I-antigens in the adherent and nonadherent fractions of lymphocytes was analyzed by flow cytometry. As shown in Fig 7a, the sialyl SSEA-1$^{high+}$ lymphocytes (indicated by arrows) were remarkably enriched in the adherent fraction of lymphocytes, and almost completely depleted in the nonadherent fraction. On the other hand, the lymphocyte population that adhered to HUVECs contained only a negligible number of I-antigen$^+$ cells (indicated by arrowheads), and essentially all I-antigen$^+$ lymphocytes were recovered from the nonadherent fraction. Adherent sialyl SSEA-1$^+$ I-antigen$^-$ cells, as expected, were identified as NK cells by surface marker analysis (data not shown).

*Adhesion of Con A blasts to endothelial cells and its relation to the expression of carbohydrate antigens.* Con A blasts, which strongly express sialyl SSEA-1, also exhibited significant E-selectin–dependent adhesion to the rIL-1β-activated HUVECs (Fig 6b). The number of adherent cells far exceeded that observed in unstimulated peripheral lymphocytes from normal individuals, even that observed in case 1. The role played by the sialyl SSEA-1/E-selectin adhesion system was significant, as is shown by liposome inhibition experiments. The other two cell adhesion systems, ie, ICAM-1/LFA1 and VCAM-1/VLA4, also seemed to participate in the reaction, judging from the further increase in the inhibition of adhesion by the addition of antibodies against these molecules. When analyzed by flow cytometry (Fig 7b), the cells adherent to HUVECs were again remarkably enriched with sialyl SSEA-1$^{high+}$ cells as indicated by an arrow, while nonadherent cells were negative or only weakly positive for sialyl SSEA-1. The I-antigen was not analyzed, because it was not expressed on these Con A blasts from the beginning of the adhesion experiments. The adherent cells were shown to be mainly CD3$^+$ T blasts (data not shown).

*Adhesion of cultured lymphocytic leukemia cells to endothelial cells.* Several cultured human lymphocytic leukemia cells also showed a significant E-selectin–dependent adhesion to the rIL-1β-activated HUVECs (Fig 8). Liposome inhibition experiments clearly indicated the participation of E-selectin in the adhesion of leukemia cells to HUVECs. The inhibition pattern obtained by the addition of antibodies against cell adhesion molecules was very similar to the pattern observed with Con A blasts; ie, addition of any one antibody never yielded complete inhibition of adhesion, and a significant contribution of E-selectin was seen only when the appropriate combination of antibodies was used.

## DISCUSSION

The I-antigen was first described as a differentiation antigen in human erythrocytes.[26-29] It is well known that erythrocytes

