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A Cancer-reactive Human Monoclonal Antibody Derived from a Colonic Cancer Patient Treated with Local Immunotherapy

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A human monoclonal antibody, YJ-37 (IgM) was generated through the fusion of human B lymphoblastoid cell line HO-323 with the regional lymph node lymphocytes from a colonic cancer patient who was treated with a local immunotherapy. This antibody was purified and conjugated with biotin, after which direct immunohistochemical staining was performed. The results revealed that YJ-37 selectively reacted with colonic cancer (7/19), gastric cancer (3/6), endometrial cancer (1/2) and colonic adenoma (7/13), but not with normal epithelia. Membrane immunofluorescence and FACS analysis also showed that YJ-37 bound to tumor cell surfaces. Furthermore, the chemical structure of the antigen defined by YJ-37 was analyzed by means of thin-layer chromatography immunostaining and ELISA. The results indicated that YJ-37 reacted with sialylated lacto-series carbohydrate chains, which have been reported to accumulate in cancer cells.

Key words: Human monoclonal antibody — Local immunotherapy — Colon cancer — Tumor cell surface — Sialylated lacto-series carbohydrate antigen

Human monoclonal antibody (HuMAb) technology not only provides us with useful tools for diagnosis and therapy of tumors but also offers us opportunities to learn more about tumor immunology. In this field, HuMAbs generated from melanoma patients have been well analyzed. It has been reported that melanoma-reactive HuMAbs were aimed at ganglio-series antigens on the cell surface, and that administration of these HuMAbs induced tumor regression in melanoma patients as well as in nude mice.[1-3] However, for most tumors of low immunogenicity, including gastrointestinal carcinomas, it is difficult to obtain HuMAbs against the antigens associated with the cell membrane,[4] and many of the HuMAbs previously raised against colonic cancer have been revealed to react with cytoplasmic or nuclear antigens.[5-7] These observations suggest that augmentation of the host's immunity against tumor-surface antigens is necessary for the generation of clinically useful HuMAbs. For this reason, active immunization of cancer patients has been attempted by several researchers. Haspel *et al.* immunized colonic cancer patients with irradiated autologous tumor cells,[8] and Koyama *et al.* reported successful establishment of HuMAbs through the immunization of a healthy volunteer with living cells of gastric cancer.[9] *In vivo* immunization of cancer patients thus appears to be effective for the generation of potent HuMAbs, but the method is not always applicable.

Recently, we reported that an effective local immunotherapy that induced the degeneration of tumors through the immune responses could be an adjunctive tool for the generation of human hybridomas.[10] The hybridomas obtained in this way have been reported to produce high-titer immunoglobulins against antigens associated with the cell surface. In this study, we analyzed one of the HuMAbs generated from a colonic cancer patient who had been treated with this local immunotherapy.

MATERIALS AND METHODS

***In vivo* activation of lymphocytes through local immunotherapy** We performed *in vivo* activation of lymphocytes in colonic cancer patients through the local immunotherapy described previously.[11] Briefly, we prepared the solution for immunotherapy by dissolving five klinische Einheiten (KE) (corresponding to 0.5 mg of lyophilized *Streptococcus pyogenes*) of OK-432 (Chugai Pharmaceutical Co., Tokyo) in 1 ml of aprotinin (1,000 kallikrein inhibitor units) and this solution was mixed with 80 mg of heat-treated human fibrinogen (Behring Werke, Marburg, Germany). Before surgery, the mixture (hereafter referred as OK-432 fibrinogen solution) was injected intratumorally under endoscopy.

Source of lymphocytes Lymphocytes were obtained from the mesenteric lymph nodes draining the tumor

region of the colonic cancer patients who had received the local immunotherapy. Isolated lymphocytes were resuspended in RPMI 1640 containing 10% fetal bovine serum (FBS) and incubated for 48 h at 37°C in 5% CO_2.

