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Characterization of Sialosylated Lewis^x as a New Tumor-associated Antigen

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ABSTRACT

A monoclonal antibody CSLEX1 which reacts with sialosyl Le^x but not with sialosyl Le^a has been produced. The CSLEX1 antigen has a tissue distribution similar to that of Le^x, appearing characteristically in the proximal tubules of the kidney and on granulocytes. It is tumor associated in that 14 of 34 (41%) of tumor lines tested reacted with the CSLEX1 antibody, and 50 of 74 (68%) of tumor tissues tested reacted with the antibody. Loss of immunoperoxidase staining of tissues after neuraminidase treatment showed that the antibody is reacting to sialyl derivatives. The antibody reacted in solid-phase radioimmunoassay to sialosyllactofucopentaosyl(III)ceramide and sialosyldifucosylganglioside (6B). These results indicate that the CSLEX1 epitope has the following structure:

$$\begin{array}{l}\text{NeuAc}\alpha 2\rightarrow 3\text{Gal}\beta 1\rightarrow 4\text{GlcNAc}\beta 1\rightarrow 3\text{Gal}\beta\rightarrow\\ \qquad\qquad\quad 3\\ \qquad\qquad\quad\uparrow\\ \qquad\qquad\text{Fuc}\alpha 1\end{array}$$

This structure had not previously been known to be tumor associated.

INTRODUCTION

Immunization to various cancers and myeloid leukemia cells has led to production of monoclonal antibodies directed against the trisaccharide hapten of lacto-*N*-fucopentose III (3, 9, 10, 13, 14–16, 31). The structure is also denoted as Le^x (13), SSEA-1 (29), and My-1 (4). A glycolipid with this carbohydrate structure was found to accumulate in various human adenocarcinomas (38), and antibodies to this structure react with preimplantation mouse embryo, mouse teratocarcinoma, human granulocytes, various human cancer tissues, and normal cells such as tubules of the kidney and gastric glands (5). The trisaccharide hapten of lacto-*N*-fucopentose III has the following structure:

$$\begin{array}{l}\text{Gal}\beta 1\rightarrow 4\text{GlcNAc}\beta 1\text{-}\\ \qquad\quad 3\\ \qquad\quad\uparrow\\ \qquad\text{Fuc}\alpha 1\end{array}$$

We describe here a new monoclonal antibody that reacts with the sialosylated form of the Le^x antigen. To our knowledge, such an antibody against sialosylated Le^x has not been described previously. We provide data on its reactivity with various cancer cell lines, distribution in both tumor and normal tissues, and evidence that the antibody CSLEX1 reacts with sialosylated Le^x.

MATERIALS AND METHODS

Immunization and Somatic Cell Hybridization. Four- to 6-week-old female BALB/c mice were immunized s.c. with a 0.5-mg membrane protein from stomach adenocarcinoma tissue (32-OP-T-ST) emulsified in complete Freund's adjuvant. Two booster injections with the same amounts of membrane protein were given at 2-week intervals. After 3 days, fusion of spleen cells was done with myeloma P3-X63-Ag8.653 (21) by a modified method of Köhler and Milstein (23). Two weeks after fusion, supernatants were analyzed for antibody production by ELISA[3] and a microcytotoxicity test. After fusion, 138 macroscopic clones were identified, 17 of which were reactive with the immunizing tissue but not with normal stomach and colon tissues. From these 17 clones, a rapidly growing clone was selected, and subcloned twice by limiting dilution and passaged into BALB/c mice to produce ascites.

Tissues. Human tumor tissue from various organs was obtained at surgery and stored at −80°. Normal human tissue was obtained from cadaver kidney donors and from patients at autopsy with no neoplastic disease, then immediately frozen in isopentane:dry ice and stored at −80°.

Cell Lines. Established stomach cancer cell lines (MKN1, MKN28, MKN45, and MKN74 by Dr. H. Hojo and KATO-III by Dr. M. Sekiguchi) were kindly provided by the First Department of Pathology in Niigata University (Professor H. Watanabe), Japan. Stomach cancer line MK-92 was established by Dr. S. Mukai and Dr. Y. Kurosu (Nippon University, Japan). Lung and colon cancer lines (PC-1, PC-3, PC-6, PC-7, PC-8, PC-9, PC-10, PC-12, PC-13, PC-14, QG-56, and C-1) were kindly supplied by Dr. Y. Hayata (Tokyo Medical College, Japan) and Dr. K. Tanaka (Kyushu University, Japan). Colon cell line M-7609 was kindly provided by Dr. M. Fukushima (Hirosaki University, Japan). Esophagus cancer lines (TE-1 and SH1) were established by Dr. T. Nishihira (Tohoku University, Japan) and Dr. Iizuka (National Cancer Center, Japan). Other cell lines used in this study were obtained from the American Type Culture collection. All cell lines were maintained in culture in RPMI 1640 supplemented with 15% fetal calf serum, penicillin, and streptomycin.

Monoclonal Antibody Screening by ELISA. For the micro-ELISA testing, Terasaki tissue culture plates (Falcon) were coated overnight at 4° with various membrane fractions (at 25 μg/ml in bicarbonate buffer, pH 9.6) which were prepared by nitrogen decompression followed by differential centrifugation. After a wash in PBS:0.05% Tween 20, the wells were coated with 1% ovalbumin in bicarbonate buffer for 1 hr at 37°. Following removal of the ovalbumin, 5 μl of sample were added and incubated for 2 hr at 37°. After 3 washings with PBS:0.05% Tween 20), 5 μl of peroxidase-labeled goat antimouse immunoglobulin (IgG + IgM) (KPL Laboratories) were allowed to react for 1 hr at 37°. After washing 5 times, 5 μl of *O*-phenylenediamine were added at room temperature for 15 min. The reaction was stopped with 2.5 M sulfuric acid. The absorbance was measured at 492 nm with a Dynatech TR200 reader.

