Novel Fucolipids Accumulating in Human Adenocarcinoma

II. SELECTIVE ISOLATION OF HYBRIDOMA ANTIBODIES THAT DIFFERENTIALLY RECOGNIZE MONO-, DI-, AND TRIFUCOSYLATED TYPE 2 CHAIN*

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A series of glycolipids having the X determinant (Galβ1→4[GalNAc]α1→3) at the terminus and a fucosyl α1→3 residue at the internal GlcNAc residue have been isolated and characterized from tumor tissues (Hakomori, S., Nudelman, E., Levery, S. B., and Kannagi, R. (1984) J. Biol. Chem. 259, 4672–4680). A series of monoclonal antibodies that differentially recognize glycolipids with mono-, di-, and trifucosylated type 2 chain have been isolated and characterized. The antibody FH4 shows a remarkable preferential reactivity towards di- or trifucosylated type 2 chain, i.e. it does not react with monofucosylated structures, including laetofucopenaicosyl(III)ceramide (III FuncnLca), monofucosyl neolactonorhexaosylceramide (y3, V FuncnLca), and monofucosyl neolactonoroctaosylceramide (z1, VII FuncnLca), but reacts well with di- and trifucosylated type 2 chain structures such as difucosyl neolactonorhexaosylceramide (III FuncnLce) and trifucosyl neolactonoroctaosylceramide (III FuncnLcd). Two other monoclonal antibodies, FH5 and ACFH18, preferentially react with trifucosylated type 2 chain structure (III FuncnLce), although cross-reactivity with difucosylated type 2 chain (III FuncnLca) was observed. They showed a minimal cross-reaction with monofucosylated type 2 chain. In contrast, the antibody FH1 does not react with III FuncnLca but reacts with VII FuncnLca, III FuncnLce, and III FuncnLcd. Two monoclonal antibodies, FH2 and FH3, do not discriminate among various glycolipids having fucosylated type 2 chain, and their reactivities are essentially identical to previously established antibodies directed to the X determinant, such as anti-SSEA-1 (2, 4, 5), WGS 29, ZWG 13, 14, 111 (6), 538 F12, 538 F8 (7), VEP8. VEP9, VEP8 (8), M-1 (9), etc. None of these monoclonal antibodies, however, can distinguish among the various fucosylated type 2 chain structures described above. Since accumulation of difucosyl neolactonorhexaosylceramide and trifucosyl neolactonoroctaosylceramide is a characteristic membrane phenotype of various human cancers, it is highly desirable to establish hybridoma antibodies that react specifically to these structures and do not react to monofucosylated type 2 chain with the X determinant at the terminus. We have selected hybridomas based on their reactivity to a purified glycolipid with a defined structure. With this procedure, we have successfully isolated several hybridoma clones showing distinctive differential reactivities towards various types of glycolipids with fucosylated type 2 chain structure. These reagents may be useful to distinguish among various types of cells at different stages of differentiation and oncogenic progression.

Accumulation of a series of glycolipids having the X determinant (Galβ1→4[GalNAc]α1→3) at the terminus and a fucosyl α1→3 residue at the internal GlcNAc residue is one of the most characteristic membrane phenotypes detected in various human adenocarcinomas (1, 2). III FuncnLce,4 was found to accumulate in some adenocarcinomas as previously described (1, 2). In addition, III FuncnLce and III VIFuncnLce have been isolated and chemically characterized as the major components accumulating in human primary liver adenocarcinoma and colonic adenocarcinoma, as described in the accompanying paper (21). Monofucosylated type 2 chain, previously designated as y3 (V FuncnLce), z1 (VII FuncnLca), and z2 (V III FuncnLca) were isolated and characterized as normal cell components (3). All these components have the X determinant structure at the terminus and, therefore, have been detected by immunostaining with monoclonal antibodies directed to the X determinant, such as anti-SSEA-1 (2, 4, 5), WGS 29, ZWG 13, 14, 111 (6), 538 F12, 538 F8 (7), VEP8. VEP9, VEP8 (8), M-1 (9), etc. None of these monoclonal antibodies, however, can distinguish among the various fucosylated type 2 chain structures described above. Since accumulation of difucosyl neolactonorhexaosylceramide and trifucosyl neolactonoroctaosylceramide is a characteristic membrane phenotype of various human cancers, it is highly desirable to establish hybridoma antibodies that react specifically to these structures and do not react to monofucosylated type 2 chain with the X determinant at the terminus. We have selected hybridomas based on their reactivity to a purified glycolipid with a defined structure. With this procedure, we have successfully isolated several hybridoma clones showing distinctive differential reactivities towards various types of glycolipids with fucosylated type 2 chain structure. These reagents may be useful to distinguish among various types of cells at different stages of differentiation and oncogenic progression.