Cell fusion The human B-lymphoblastoid cell line, HO-323, was maintained in RPMI 1640 medium supplemented with 10% FBS, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 300 μg/ml of L-glutamine (Gibco, New York, USA). This cell line has been proved to produce no immunoglobulin,[12] and was used as the source of parent cells for cell fusion.

Cell fusion between HO-323 and the lymphocytes from the regional lymph nodes was performed in the presence of 50% polyethylene glycol as described elsewhere.[13] Hybridomas were generated two or three weeks after fusion, and enzyme-linked immunosorbent assay (ELISA) was used to test the culture supernatant of the grown hybridomas for the presence of human immunoglobulins. Hybridomas producing $\geqq 100$ ng/ml of human immunoglobulins were selected and analyzed according to the methods described below.

Determination of immunoglobulin class The immunoglobulin class of the HuMAbs was determined by immunocytochemical assay. The established clones were cytocentrifuged on glass slides and immediately fixed by microwave (MW) irradiation at 500 W for 10 s, as described previously.[14] After blocking with 1% normal rabbit serum for 30 min, the slides were incubated for 30 min with mouse antibody against human IgG, IgM, IgA, IgE, or IgD. After washing in PBS, the slides were incubated with biotinylated rabbit anti-mouse immunoglobulin for 30 min, reacted with avidin-biotin-peroxidase complex (ABC) for 15 min with the aid of a kit (Histofine, Nichirei Co., Tokyo) for immunostaining, and then stained for each of the immunoglobulins in a solution of 0.005% hydrogen peroxide and 0.5 m*M* diaminobenzidine (DAB). Microscopic examination of the slides showed the immunoglobulin class to be IgM.

Screening and selection of a potent HuMAb (YJ-37) Screening for reactivity of HuMAbs to cancer cells was performed immunohistochemically. For this purpose, we prepared the human colon cancer cell lines, LS174T and LoVo. The cells were maintained in Dulbecco's MEM supplemented with 10% FBS, and xenografted subcutaneously to nude mice. The resulting tumors of approximately 5 mm in diameter were fixed with MW irradiation as described above, and paraffin-embedded sections (4 μm) were prepared. After deparaffinization, the sections were incubated for 2 h at room temperature with one of the HuMAbs, and reacted with biotinylated goat anti-human IgM (E·Y Labs, Inc., San Mateo, USA) as a second antibody, followed by reaction with ABC. Based on the results of immunostaining, we selected one clone of HuMAb (YJ-37), which reacted strongly with the xenografted tumors.

In order to elucidate whether YJ-37 recognizes cell-surface antigens, membrane immunofluorescence was performed on the colon cancer cell line, LS174T. In brief, the cells were incubated with HuMAb YJ-37 in a glass tube for 30 min at 4°C, and then incubated with fluorescein-conjugated goat anti-human IgM (E·Y Labs, Inc.) for 30 min at 4°C. After washing, the cells were resuspended in PBS, and membrane immunofluorescence was analyzed by using a fluorescence-activated cell sorter (FACS) or by fluorescence microscopy (Olympus Co., Tokyo). For the negative control, we replaced the primary antibody, YJ-37, with a polyclonal human IgM (Midori Juji, Osaka).

Purification and biotinylation of HuMAb YJ-37 (IgM) A hybridoma producing HuMAb YJ-37 was cultured in a serum-free medium, as described previously.[12] The culture supernatant was concentrated by ultrafiltration using a MINITAN^{TN} system (Nihon Millipore Ltd., Tokyo) and then applied to a Sephacryl S-400 column (Pharmacia, Freiburg, Germany) in order to separate IgM. The eluted solution was concentrated, then diluted with 20 m*M* 2-(N-morpholino)ethanesulfonic acid (pH 6.0) (MES) and applied to an ion exchange column (ABx®, J.T. Baker, Phillipsburg, USA). After washing with MES, the column was eluted with 500 m*M* ammonium sulfate and 20 m*M* sodium acetate (pH 7.0). The eluted fraction was then concentrated and applied to a ConA agarose column (Seikagaku Kogyo, Tokyo). Finally, the IgM was eluted with a PBS containing 0.2 *M* methyl-α -D-mannopyranoside. Purity was determined by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. The purified IgM was biotinylated according to the method of Bonnard *et al.*[15] using a biotinylation Kit (Amersham Japan, Inc., Tokyo). We performed an additional immunohistological study by applying biotinylated YJ-37 to MW-fixed sections of various human tissues (colon, stomach, liver, breast, thyroid, uterus and ovary).