Microcytotoxicity Test. The complement-dependent microcytotoxicity test was performed according to standard microtechniques (30).

Indirect Immunofluorescent Assay. Indirect immunofluorescence was performed by reacting cells with 50 μl of appropriately diluted antibodies at room temperature for 30 min. After washing 3 times with PBS-0.01% sodium azide, the cells were incubated in 50 μl of fluorescein

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[3] The abbreviations used are: ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; TLC, thin-layer chromatography.

isothiocyanate conjugated goat antimouse IgM for another 30 min at 4°, followed by washing 3 times. The cells were examined by fluorescence microscopy. We used mouse myeloma IgM (10 to 20 μg/ml) as negative controls.

Immunoperoxidase Staining. Normal and neoplastic fresh tissues were used to examine immunochemical localization of the reactive antigens by immunoperoxidase staining. Cryostat-prepared tissue sections fixed in 4% formalin in Tris-buffered solution were incubated with 1:2000 diluted monoclonal antibodies (and mouse myeloma protein as a negative control) for 1 hr at room temperature. After washing in PBS, peroxidase-conjugated goat antimouse IgG + IgM F(ab)'$_2$ (Jackson Immuno-Research) was added to the tissue section for 45 min at room temperature. After being washed in PBS, the slides were treated for 6 min in 0.021% w/v 3-amino-9-ethylcarbozole (Sigma Chemical Co., St. Louis, MO) in 0.02 M sodium acetate buffer at pH 5.2 and 0.01% H_2O_2, then counterstained with hematoxylin, and mounted in glycerol:PBS.

Tissue sections were scored by staining intensity: ++, uniformly very dark; +, moderately dark; and ±, slightly greater than control staining. Control tissues in all cases were completely negative (−).

Enzyme Treatment. Enzymatic treatment of the stomach cancer tissue used for immunization was done by the standard ELISA technique, using Pronase (60 μg/0.1 ml; Calbiochem-Behring, San Diego, CA), trypsin (500 μg/0.1 ml; Worthington Biochemical, Freehold, NJ), ficin (10 μg/0.1 ml; Sigma), neuraminidase (0.5, 0.1, and 0.02 IU/ml; from *Vibrio cholerea*, Calbiochem), and sodium periodate (5 mM). The enzymatic reactions took place at 37° for 1 hr. Sodium periodate was incubated at 4° for 1 hr.

Neuraminidase treatment by immunoperoxidase was also done using neuraminidase (from *Arthrobactor ureafaciens*, Calbiochem) with incubations at 37° for periods of 2 hr.

Glycolipid Samples. Gangliosides, 6B and 6C, were prepared from human colonic adenocarcinoma as described in a preliminary note (12). A ganglioside, 7B, was prepared from human colonic adenocarcinoma.[4] The fraction containing sialosyl Le^a ganglioside (24) was prepared from human colonic cancer metastatic to liver. The major band in this fraction was identified as sialosyl Le^a because it released Le^a-active glycolipid on treatment with *Clostridium perfringens* sialidase in the absence of detergent (24). The Le^a-active core glycolipid released on sialidase treatment was a ceramide pentasaccharide with the same mobility as Le^a-active glycolipids isolated from human erythrocytes (x4b glycolipids). Sialosyllactofucopentaosyl(III)ceramide (27) was prepared from human kidney and is a gift from Dr. Heikki Rauvala, Department of Biochemistry, University of Helsinki, Helsinki, Finland. Difucosyllacto-*nor*-hexaosylceramide was prepared from human colonic cancer as described previously (11). Sialosylparagloboside was prepared from human erythrocyte membrane as described previously (28).

Solid-Phase Radioimmunoassay. Determination was made according to the procedure described by Kannagi *et al.* (20). Each well was coated with 10 ng of glycolipid together with 50 ng of lecithin and 30 ng of cholesterol.

TLC Immunostaining. TLC immunostaining was done on a Baker's high-performance TLC miniplate (5 × 6 cm) using the method of Magnani *et al.* (25), as modified by Kannagi *et al.* (19). Antibody was diluted 300 times and applied on a TLC plate in order to minimize nonspecific staining.

RESULTS

Reactivity against Normal Peripheral Blood Cells and Various Cell Lines. The CSLEX1 monoclonal antibody was tested for cytotoxic activity with normal panel cells as shown in Table 1. The IgM antibody (ascites titer 1:10^4) was cytotoxic to granulocytes and noncytotoxic to lymphocytes, monocytes, platelets, and RBC tested, although monocytes did react with CSLEX1 in immunofluorescence tests. Various cell lines were examined for reactivity by microcytotoxicity, immunofluorescence, and immunoperoxidase staining. Among leukemia-lymphoma lines tested, CSLEX1 reacted only with APL line HL-60 and histiocytic lymphoma line U-937, but not to T-ALL lines 8402, CEM, MOLT-4, or HPB-MLT; B-lymphoma lines Daudi, Ramos, Raji, or Wel; cALL lines KM-3 or Reh; or CML line K-562.