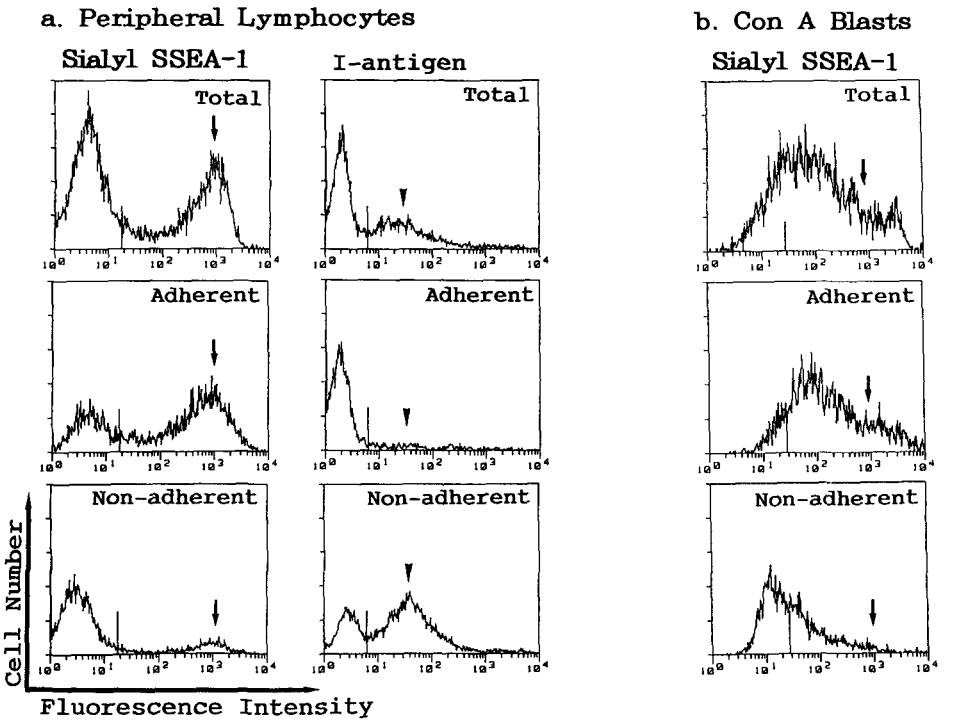


**Fig 7. Expression of the sialyl SSEA-1 and I-antigens on normal peripheral lymphocytes (a) or Con A blasts (b) that were adherent or nonadherent to rIL-1β–activated HUVECs. Upper panel, normal peripheral lymphocytes or Con A blasts before adhesion experiments; middle panel, adherent fraction; lower panel, nonadherent fraction. Sialyl SSEA-1 was stained with the FH-6 antibody that is specific to the sialyl $Le^x$-i hapten, and the I-antigen was stained with the C6 antibody. Arrows indicate sialyl SSEA-$1^{high+}$ cells and arrowheads indicate I-$antigen^+$ cells.**

undergo a developmental change from i-antigenic cord erythrocytes to I-antigenic adult erythrocytes.[27,28] Most leukocytes are known to express the i-antigen but not I-antigen, and it has been reported that some lymphocytes as well as CLL leukemia cells exceptionally express the I-antigen.[30-34] Our present results confirmed and expanded the knowledge on the distribution of I-antigen in human lymphoid cells, using a specific murine MoAb instead of classical human sera containing cold agglutinins. The I-antigen is expressed preferentially by lymphoid cells having relatively well-differentiated characteristics. The distribution pattern of the I-antigen in lymphoid cells is in clear contrast to the distribution of sialyl SSEA-1, which is expressed in cells having immature properties. It is noteworthy that sialyl SSEA-1 is closely related to the i-antigen;[35] ie, SSEA-1 is the i-antigen that is modified by fucose residues in its carbohydrate structure.[10]

Expression of the sialyl SSEA-1 and I-antigens in normal human lymphocytes is dependent on the cell lineage. Resting mature T and B cells are usually I-$antigen^+$ sialyl SSEA-$1^-$, while only NK cells are I-$antigen^-$ sialyl SSEA-$1^+$. The expression of the I- and sialyl SSEA-1 antigens is not fixed in normal T and B cells, but is reversible depending on the activation status of the cells. Resting I-$antigen^+$ sialyl SSEA-$1^-$ T and B cells become I-$antigen^-$ sialyl SSEA-$1^+$ on activation or blastogenesis. It was also observed that Con A blasts convert gradually from I-$antigen^-$ sialyl SSEA-$1^+$ status to resting I-$antigen^+$ sialyl SSEA-$1^-$ status on prolonged culture in vitro (data not shown).

Judging from the expression pattern in lymphocytic leukemia cells, lymphocytes of T- and B-cell lineages seem to undergo an antigenic shift generally from immature I-$antigen^-$ sialyl SSEA-$1^+$ status to I-$antigen^+$ sialyl SSEA-$1^-$ status on differentiation, while NK cells always show the I-$antigen^-$ sialyl SSEA-$1^+$ pattern. The putative sequence of the differentiation-dependent expression of these antigens in normal lymphoid cells is illustrated in Fig 9. The ratio of the I-an-

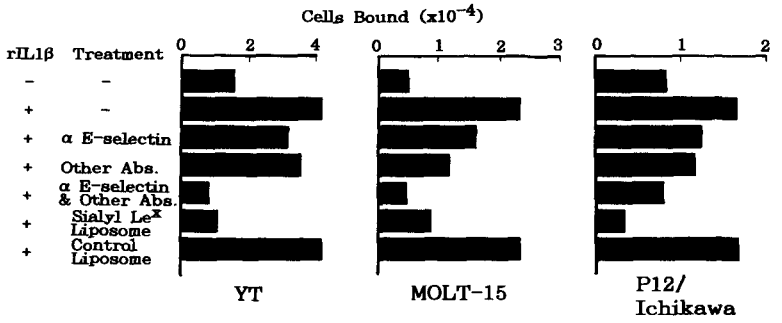


**Fig 8. Adhesion of cultured lymphocytic leukemia cells (YT, MOLT-15, and P12/Ichikawa) to rIL-1β–activated HUVECs, and effect of pretreatment with MoAbs directed to cell adhesion molecules or liposomes containing sialyl $Le^x$ determinant. "Other antibodies" indicates pretreatment of cells with anti–ICAM-1, anti-LFA1, and anti-VLA4 antibodies (50 μg/mL of each antibody). For details, see the legend for Fig 7.**