Enzymatic digestions and periodate oxidation of tissue sections To assess the chemical properties of the antigen defined by YJ-37, xenografted tumor sections were treated with the following reagents prior to immunohistochemical staining: (a) 0–0.25% trypsin (Research Institute for Microbial Diseases, Osaka) for 20 min at 37°C; (b) 0–1.0 unit/ml of neuraminidase from *Clostridium perfringens* (Sigma, St. Louis, USA) for 1 h at 37°C; (c) 0–10 m*M* periodate (Wako, Osaka) in 50 m*M* sodium acetate buffer pH 4.5 (SAB) for 1 h at room temperature.

TLC immunostaining for glycolipids To test the immunoreactivity of YJ-37 to various types of glycolipids, thin-layer chromatography (TLC) immunostaining was

performed. We prepared neutral and acidic glycolipids from human placenta and erythrocytes of blood type O as described elsewhere.[16] TLC immunostaining for the prepared glycolipids was performed with high-performance TLC (Baker Chemical Co., Phillipsburg, NJ), according to a modification[17,18] of a procedure originally described by Magani *et al.*[19] Briefly, after development of glycolipids, the HPTLC plate was reacted with YJ-37 at 4°C overnight, then with peroxidase-labeled anti-human IgM (KPL, Maryland, USA) at room temperature for 1 h as a substrate, and stained with 4-chloro-1-naphthol (Konica).

ELISA for glycolipids To analyze the reactivity of YJ-37 to the glycolipids demonstrated on TLC immunostaining, ELISA for purified glycolipids was performed according to a previously described method.[20] The following glycolipids were used for ELISA: synthetic 2→3 sialyllactotetraosylceramide (IV^3 NeuAcα-Lc$_4$) (Wako), synthetic 2→3 sialylneolactotetraosylceramide (IV^3 NeuAcα-nLc$_4$) (Wako), synthetic sialyl Lewis X (Wako), GM$_3$ ganglioside from bovine (Sigma), and GM$_{1a}$ ganglioside from bovine brain (Sigma). The structures of these glycolipids are shown in Table I. Glycolipids were dissolved at various concentrations in 100% methanol, and an aliquot of 10 μl of each solution was added to 96-well plastic plates. After air drying, non-specific binding was blocked with 3% BSA in PBS and the glycolipids were then incubated for 2 h at 37°C in the presence of 100 μl of YJ-37. For the negative control, the primary antibody, YJ-37, was replaced with a polyclonal human IgM (Midori Juji) at a matched concentration. The plates were washed in PBS, then peroxidase-labeled anti-human IgM (KPL) was applied, and the reactivity was determined by color reaction in *ortho*-phenylenediamine (Wako) solution.

RESULTS

Generation and screening of HuMAbs Four fusions were performed with lymphocytes of regional lymph nodes from four different patients who received the local immunotherapy with OK-432/fibrinogen solution from one to seven days before surgery. Examination of a total of 353 generated hybridomas by ELISA revealed that 116 of them secreted immunoglobulins, and immunohistochemical assay showed that 31 of these secreted immunoglobulins reactive to xenografts of LS174T and LoVo. From the HuMAbs with reactivity to xenografted tissue, we selected the HuMAb YJ-37 (IgM) because it most intensely stained the tumor cells. The hybridoma producing YJ-37 consistently secreted 2–3 μg/ml IgM for more than one year.