Among 34 various solid tumor cell lines examined, CSLEX1 yielded positive results with 2 stomach carcinoma lines (KATO-III and MKN28), one lung adenocarcinoma line (PC-3), 3 lung squamous cell carcinoma lines (PC-1, PC-9, and QG-56), 5 colon adenocarcinoma lines (C-1, M7609, COLO 205, WiDr, and SK-CO-1), 2 breast carcinoma lines (SK-BR-2 III and BT-20), and one esophagus tumor line (TE-1). A total of 14 of 34 cell lines (41%) showed a positive reaction. An especially high frequency of positive reactivity was observed in the colon adenocarcinoma lines (5 of 7 or 71%).

Tissue Distribution of the CSLEX1 Antigen in Normal and Malignant Tissues. The tissue distribution of CSLEX1 antibody-reactive antigen was examined by immunoperoxidase staining. Strong positive staining of normal tissues was observed in the glands and mucosa of the esophagus, as well as in the proximal tubules and descending loops of Henle of the kidney. Weaker staining was observed in very limited parts of some deep crypts of the colon, alveolar macrophages, some acinar cells of the pancreas, hepatic cells, and Kupffer cells in the liver, ureter, and granulocytes. The antigen was not detected in RBC, lymphocytes, stomach, lung parenchymal cells, bronchi, bile ducts, pancreatic ducts, brain, peripheral nerves, thymus, epidermis, sweat glands, arteries, veins, ovary, uterus, adrenal glands, muscle, or connective tissues. This reaction pattern is almost identical to that of Le^x, except that Le^x is expressed in stomach and brain, and CSLEX1 antigen is not.

Seventy-four various tumor tissues tested are shown in Table 2. Surprisingly, the antigen recognized by CSLEX1 antibody could be detected in many carcinomas: 16 of 17 stomach adenocarcinomas; 13 of 17 colon adenocarcinomas; 10 of 16 lung tumors; 2 of 4 esophagus tumors; 3 of 3 pancreas adenocarcinomas; 2 of 8 breast tumors; and 3 of 6 ovary tumors. Mouse myeloma IgM (5 to 10 μg/ml) was used as a control and did not react with any of these tissues. All samples contained only tumor tissue, except for 6 (of 17) colon adenocarcinoma samples, which contained both tumor tissue and adjacent normal tissue. In 5 of these 6 samples, the normal tissue portions were not stained. Cancerous portions of the positively reacting colon adenocarcinoma samples showed staining in the apical cytoplasm of the cancer tubules and in the luminal contents. Three of 4 stomach and 8 of 8 colon samples containing mucin lakes showed positive reactivity with this antibody, probably due to the presence of antigen on mucin. A high frequency of positive staining of tumor tissues by CSLEX1 was observed in adenocarcinomas such as stomach, colon, and lung without regard to differentiating grade of the cancer cells. Positive staining was also observed in some squamous cell carcinoma samples. The CSLEX1 antibody reacted with 50 of 74 (68%) tumors tested.

Enzyme Treatment of CSLEX1 Reactive Antigens. Treatment of the immunizing stomach adenocarcinoma with neuraminidase and sodium periodate competely diminished binding of CSLEX1. Treatment with Pronase partly decreased binding (Table 3A). These results suggest that the antigen on the immunizing tissue may be a sialosylated glycoprotein.

Immunoperoxidase staining of normal kidney tubules and

[4] E. Nudelman and S. Hakomori, unpublished data.

Table 1

Reactivity of monoclonal antibody CSLEX1 against normal panel cells and cancer cell lines

Cell tested	Origin	Cytotoxicity	Immuno-fluorescence	Immuno-peroxidase
Normal				
T-lymphocytes	Peripheral blood	0/110[a]	NT[b]	NT
B-lymphocytes	Peripheral blood and spleen	0/55	NT	NT
Monocytes	Peripheral blood	0/21	11/11	NT
Granulocytes	Peripheral blood	20/20 (1:10^4)[c]	20/20	NT
Platelets	Peripheral blood	0/15	NT	NT
RBC				
A	Peripheral blood	0/pooled	NT	NT
B	Peripheral blood	0/pooled	NT	NT
O	Peripheral blood	0/8	NT	NT
Leukemia-lymphoma				
8402	T-ALL	–	–	–
CEM	T-ALL	–	–	–
MOLT-4	T-ALL	–	–	–
HPB-MLT	T-ALL	–	–	–
Daudi	B-lymphoma	–	–	–
Ramos	B-lymphoma	–	–	–
Raji	B-lymphoma	–	–	–
Wel	B-lymphoma	–	–	–
KM-3	Common ALL	–	–	–
Reh	Common ALL	–	–	–
HL-60	Acute promyelocytic leukemia	+ (1:10^5)	+	+
U937	Histiocytic lymphoma	+ (1:10^5)	+	+
K562	Chronic myeloid leukemia	–	–	–
Stomach				
KATO-III	Signet-ring cell carcinoma	+ (1:10^4)	+	+
MKN28	Adenocarcinoma (well)	NT	+	+
MKN1	Adenosquamous cell carcinoma	NT	–	–
MKN45	Adenocarcinoma (poor)	–	–	–
MKN74	Adenocarcinoma (well)	–	–	–
MK-92	Signet-ring cell carcinoma	–	–	–
Lung				
PC-1	Squamous cell carcinoma (poor)	NT	+	+
PC-3	Adenocarcinoma (mod)	NT	+	+
PC-6	Small cell carcinoma	–	–	–
PC-7	Adenocarcinoma (poor)	–	–	–
PC-8	Adenocarcinoma (poor)	–	–	–
PC-9	Squamous cell carcinoma (well)	+ (1:10^5)	+	+
PC-10	Squamous cell carcinoma (mod)	–	–	–
PC-12	Adenocarcinoma (well)	–	–	–
PC-13	Large cell carcinoma	–	–	–
PC-14	Adenocarcinoma (poor)	–	–	–
QG-56	Squamous cell carcinoma	± (1:10^4)	+	+
A-549	Lung carcinoma	–	–	–
SK-LU-1	Adenocarcinoma (poor)	–	–	–
Colon				
C-1	Adenocarcinoma (poor)	NT	+	+
M-7609	Adenocarcinoma (poor)	± (1:10^4)	+	+
S-7512	Simple cancer	–	–	–
COLO 205	Adenocarcinoma	+ (1:10^4)	+	+
WiDr	Adenocarcinoma	± (1:10^4)	±	+~±
SK-CO-1	Adenocarcinoma	NT	+	+
COLO 320HSR	Adenocarcinoma	–	–	–
Breast				
SK-BR-1 III	Adenocarcinoma	–	–	–
SK-BR-2 III	Adenocarcinoma	+ (1:10^5)	+	+
BT-20	Adenocarcinoma	+ (1:10^4)	+	+
Esophagus				
TE-1	Squamous cell carcinoma (well)	NT	+	+
SH1	Squamous cell carcinoma	–	–	–
Liver				
SK-HEP-1	Adenocarcinoma	–	–	–
Bladder				
J82	Transitional cell carcinoma (poor)	–	–	–
SCaBER	Squamous cell carcinoma	–	–	–