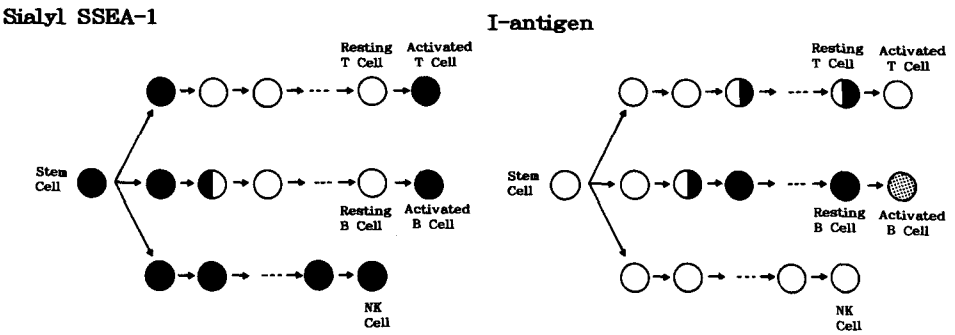


**Fig 9. Schematic presentation of expression of the sialyl SSEA-1 and I-antigens in human lymphoid cells. See text for details.**

tigen$^+$ blasts to the sialyl SSEA-1$^+$ blasts in patients with lymphocytic leukemia may serve as a "carbohydrate maturation index" that helps to evaluate the degree of differentiation of leukemia cells.

The sialyl SSEA-1 and I-antigens both belong to the type 2 chain polylactosamines in their carbohydrate structure (Table 1). The differentiation-dependent change from I-antigen$^-$ sialyl SSEA-1$^+$ status to I-antigen$^+$ sialyl SSEA-1$^-$ status is most probably mediated by alterations in the modifications of the polylactosamine backbones; loss of sialylation and fucosylation, followed by branch formation with GlcNAc$\beta$1 → 6 linkage.[12,36] Conversely, the restoration of I-antigen$^-$ sialyl SSEA-1$^+$ status from I-antigen$^+$ sialyl SSEA-1$^-$ status on blastogenesis is mediated by reduced branch formation, accompanied by increased sialylation and fucosylation. Our results indicate that these metabolic changes are reversible, and frequently occur in either direction in human lymphocytes during the course of differentiation or activation. It will be interesting to see whether changes occur only in the carbohydrate side chains carried by glycoproteins and glycolipids, or are accompanied by altered synthesis of the entire glycoprotein and glycolipid molecules involving the core protein and lipid moieties.

Sialyl SSEA-1 carries the terminal sialyl Le$^x$-hapten structure, which has recently been identified as a specific ligand for E-selectin, the cell adhesion molecule that appears in cytokine-activated endothelial cells.[4-7] Therefore, the carbohydrate antigen status at the lymphocyte surface may affect the behavior of the cells toward the vascular endothelial cells in vivo. Our results clearly indicated that the sialyl SSEA-1/E-selectin cell adhesion system plays a significant role in the binding of human lymphocytes to rIL-1$\beta$–activated HUVECs, in addition to other well-known systems such as ICAM-1/LFA1 and VCAM-1/VLA4. Sialyl SSEA-1$^+$ I-antigen$^-$ lymphocytes preferentially bound to activated endothelial cells, while most I-antigen$^+$ sialyl SSEA-1$^-$ lymphocytes did not.