Cell surface staining Using HuMAb YJ-37, membrane immunofluorescence staining and FACS were performed on LS174T cells. The results showed that YJ-37 is directed at a cell surface antigen of tumor cells (Fig. 1).

Immunostaining of biotinylated HuMAb YJ-37 HuMAb YJ-37 was purified and biotinylated to prevent non-specific reaction caused by endogenous immunoglobulin in human tissue sections. Direct immunostaining on MW-fixed, paraffin-embedded sections of various human tissues revealed that YJ-37 reacted with colonic cancer (7/19), colonic adenoma (7/15), gastric cancer (3/6) and endometrial cancer (1/2), but not with normal epithelia of the alimentary tract (Table II and Fig. 2). The histological type of the colonic and gastric cancers reactive with YJ-37 was well-differentiated adenocarcinoma. In a few cases of gastric mucosa, intestinal metaplasia showed weak staining.

Chemical characterization of the epitope structure defined by HuMAb YJ-37 Treatment of the MW-fixed

Table I. Structures of Glycolipids Used in the ELISA and Structures Closely Related to HuMAb YJ-37

Name	Structure
GM$_3$	NeuAcα2→3Ga1β1→4Glc→Cer
GM$_{1a}$	Ga1β1→3GalNAcβ1→4Galβ1→4Glc→Cer 3 ↑ NeuAcα2
2→3 Sialyllactotetraosylceramide (IV^3 NeuAcα-Lc$_4$)	NeuAcα2→3Galβ1→3GlcNAcβ1→3Galβ1→4Glc→Cer
2→3 Sialylneolactotetraosylceramide (IV^3 NeuAcα-nLc$_4$)	NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc→Cer
Sialyl Lewis X	NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc→Cer 3 ↑ Fucα1
2→3 Sialyllactohexaosylceramide (VI^3 NeuAcα-Lc$_6$)	NeuAcα2→3Galβ1→3GlcNAcβ1→3Galβ1→3GlcNAcβ1→3Galβ1→4Glc→Cer
2→3 Sialyllacto*nor*hexaosylceramide (VI^3 NeuAcα-nLc$_6$)	NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc→Cer

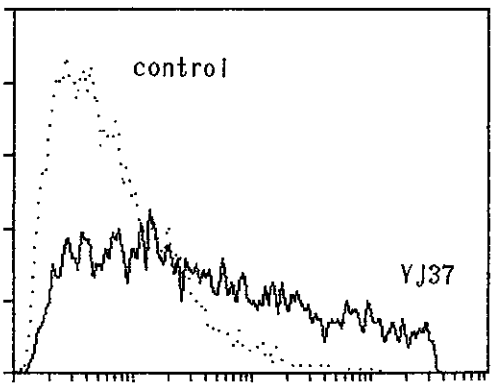

Fig. 1. Result of indirect immunofluorescence flow cytometry experiment with YJ-37. The x- and y-axis represent the fluorescence intensity on a log scale and the number of reacting cells, respectively. Colonic cancer cells were preincubated with HuMAb YJ-37 (—) or polyclonal human IgM (···) as controls, followed by FITC-labeled anti-human IgM, and analyzed by flow cytometry.

Table II. Reactivity of Biotinylated HuMAb YJ-37 to Human Tissues

Tumor	Positive/tested	Normal epithelium	Positive/tested
Colonic cancer	7/19	Colon	0/15
adenoma	7/13		
Gastric cancer	3/6	Stomach	0/7
Esophageal cancer	0/2	Esophagus	0/2
Hepatoma	0/2	Liver	0/4
Gallbladder cancer	0/2	Gallbladder	0/2
Breast cancer	0/4	Breast	0/2
Thyroid cancer	0/3		
Uterine cancer	1/3	Uterus	0/4
Ovarian cancer	0/2	Ovary	0/2
		Placenta	0/2
		Lung	0/2
		Skin	0/2

Determined by direct immunoperoxidase assay on microwave-fixed specimens.