[a] Number of positive per number of tested samples.

[b] NT, not tested; –, negative; ±, weakly positive; +, moderately or strongly positive; well, well differentiated; mod, moderately differentiated; poor, poorly differentiated.

[c] Reciprocal titer.

Table 2

Reactivity of the CSLEX1 antibody against tumor tissues

Cancer	No. of tumor tissues tested	% of positive samples	Reactivity +++[a]	++	+	±	Mucin[b]
Stomach	17	94	8	1	4	3	3/4
Colon	17	76	2	1	6	6	8/8
Lung							
AD	9	78	5	1	0	1	
SQ	4	50	1	0	1	0	
UD	3	33	0	0	0	1	
Total	16	63	6	1	1	2	
Esophagus	4	50	0	2	0	0	
Ovary	6	50	3	0	0	0	
Breast	8	25	0	1	0	1	
Bladder	1	100	1	0	0	0	
Kidney	1	0	0	0	0	0	
Pancreas	3	100	1	1	0	1	
Uterus	1	0	0	0	0	0	
Total	74	68					

[a] +++, diffuse; ++, 40 to 80%; +, 10 to 40%; ±, 1 to 10%; AD, adenocarcinoma; SQ, squamous cell carcinoma; UD, undifferentiated carcinoma.
[b] Number of positive per number of tested samples containing mucin lakes.

Table 3

Effect of enzyme treatment

A. ELISA of CSLEX1 with enzyme-treated immunizing tissue.

	Absorbance[a]
Control	1.55
Pronase	0.42
Trypsin	0.88
Ficin	1.39
$NaIO_4$	0.10
Neuraminidase[b]	
0.5 unit	0.14
0.1 unit	0.17
0.02 unit	0.35

B. Effect of neuraminidase on the expression of CSLEX1-reactive antigens by immunoperoxidase assay.

Tissue tested	Monoclonal antibody	Neuraminidase[c] treatment 0.5 unit	0.1 unit	0.01 unit	PBS
Kidney (1KD580)	CSLEX1	−	−	±[d]	++
	CLEX1	++	++	++	++
	Mouse myeloma IgM	−	−	−	−
Esophagus (1ES390)	CSLEX1	−	−	±	++
	CLEX1	++	++	++	++
	Mouse myeloma IgM	−	−	−	−

[a] Negative, <0.2 at A_{450}.
[b] Neuraminidase from *Vibrio cholerae* (Calbiochem).
[c] Neuraminidase from *Arthrobacter ureafaciens* (Calbiochem).
[d] For scoring of slides, see "Materials and Methods."

esophageal glands with CSLEX1 was abolished by neuraminidase treatment (Table 3B), whereas reactivity of antibody (CLEX1) directed against Le^x was not affected or increased by neuraminidase treatment.

Reactivity of the CSLEX1 with Various Gangliosides. Table 4 shows the structures of the various glycolipids used in the solid-phase radioimmunoassay and/or the TLC immunostaining assay. The structure of 6B-ganglioside has just recently been identified.[5] The reactivities with gangliosides at different antibody dilutions by solid-phase immunoradioassay are shown in Chart 1. It is obvious that the antibody reacted with sialosyllactofucopentaosyl(III)ceramide (Rauvala), sialosyl difucosyllacto-*nor*-hexaosylceramide (6B), and sialosyl monofucosyllacto-*nor*-octaosylceramide (7B), but not with others. Fig. 1 shows the TLC immunostaining pattern of gangliosides with the CSLEX1 antibody. CSLEX1 reacted with both 6B and 7B gangliosides and Rauvala's ganglioside, but with neither the sialosyl Le^a fraction nor other gangliosides.

Table 4 shows the reactivity of the CSLEX1 antibody with known novel fucogangliosides tested. It can be seen that the monoclonal antibody reacted with the first 3 gangliosides, which contain the sialosyl Le^x structure. The antibody did not react with similar derivatives having chemical structures which were slightly different (Table 4).