The differential expression of the two antigens in various lymphocyte subpopulations would be quite beneficial to host defense mechanisms in inflammatory reactions. Among peripheral lymphocytes, only NK cells are known to have an effector function in the absence of any specific antigenic stimulation, and our results suggest that these cells may be preferentially recruited to the vessel wall at the site of inflammation, since they are sialyl SSEA-1$^+$ I-antigen$^-$. On the other hand, most resting T and B cells, which do not usually have any direct effector functions in the absence of antigenic stimulation, are mostly I-antigen$^+$ sialyl SSEA-1$^-$ according to our analysis, and would remain in the circulating blood. Once activated by specific antigens, these cells undergo blastogenesis and become sialyl SSEA-1$^+$ I-antigen$^-$, and this would lead to the recruitment of these cells to the vessel wall at the inflammatory site. Therefore, the sialyl SSEA-1/E-selectin system seems to play a role as a precise cell selection system at the vessel wall, by which the lymphocyte populations, expected to exert beneficial effector functions in inflammatory reactions, are specifically sorted out from the vast majority of resting lymphocytes. The continuous change in the sialyl SSEA-1 and I-antigenic status at the surface of lymphocytes may serve as an important regulatory mechanism in this cell selection system, and contribute to host defenses.

Recently a distinct subset of the CD4$^+$ memory T cells has been shown to express a carbohydrate antigen defined by the HECA-452 antibody, and this antigen is proposed to be one of the carbohydrate ligands for E-selectin. The specificity of the antibody HECA-452 seems to be broad, as it is reactive with both the sialyl Le$^x$ and sialyl Le$^a$ antigens.[37] It is proposed that the antigen detected on the surface of the subset of memory T cells has a structure related to these antigens.[37,38] However, the anti-sialyl SSEA-1 antibody FH-6 used in this report did not react with resting T cells in healthy individuals (ref 11 and this report). The anti-sialyl Le$^x$ antibody SNH3 was also not reactive with the memory T cells (data not shown). It is also reported that another anti-sialyl SSEA-1 antibody, CSLEX-1, is not reactive with memory T cells.[38] Further studies are necessary to identify the HECA452-reactive carbohydrate antigen expressed on memory T cells.

The acquisition and constant expression of sialyl SSEA-1 on lymphocytic leukemic cells reflect the immature characteristics of the malignant cells, and must be also related to the behavior of the leukemic cells in blood vessels. I-antigen$^-$ sialyl SSEA-1$^+$ leukemia cells probably possess significant adhesive activity to E-selectin–positive endothelial cells, leading to enhanced extravascular infiltration of leukemia cells. It is ironic that this antigen, which is designed to serve as an important component of self-defense mechanisms under normal conditions, mediates the expansion and progression

of malignant disorders via the very same molecular mechanism, once expressed on leukemic cells. In this sense, evaluation of the sialyl SSEA-1 and I-antigenic status of leukemic cells, the "carbohydrate maturation index," may serve as a prognostic marker in patients with lymphoid malignancies.

## ACKNOWLEDGMENT

We thank Drs Sen-itiroh Hakomori and Bruce A. Fenderson for their gifts of the MoAbs. We also thank Mikiko Sawada and Yoko Yoshinobu for their technical and secretarial assistance in preparing the manuscript.

## REFERENCES

1. Springer TA, Lasky LA: Sticky sugars for selectins. Nature 349: 196, 1991

2. Drickamer K: Two distinct classes of carbohydrate-recognition domains in animal lectins. J Biol Chem 263:9557, 1988

3. Brandley BK, Swiedler SJ, Robbins PW: Carbohydrate ligands of the LEC cell adhesion molecules. Cell 63:861, 1990

4. Lowe JB, Stoolman LM, Nair RP, Larsen RD, Berhend TL, Marks RM: ELAM-1–dependent cell adhesion to vascular endothelium determined by a transfected human fucosyltransferase cDNA. Cell 63:475, 1990

5. Phillips ML, Nudelman E, Gaeta FCA, Perez M, Singhal AK, Hakomori S, Paulson JC: ELAM-1 mediates cell adhesion by recognition of a carbohydrate ligand, sialyl-$Le^x$. Science 250:1130, 1990

6. Waiz G, Aruffo A, Kolanus W, Bevilacqua M, Seed B: Recognition by ELAM-1 of the sialyl-$Le^x$ determinant on myeloid and tumor cells. Science 250:1132, 1990

7. Tiemeyer M, Swiedler SJ, Ishihara M, Moreland M, Schweingruber H, Hirtzer P, Brandley BK: Carbohydrate ligands for endothelial-leukocyte adhesion molecule 1. Proc Natl Acad Sci USA 88: 1138, 1991