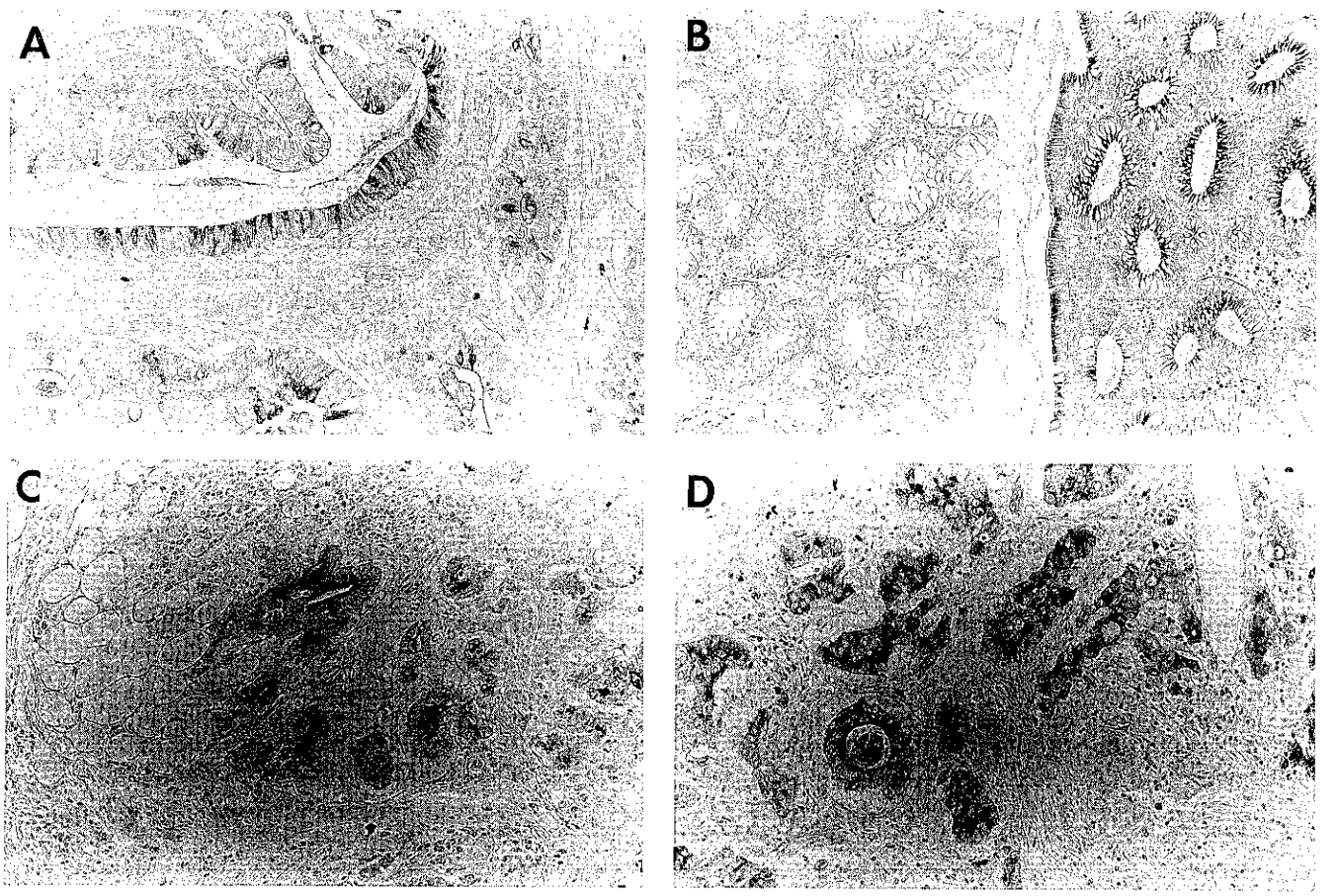

Fig. 2. Direct immunostaining of microwave-fixed human tissues by YJ-37. A: Colonic cancer (×200); B: colonic adenoma (×200); C: gastric cancer (×200); D: endometrial cancer (×200). Note that the normal epithelium adjacent to the tumors is completely negative, and the staining is restricted to the apical portion of the tumor cells.