DISCUSSION

A monoclonal antibody, CSLEX1, is described here which defines a new tumor-associated antigen, a sialosylated Le^x antigen. This antigen has been found to have a tissue distribution similar to, but more restricted than, that reported previously for the nonsialosylated Le^x antigen (5). Among normal tissues, both Le^x antigen and the antigen detected by CSLEX1 are found on granulocytes, the proximal tubules of the kidney, and the descending loops of Henle. Among tumor cell lines, both antigens are found on HL-60 (an acute promyelocytic leukemia line) and U-937 (derived from a histiocytic lymphoma). This reaction pattern suggests that the distribution of CSLEX1 antigen is similar to that of Le^x. However, unlike Le^x, the antigen detected by CSLEX1 is neuraminidase sensitive. When tested against known fucogangliosides, the CSLEX1 antibody reacted with sialosyllactofucopentaosyl(III)ceramide, sialosyldifucosylganglioside (6B) and sialosylmonofucosyllacto-*nor*-hexaosyl ceramide (7B), but not with sialosyl Le^a, 6C ganglioside, difucosyllacto-*nor*-hexaosylceramide, or sialsylparagloboside. These results indicate that the epitope structure detected by CSLEX1 is sialosylated Le^x, a structure first detected in the human kidney and described by Rauvala (27).

Many of the monoclonal antibodies raised against cancer cells react primarily with terminal carbohydrate structures such as sialylated Le^a (24), Le^b (2), and Le^x (13). In fact, antibodies against the Le^x structure have been the most commonly produced monoclonal antibody following immunization with human cancer cells (3, 14, 15). This, along with the fact that monoclonal antibodies have been produced against other terminal carbohydrate groups such as RBC Group A (34), RBC Group B (14), RBC Group H type 2 (22), and A_1 Le^d (17), attests to the strong immunogenicity of the terminal carbohydrate structure when human cancer cells are used as immunogens in mice.

The Le^x structure is clearly stage-specific in mouse preimplantation embryo, expressed maximally at morulae, disappears during further development (29), and is then expressed again in various mouse adult tissues (6). The Le^x antigen or its di- or trimeric form is clearly tumor-associated in humans in that many human adenocarcinomas accumulate a large quantity of this antigen bound to a novel ceramide (11, 38). During development of the human embryo and fetus, maximum expression of Le^x and its di- or trimeric structure occurred between 38 and 85 days of gestation at the epithelia of various organs, particularly gastrointestinal epithelia. Expression of dimeric Le^x declined

[5] Y. Fukushi, E. Nudelman, S. B. Levery, S. Hakomori, and H. Rauvala, submitted for publication.

Table 4

Structures of novel fucogangliosides and their reactivity with the CSLEX1 monoclonal antibody

		Reactivity
Sialosyldifucosylganglioside (6B ganglioside) (12)	Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer 3 3 3 ↑ ↑ ↑ NeuAcα2 Fucα1 Fucα1	++
Sialosyllactofucopentaosyl(III)ceramide (Rauvala's ganglioside) (27)	Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer 3 3 ↑ ↑ NeuAcα2 Fucα1	++
Sialosylmonofucosyllacto-*nor*-octaosylceramide (7B ganglioside)[4]	Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer 3 3 ↑ ↑ NeuAcα2 Fucα1	++
Sialosyl Le^a (24)	Galβ1→3GlcNAcβ1→3Galβ1→4Glcβ1→1Cer 3 4 ↑ ↑ NeuAcα2 Fucα1	–
6C ganglioside (12)	Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer 6 3 ↑ ↑ NeuAcα2 Fucα1	–
Difucosyllacto-*nor*-hexaosylceramide (11)	Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer 3 3 ↑ ↑ Fucα1 Fucα1	–
Sialosylparagloboside (28)	Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer 3 ↑ NeuAcα2	–

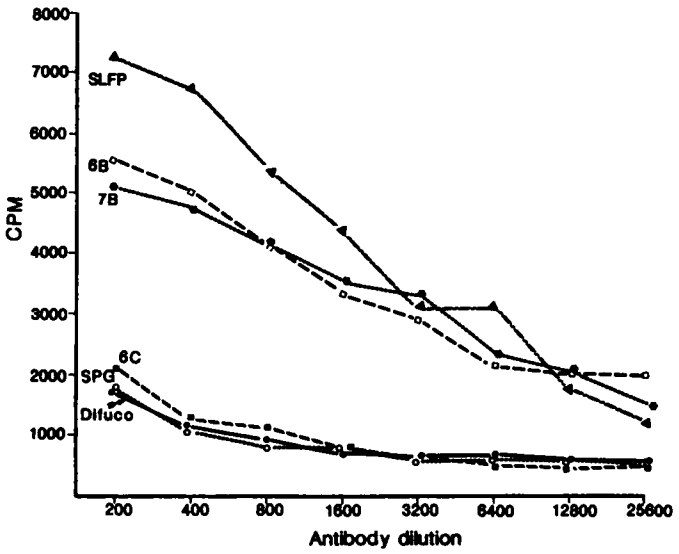

Chart 1. Reactivity of the CSLEX1 antibody by solid-phase radioimmunoassay with sialosyllactofucopentaosyl(III)ceramide (*SFLP*, ▲), sialosyldifucosylganglioside (*6B*, □), sialosylmonofucosyllacto-*nor*-octaosylceramide (*7B*, *), 6C ganglioside (*6C*, ■), sialosylparagloboside (*SPG*, ○), and difucosyllacto-*nor*-hexaosylceramide (*Difuco*, ●).

greatly on further development.[6] Interestingly, no Le^x antigen was observed to be expressed in various mouse cancers.[7] Thus, Le^x and its di- or trimeric structure are characteristic human oncofetal antigens. The nonsialosylated Le^x antigen has been shown to be present in 93% (52 of 56) of gastric carcinomas (35). The sialosylated form of Le^x antigen detected by CSLEX1 has been demonstrated here to be present in 94% (16 of 17) of stomach cancers, 76% (13 of 17) of colon cancers, and also in other adenocarcinomas and squamous cell carcinomas as shown in Table 2. Sialosylated Le^x, like Le^x, is tumor associated.