8. Solter D, Knowles BB: Monoclonal antibody defining a stage-specific embryonic antigen (SSEA-1). Proc Natl Acad Sci USA 75: 5565, 1978

9. Gooi HC, Feizi T, Kapadia A, Knowles BB, Solter D, Evans JM: Stage-specific embryonic antigen involves $\alpha 1 \rightarrow$ 3fucosylated type 2 blood group chains. Nature (Lond) 292:156, 1981

10. Kannagi R, Nuedlman E, Levery SB, Hakomori S: A series of human erythrocyte glycosphingolipids reacting to the monoclonal antibody directed to a developmentally-regulated antigen, SSEA-1. J Biol Chem 257:14865, 1982

11. Ohmori K, Yoneda T, Shigeta K, Hirashima K, Kanai M, Itai S, Sasaoki T, Arii S, Arita H, Kannagi R: Sialyl SSEA-1 antigen as a carbohydrate marker of human natural killer cells and immature lymphoid cells. Blood 74:255, 1989

12. Watanabe K, Hakomori S, Childs RA, Feizi T: Characterization of a blood group I-active ganglioside: Structural requirements for I and i specificities. J Biol Chem 254:3221, 1979

13. Fukushi Y, Nudelman E, Levery SB, Hakomori S: Novel fucolipids accumulating in human cancer. III, A hybridoma antibody (FH-6) defining a human cancer-associated difucoganglioside ($VI^3NeuAcV^3III^3Fuc_2nLc_6$). J Biol Chem 259:10511, 1984

14. Kannagi R, Fukushi Y, Tachikawa T, Noda A, Shin S, Shigeta K, Hiraiwa N, Fukuda Y, Inamoto T, Hakomori S, Imura H: Quantitative and qualitative characterization of cancer-associated serum glycoprotein antigens expressing fucosyl or sialosyl-fucosyl type 2 chain polylactosamine. Cancer Res 46:2619, 1986

15. Fenderson BA, Nichols EJ, Clausen H, Hakomori S: A monoclonal antibody defining a binary N-acetyllactosaminyl structure in lacto*iso*octaosylceramide ($IV^6Gal\beta 1 \rightarrow 4GlcNAcnLc_6$): A useful probe for determining differential glycosylation patterns between normal and transformed human fibroblasts. Mol Immunol 23:747, 1986

16. Lanier LL, Le AM, Phillips JH, Warner NL, Babcock GF: Subpopulation of human natural killer cells defined by expression of the Leu-7 (HNK-1) and Leu-11 (NK-15) antigens. J Immunol 131:1789, 1983

17. Minowada J, Minato K, Srivastava BIS, Nakazawa S, Kubonishi I, Tatsumi E, Ohnuma T, Ozer H, Freeman AI, Henderson ES, Gallo RC: A model scheme of human hematopoietic cell differentiation as determined by leukemia-lymphoma study: T-cell lineages, in Serrou B (ed): Current Concepts in Human Immunology and Cancer Immunomodulation. New York, NY, Elsevier, 1982, p 75

18. Doi S, Ohno H, Tatsumi E, Arita Y, Kamesaki H, Fukuhara H, Nishikori M, Miwa H, Kita K, Hatanaka M, Honjo T, Fujii H, Uchino H: Lymphoma cell line (FL-18) and Epstein-Barr virus (EBV) carrying cell line (FL-18-EB) obtained from a patient with follicular lymphoma: Their monoclonal derivation and different properties. Blood 70:1619, 1987

19. Yodoi J, Teshigawara K, Nikaido T, Fukui K, Noma T, Honjo T, Takigawa M, Sasaki M, Minato N, Tsudo M, Uchiyama T, Maeda M: TCGF (IL 2)-receptor inducing factor(s). I. Regulation of IL 2 receptor on a natural killer-like cell line (YT cells). J Immunol 134: 1623, 1985

20. Bevilacqua MP, Pober JS, Mendrick DL, Cotran RS, Gimbrone MA Jr: Identification of an inducible endothelial-leukocyte adhesion molecule. Proc Natl Acad Sci USA 84:9238, 1987