sections of xenograft (LS174T) with periodate or neuraminidase diminished the reactivity of YJ-37 to tumor tissue, but the trypsinization of tissue sections produced no change (Fig. 3). These findings suggest that a sialylated carbohydrate chain is involved in the antigen recognized by YJ-37.

For elucidation of the chemical properties of the antigen defined by YJ-37, glycolipids of erythrocytes and placenta were separated by HPTLC and immunostained with YJ-37. The results showed that YJ-37 reacted with one major and one minor band the entire acidic glycolipid extracts of erythrocytes and placenta. In terms of their migration rates, the major band corresponded to VI^3 NeuAcα-nLc$_6$ or VI^3 NeuAcα-Lc$_6$, and the minor band to IV^3 NeuAcα-nLc$_4$ (Fig. 4). Furthermore, the reactivity of YJ-37 with related purified glycolipids was tested by solid-phase ELISA. The results showed that YJ-37 reacted with highly concentrated IV^3 NeuAcα-Lc$_4$ and IV^3 NeuAcα-nLc$_4$, but not with sialyl Lewis X, GM$_3$ ganglioside or GM$_{1a}$ ganglioside (Fig. 5). The findings obtained with TLC immunostaining and ELISA suggest that the essentially reactive structure of YJ-37 is (NeuAcα2→3Galβ1→3(or4)GlcNAc) and the internal sugar residues might affect the intensity of antibody binding.

DISCUSSION

We demonstrated that one of the HuMAbs, YJ-37, generated from the colonic cancer patients treated with local immunotherapy selectively reacted with sialylated lacto-series carbohydrate antigens.

The lacto-series carbohydrate chains (Galβ1→3(or4)-GlcNAcβ1→3Gal) are known to be the major carriers of blood group antigen (BGA). Normal adult colonic mucosae express a large amount of fucosylated forms of