The Le^x determinants can be expressed in both glycolipid and glycoprotein molecules of a cell (31). Sialosylated Le^x has been detected on a glycolipid present in the human kidney (27). The presence of sialosyl Le^x in the luminal content of tubules, mucin lakes in stomach, and colon adenocarcinomas, as well as the observed partial reduction of CSLEX1 activity by Pronase suggest that the sialosylated form of Le^x is present on mucins (mucus glycoproteins).

The significance of sialylation in neoplasma has been the subject of many reports (8, 32, 36, 37, 39). An increase in sialyltransferase activity in cancer tissues (1) and in the sera of cancer patients has been reported (7). The presence of sialic acid on cell surfaces protects against proteolytic digestion (33), serves as a receptor, attenuates immunogenicity by masking carbohydrates, plays a role in adhesion (18), tends to increase solubility, or appears to correlate with metastatic ability (39). Sialosylated Le^x antigen was observed in both the apical cytoplasma of cancer tubules and the luminal contents, while the nonsialosylated form of Le^x antigen was detected only in the cytoplasma of the cancer cells, not in the luminal contents. These results indicate that the activity of sialyltransferase may be increased in the surface membrane of cancer cells and the sialosylated form of Le^x may be solubilized, then secreted in the lumen.

By immunofluorescence, the sialosylated Le^x epitope appears to be on monocytes as shown by strong fluorescence in 11 of 11 samples tested. Yet by cytotoxicity, the antibody failed to kill monocytes from 21 blood samples. Nonspecific staining of monocytes is unlikely, since many other monoclonal antibodies directed against other specificites failed to stain monocytes. Although monocytes are more resistant to cytotoxic lysis than lymphocytes, several monoclonal antibodies other than CSLEX1 were shown to readily lyse monocytes. The discrepancy remains unexplained at the present time.

In a separate study, the CSLEX1 antibody was shown to react with 45% (23 of 51) of lung adenocarcinoma patients' sera and

[6] Y. Fukushi, T. Shepart, and S. Hakomori, unpublished observations.
[7] Y. Fukushi and S. Hakomori, unpublished observations.

23% (72 of 313) of all cancer patients' sera, while not reacting with any of 80 normal sera or 41 sera from patients with benign diseases (40). As in the case of sialosyl Le^a (26), sialosylated Le^x is present in cancer patients' sera, but not in normal sera, probably because of: (*a*) the increased activity of sialyltransferase in cancer tissues; (*b*) the increased solubility of the sialosylated form; and (*c*) the prevention of uptake of the sialosylated form by the blood clearance system (18).

The sialosylated Le^x antigen appears to be a useful marker of cancer antigens. CSLEX1 should therefore be of practical value in diagnostic tests and in monitoring various cancer treatments.