21. Bevilacqua MP, Stengelin S, Gimbrone MA Jr, Seed B: Endothelial leukocyte adhesion molecule 1: An inducible receptor for neutrophils related to complement regulatory proteins and lectins. Science 243:1160, 1989

22. Takada A, Ohmori K, Takahashi N, Tsuyuoka K, Yago K, Zenita K, Hasegawa A, Kannagi R: Adhesion of human cancer cells to vascular endothelium mediated by a carbohydrate antigen, sialyl Lewis A. Biochem Biophys Res Commun 179:713, 1991

23. Kameyama A, Ishida H, Kiso M, Hasegawa A: Total synthesis of sialyl Lewis X. Carbohydr Res 209:c1, 1991

24. Springer TA: Adhesion receptors of the immune system. Nature 346:425, 1990

25. Graber N, Gopal TV, Wilson D, Beall LD, Polte T, Newman W: T cells bind to cytokine-activated endothelial cells via a novel, inducible sialoglycoprotein and endothelial leukocyte adhesion molecule-1. J Immunol 145:819, 1990

26. Wiener AS, Unger LJ, Cohen L, Feldman J: Type-specific cold autoantibodies as a cause of acquired hemolytic anemia and hemolytic transfusion reactions: Biologic test with bovine red cells. Ann Intern Med 44:221, 1956

27. Marsh WL, Jenkins WJ: Anti-i: A new cold antibody. Nature (Lond) 188:753, 1960

28. Hakomori S: Blood group ABH and Ii antigens of human erythrocytes: Chemistry, polymorphism, and their developmental change. Semin Hematol 18:39, 1981

29. Kannagi R, Papaayannopoulou Th, Nakamoto B, Cochran NA, Yokochi T, Stamatoyannopoulos G: Carbohydrate antigen profile of human erythroleukemia cell lines, HEL and K562. Blood 62: 1230, 1983

30. Shumak KH, Rachkewich RA, Greaves MF: I and i antigens on normal human T and B lymphocytes and on lymphocytes from patients with chronic lymphocytic leukemia. Clin Immunol Immunopathol 4:241, 1975

31. Pruzanski W, Farid N, Keystone E, Armstrong M, Greaves MF: The influence of homogeneous cold agglutinins on human B and T lymphocytes. Clin Immunol Immunopathol 4:248, 1975

32. Childs RA, Kapadia A, Feizi T: Expression of blood group I and i active carbohydrate sequences on cultured human and animal cell lines assessed by radioimmunoassays with monoclonal cold agglutinins. Eur J Immunol 10:379, 1980

33. Childs RA, Feizi T: Differences in carbohydrate moieties of high molecular weight glycoproteins of human lymphocytes of T and B origins revealed by monoclonal autoantibodies with anti-I and anti-i specificities. Biochem Biophys Res Commun 102:1158, 1981

34. Pruzanski W, Katz A: Cold agglutinins-antibodies with biological diversity. Clin Immunol Rev 3:131, 1984

35. Niemann H, Watanabe K, Hakomori S, Childs RA, Feizi T: Blood group i and I activities of "lacto-N-*nor*hexaosylceramide" and its analogues: The structural requirements for i-specificities. Biochem Biophys Res Commun 81:1286, 1978

36. Holmes EH, Ostrander GK, Hakomori S: Biosynthesis of the sialyl-$Le^x$ determinant carried by type 2 chain glycosphingolipids ($IV^3NeuAcIII^3FucnLc_4$, $VI^3NeuAcV^3FucnLc_6$, and $VI^3NeuAcIII^3$-$V^3Fuc_2nLc_6$) in human lung carcinoma PC9 cells. J Biol Chem 261: 3737, 1986

37. Berg EL, Robinson MK, Mansson O, Butcher EC, Magnani JL: A carbohydrate domain common to both sialyl $Le^a$ and sialyl $Le^x$ is recognized by the endothelial cell leukocyte adhesion molecule ELAM-1. J Biol Chem 266:14869, 1991

38. Berg EL, Yoshino T, Rott LS, Robinson MK, Warnock A, Kishimoto TK, Picker LJ, Butcher EC: The cutaneous lymphocyte antigen is a skin lymphocyte homing receptor for the vascular lectin endothelial cell-leukocyte adhesion molecule 1. J Exp Med 174:1461, 1991