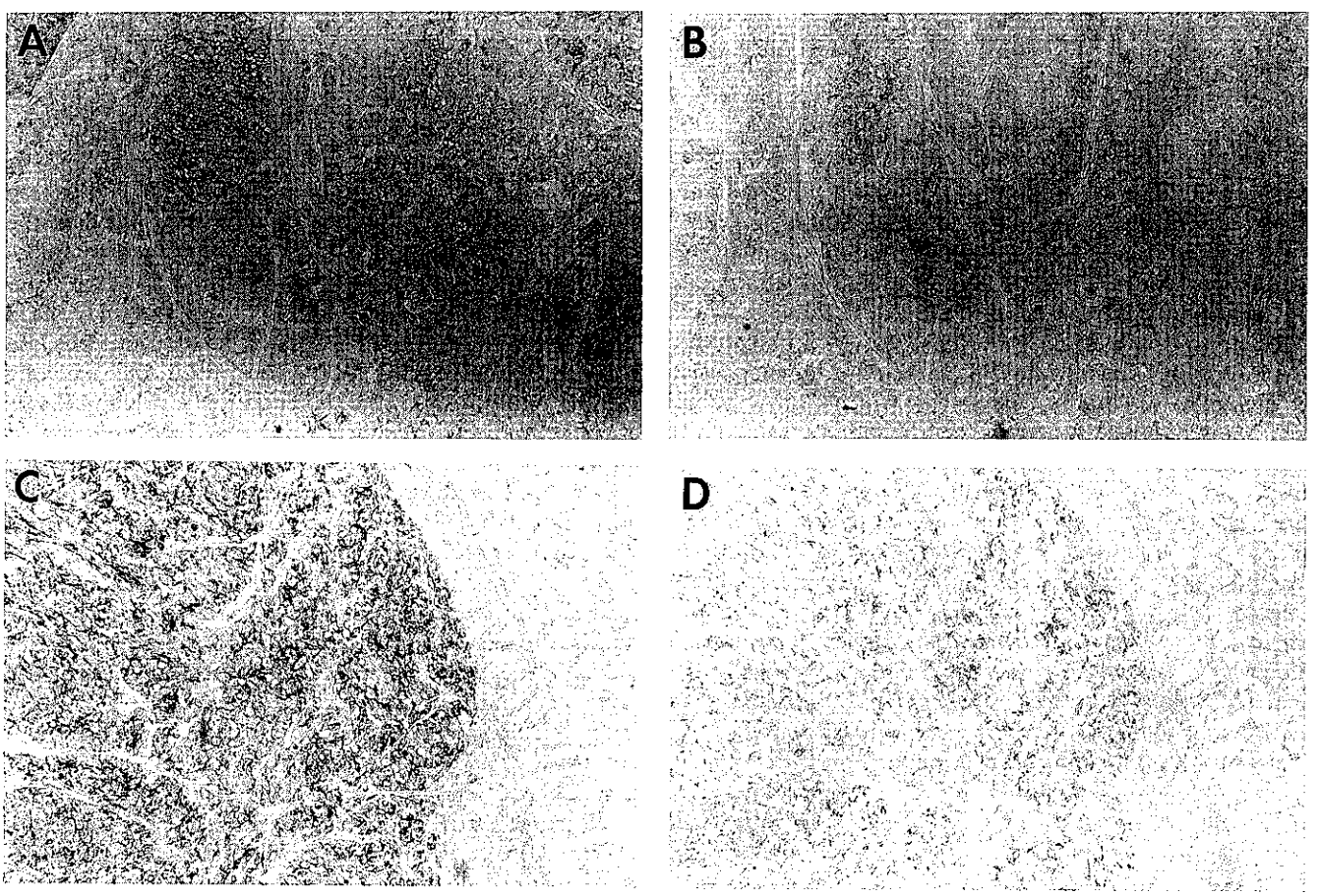

Fig. 3. Effect of periodate oxidation and neuraminidase digestion of tissue sections on reactivity of YJ-37. Reactivity of YJ-37 to tumor sections of xenografted LS174T(A, C) is greatly reduced after treatment with 10 m*M* periodate (B) or with 10 U/ml neuraminidase (D), (×100).

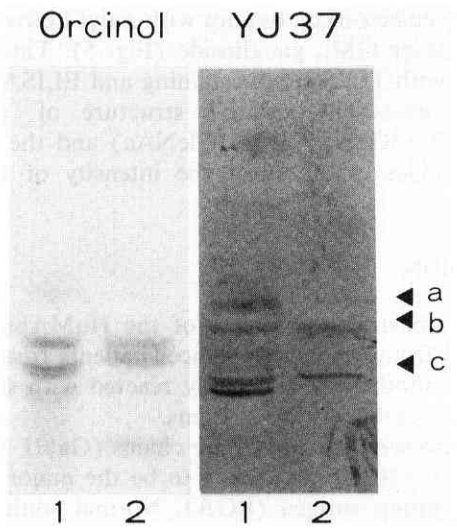

Fig. 4. Reactivities of YJ-37 to glycolipids extracted from erythrocytes and placenta by HPTLC. Orcinol/sulfuric acid staining (left) and TLC immunostaining with YJ-37 (right) are shown. Lane 1: acidic fraction of glycolipids from O erythrocytes; Lane 2: acidic fraction of glycolipids from human placenta. a: GM_3; b: IV^3 NeuAcα-nLc_4; c: VI^3 NeuAc-nLc_6 or VI^3 NeuAc-Lc_6.