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REFERENCES

1. Bosmann, H. B., and Hall, T. C. Enzyme activity in invasive tumors of human breast and colon. Proc. Natl. Acad. Sci. USA, *71:* 1883–1887, 1974.
2. Brockhaus, M., Magnani, J. L., Blaszczyk, M., Steplewski, Z., Koprowski, H., Karlsson, K-A., Larson, G., and Ginsburg, V. Monoclonal antibodies directed against the human Le^b blood group antigen. J. Biol. Chem., *256:* 13223–13225, 1981.
3. Brockhaus, M., Magnani, J. L., Herlyn, M., Blaszczyk, M., Steplewski, Z., Koprowski, H., and Ginsburg, V. Monoclonal antibodies directed against the sugar sequence of lacto-*N*-fucopentaose III are obtained from mice immunized with human tumors. Arch. Biochem. Biophys., *217:* 647–651, 1982.
4. Civin, C. I., Mirro, J., and Banquerigo, M. L. My-1, a new myeloid-specific antigen identified by a mouse monoclonal antibody. Blood, *57:* 842–845, 1982.
5. Fox, N., Damjanov, I., Knowles, B. B., and Solter, D. Immunohistochemical localization of the mouse stage-specific embryonic antigen 1 in human tissues and tumors. Cancer Res., *43:* 669–678, 1983.
6. Fox, N., Damjanov, I., Martinez-Hernandez, A., Knowles, B. B., and Solter, D. Immunohistochemical localization of the early embryonic antigen (SSEA-1) in postimplantation mouse embryos and fetal and adult tissues. Dev. Biol., *83:* 391–398, 1981.
7. Ganzinger, V., and Deutsch, E. Serum sialyltransferase levels as a parameter in the diagnosis and followup of gastrointestinal tumors. Cancer Res., *40:* 1300–1304, 1980.
8. Glick, M. C. Membrane glycopeptides from virus-transformed hamster fibroblasts and the normal counterpart. Biochemistry, *18:* 2525–2532, 1979.
9. Gooi, H. C., Feizi, T., Kapadia, A., Knowles, B. B., Solter, D., and Evans, M. J. Stage-specific embryonic antigen involves α1→3 fucosylated type 2 blood group chains. Nature (Lond.), *292:* 156–158, 1981.
10. Gooi, H. C., Thorpe, S. J., Hounsell, E. F., Rumpold, H., Kraft, D., Föser, O., and Feizi, T. Marker of peripheral blood granulocytes and monocytes of man recognized by two monoclonal antibodies VEP8 and VEP9 involves the trisaccharide 3-fucosyl-*N*-acetyllactosamine. Eur. J. Immunol., *13:* 306–312, 1983.
11. Hakomori, S., Nudelman, E. Levery, S. B., and Kannagi, R. Novel fucolipids accumulating in human adenocarcinoma. I. Glycolipids with di- or trifucosylated type 2 chain. J. Biol. Chem., *259:* 4672–4680, 1984.
12. Hakomori, S., Nudelman, E., Levery, S. B., and Patterson, C. M. Human cancer-associated ganglioside defined by a monoclonal antibody (IB9) directed to sialosyl α2→6 galactosyl residue: a preliminary note. Biochem. Biophys. Res. Commun., *113:* 791–798, 1983.
13. Hakomori, S., Nudelman, E., Levery, S., Solter, D., and Knowles, B. B. The hapten structure of a developmentally regulated glycolipid antigen (SSEA-1) isolated from human erythrocytes and adenocarcinoma: a preliminary note. Biochem. Biophys. Res. Commun., *100:* 1578–1586, 1981.
14. Hansson, G. C., Karlsson, K-A., Larson, G., McKibbin, J. M., Blaszczyk, M., Herlyn, M., Steplewski, Z., and Koprowski, H. Mouse monoclonal antibodies against human cancer cell lines with specificities for blood group and related antigens. J. Biol. Chem., *258:* 4091–4097, 1983.
15. Huang, L. C., Brockhaus, M., Magnani, J. L., Cuttitta, F., Rosen, S., Minna, J. D., and Ginsburg, V. Many monoclonal antibodies with an apparent specificity for certain lung cancers are directed against a sugar sequence found in lacto-*N*-fucopentaose III. Arch. Biochem. Biophys., *220:* 318–320, 1983.
16. Huang, L. C., Civin, C. I., Magnani, J. L., Shaper, J. H., and Ginsburg, V. My-1, the human myeloid-specific antigen detected by mouse monoclonal antibodies, is a sugar sequence found in lacto-*N*-fucopentaose III. Blood, *61:* 1020–1023, 1983.
17. Iwaki, Y., Kasai, M., Terasaki, P. I., Bernoco, D., Park, M., Cicciarelli, J., Heintz, R., Saxton, R. E., Burk, M. W., and Morton, D. L. Monoclonal antibody against A_1 Lewis d antigen produced by the hybridoma immunized with a pulmonary carcinoma. Cancer Res., *42:* 409–411, 1982.
18. Jeanloz, R. W., and Codington, J. F. The biological role of sialic acid at the surface of the cell. *In:* A. Rosenberg and C-L. Schengrund (eds.), Biological Roles of Sialic Acid, pp. 201–238. New York: Plenum Publishing Corp., 1976.
19. Kannagi, R., Nudelman, E., Levery, S. B., and 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:* 14865–14874, 1982.
20. Kannagi, R., Stroup, R., Cochran, N. A., Urdal, D. L., Young, W. W., Jr., and Hakomori, S. Factors affecting expression of glycolipid tumor antigens: influence of ceramide composition and coexisting glycolipid on the antigenicity of gangliotriaosylceramide in murine lymphoma cells. Cancer Res., *43:* 4997–5005, 1983.
21. Kearney, J. F., Radbruch, A., Liesegand, B., and Rajewsky, K. A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. J. Immunol., *123:* 1548–1550, 1979.
22. Knowles, R. W., Bai, Y., Daniels, G. L., and Watkins, W. Monoclonal anti-type 2 H: an antibody detecting a precursor of the A and B blood group antigens. J. Immunogenetics, *9:* 69–76, 1982.
23. Köhler, G., and Milstein, C. Continuous culture of fused cells secreting antibody of predefined specificity. Nature (Lond.), *256:* 495–497, 1975.
24. Magnani, J. L., Nilsson, B., Brockhaus, M., Zopf, D., Steplewski, Z., Koprowski, H., and Ginsburg, V. A monoclonal antibody-defined antigen associated with gastrointestinal cancer is a ganglioside containing sialylated lacto-*N*-fucopentaose II. J. Biol. Chem., *257:* 14365–14369, 1982.
25. Magnani, J. L., Smith, D. F., and Ginsburg, V. Detection of gangliosides that bind cholera toxin: direct binding of ^{125}I-labeled toxin to thin-layer chromatograms. Anal. Biochem., *190:* 399–402, 1980.
26. Magnani, J. L., Steplewski, Z., Koprowski, H., and Ginsburg, V. Identification of the gastrointestinal and pancreatic cancer-associated antigen detected by monoclonal antibody 19-9 in the sera of patients as a mucin. Cancer Res., *43:* 5489–5492, 1983.
27. Rauvala, H. Gangliosides of human kidney. J. Biol. Chem., *251:* 7517–7520, 1976.
28. Siddqui, B., and Hakomori, S. A ceramide tetrasaccharide of human erythrocyte membrane reacting with anti-type XIV pneumococcal polysaccharide antiserum. Biochim. Biophys. Acta., *330:* 147–155, 1973.
29. Solter, D., and Knowles, B. B. Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). Proc. Natl. Acad. Sci. USA *75:* 5565–5569, 1978.
30. Terasaki, P. I., Bernoco, D., Park, M. D., Ozturk, G., and Iwaki, Y. Microdroplet testing for HLA-A, -B, -C, and -D antigens. Am. J. Clin. Pathol., *69:* 103–120, 1978.
31. Urdal, D. L., Brentrall, T. A., Bernstein, I. D., and Hakomori, S-I. A granulocyte reactive monoclonal antibody, 1G10, identifies the Galβ-4 (Fuc α 1-3) GlcNAc (X determinant) expressed in HL-60 cells on both glycolipid and glycoprotein molecules. Blood, *62:* 1022–1026, 1983.
32. Van Beek, W. P., Emmelot, P., and Homburg, C. Comparison of cell-surface glycoproteins of rat hepatomas and embryonic rat liver. Br. J. Cancer, *36:* 157–165, 1977.
33. Velican, C., Velican, D., Marcus, N., and Tacorian, S. Increased susceptibility to proteolytic action of gastric superficial mucosa after sialic acid release. Am. J. Dig. Dis., *15:* 413–418, 1970.
34. Voak, D., Sacks, S., Alderson, T., Takei, F., Lennox, E., Jarvis, J., Milstein, C., and Darnborough, J. Monoclonal anti-A from a hybrid-myeloma: evaluation as a blood grouping reagent. Vox Sang, *39:* 134–140, 1980.
35. Wang, S-M., Huang, T-W., and Hakomori, S. Immunohistochemistry of two glycolipid tissue antigens in human gastric carcinoma. Cancer (Phila.), *52:* 2072–2076, 1983.
36. Warren, L., Buck, C. A., and Tuszynski, G. P. Glycopeptide changes and malignant transformation. A possible role for carbohydrate in malignant behavior. Biochim. Biophys. Acta, *516:* 97–127, 1978.
37. Warren, L., Fuhrer, J. P., and Buck, C. A. Surface glycoproteins of normal and transformed cells: a difference determined by sialic acid and a growth-dependent sialyl transferase. Proc. Natl. Acad. Sci. USA, *69:* 1838–1842, 1972.
38. Yan, H-J., and Hakomori, S. A sphingolipid having a novel type of ceramide and "lacto-*N*-fucopentaose III." J. Biol. Chem., *246:* 1192–1200, 1971.
39. Yogeeswaran, G., and Tao, T-W. Cell surface sialic acid expression of lectin-resistant variant clones of B16 melanoma with altered metastasizing potential. Biochem. Biophys. Res. Commun., *95:* 1452–1460, 1980.
40. Iguro, T., Wakisaka, A., Terasaki, P. I., Hirota, M., Suyama, N., Fukushima, K., and Chia, D. Sialylated $Lewis^x$ antigen detected in the sera of cancer patients. Lancet, in press, 1984.

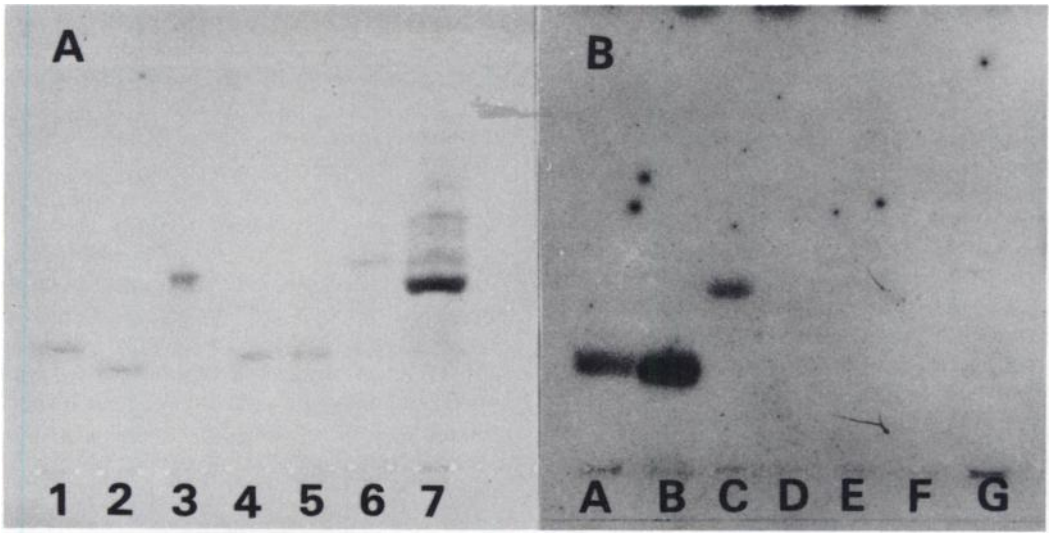

Fig. 1. Immunostaining pattern of gangliosides detected by the antibody CSLEX1. Bands in *plate A* (*Lanes 1 to 7*) were detected by orcinol:sulfuric acid reaction. Bands in *plate B* (*Lanes A to G*) were detected by immunostaining with CSLEX1. *Lane 1*, 6B ganglioside; *Lane 2*, 7B ganglioside; *Lane 3*, sialosyllactofucopentaosyl(III)ceramide; *Lane 4*, 6C ganglioside; *Lane 5*, difucosyllacto-*nor*-hexaosylceramide; *Lane 6*, sialosylparagloboside; *Lane 7*, sialosyl-Le^a fraction. *Lanes A to G* (*B*), autoradiogram of the same gangliosides in *Lanes 1 to 7* (*A*), respectively, but developed on a separate plate. The major band detected by autoradiogram of *Lanes A*, *B*, and *C* coincides exactly with the major band chemically detected by orcinol:sulfuric acid reaction. Baker's high-performance TLC plate with a solvent of chloroform:methanol:water (50:40:10, v/v/v) containing 0.02% $CaCl_2$ was used.