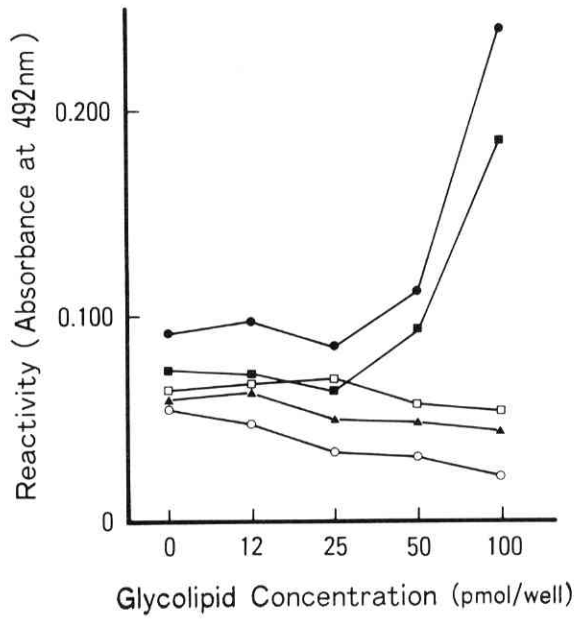

Fig. 5. Antibody binding activity of various glycolipids with HuMAb YJ-37 examined by ELISA. (●): IV^3 NeuAcα-Lc_4; (■): IV^3 NeuAcα-nLc_4; (□): sialyl Lewis X; (▲): GM_3 ganglioside; (○): GM_{1a} ganglioside.

BGA, including H, Le^a, and Le^b, with a concomitant expression of lesser amounts of BGA A, B, Le^x and Le^y.

In colonic cancer tissues, however, the profile of BGA expression shows a substantial change and abnormal sialylation of BGA has been observed.[21] Sialylated Le^a (CA19-9), which is derived from type 1 carbohydrate chain, is one of the most useful markers for the diagnosis of malignancy. Another sialylated form of BGA, sialyl Le^x, is a derivative of the type 2 carbohydrate chain, and has been identified as a ligand of ELAM-1, which plays an important role in cell adhesion.[22, 23]

ELISA for YJ-37 showed no reactivity with sialyl Le^x, and TLC immunostaining suggested that YJ-37 did not react to sialyl Le^a (deta not shown). Thus, the antigen defined by YJ-37 seemed to comprise the precursors of sialyl-Le^x and sialyl-Le^a, which are the sialylated but not fucosylated forms of type 1 and type 2 carbohydrate chains. Nilsson *et al.* and Retting *et al.* have reported that mouse monoclonal antibodies against sialylated type 1 chain could be established by immunization with colonic cancer or teratocarcinoma cells,[24–26] but HuMAbs directed to these carbohydrate antigens have not been reported. The reason for the difficulties of establishing HuMAbs against such carbohydrate antigens as the sialylated lacto-series is unclear, but we assume that the antigens shed by tumor cells may be small and/or have a low immunogenicity in humans.

Successful establishment of a HuMAb, YJ-37, directed to the sialylated lacto-series in this study may be the result of degradation of the tumors by the local immunotherapy, which simultaneously enhanced the host's immunity. A massive destruction of tumors may have resulted in the influx of tumor-associated antigens, including the sialylated lacto-series, into the draining lymph nodes, while the potentiation of lymphocytes through the local immunotherapy may have contributed to adequate sensitization to the antigens.[27] The regional lymph nodes from the patients treated with the effective local immunotherapy thus seem to be a promising source of lymphocytes for potentially useful HuMAbs.

The selective reactivity of YJ-37 to tumor tissues revealed by immunohistochemical analysis may indicate the possibility of clinical use of this HuMAb, although further analysis of the physiological function of YJ-37 in tumor-bearing animal models in needed.

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