MATERIALS AND METHODS

The general procedure for establishing hybridomas producing monoclonal antibodies was followed according to a modification (10) of the procedure described by Kohler and Milstein (11). The glycolipids designated III FuncnLce, III FuncnLce, and III VIFuncnLce were prepared from human adenocarcinoma as described in the accompanying paper (21). The y3 (V FuncnLca) and z2 (VIII FuncnLca) glycolipids were prepared from human adenocarcinoma and human erythrocytes (3). Solid phase radioimmunoassay was performed on a detachable vinyl strip (Costar, Cambridge, MA) as described previously (12).

Immunization was performed in one series of experiments with a membrane fraction and in another series of experiments with glycolipids adsorbed to Salmonella minnesota (10, 13). The membrane fraction that was used for immunization was prepared by the following procedure. 1.5–2 g of tumor tissue or cells (TG113 tumor or MKN75 cells) were homogenized in the Dounce homogenizer with 10 ml of distilled water containing 10 kalikrein inhibitor units of "aprotinin"

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1 The glycolipid designations used, according to the recommendations of the Nomenclature Committee of the International Union of Pure and Applied Chemistry (20), are: III FuncnLce, laetofucopenaicosyl(III)ceramide; III FuncnLce, difucosyl neolactonorhexaosylceramide; III VIFuncnLce, trifucosyl neolactonoroctaosylceramide; V FuncnLca, monofucosyl neolactonorhexaosylceramide; VII FuncnLca, monofucosyl neolactonoroctaosylceramide; VIII FuncnLca, difucosyl neolactonoroctaosylceramide.
TABLE I
Properties of monoclonal antibodies directed to various fucosylated type 2 chain structures

<table>
<thead>
<tr>
<th>Hyridoma</th>
<th>Ig class</th>
<th>Method of preparation</th>
<th>Reactivities with</th>
<th>Reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td>FH1</td>
<td>IgM</td>
<td>TG115 membrane; intraperitoneal injection; 4 x selected by glycolipid</td>
<td>- + - + + -</td>
<td>IIIpFucnLcE, VpFucnLcE</td>
</tr>
<tr>
<td>FH2</td>
<td>IgM</td>
<td>As above</td>
<td>+ + - + + -</td>
<td>IIIpFucnLcE, VpFucnLcE</td>
</tr>
<tr>
<td>FH3</td>
<td>IgG3</td>
<td>IIIpVpFucnLce H. minnesota; intravenous injection; 4 x selected by glycolipid</td>
<td>+ + - + + -</td>
<td>IIIpFucnLcE, VpFucnLcE</td>
</tr>
<tr>
<td>FH4</td>
<td>IgG3</td>
<td>As above</td>
<td>- - - ++ ++ -</td>
<td>IIIpFucnLcE, VpFucnLcE</td>
</tr>
<tr>
<td>FH5</td>
<td>IgM</td>
<td>TG115 membrane; intravenous injection; 1 x selected by glycolipid</td>
<td>- + - - + + -</td>
<td>IIIpFucnLcE, VpFucnLcE</td>
</tr>
<tr>
<td>ACFH18</td>
<td>IgM</td>
<td>MKN74 cell membrane; intraperitoneal injection; 4 x selected by glycolipid</td>
<td>- + - - + + -</td>
<td>IIIpFucnLcE, VpFucnLcE</td>
</tr>
</tbody>
</table>

Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer

Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer

Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer

Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer

Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer

Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer

Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer

Graphs showing the reactivity of monoclonal antibodies FH1, 2, 3, 4, and 5 with various glycolipid antigens at different concentrations of antibodies. The assay was made on vinyl strips coated with glycolipids, cholesterol, and lecithin. The quantity of glycolipid per well was 10 ng with 30 ng of cholesterol and 50 ng of lecithin. A-E show the reactivity of FH1, 2, 3, 4, and 5, respectively. FVpFucnLcE, △, VpFucnLcE, ○, IIpFucnLcE; ○, IIpVpFucnLcE, ▲, IIIpFucnLcE. ACFH18, IIIpFucnLcE.
(protease inhibitor; Sigma). After about 50 strokes in an ice-water bath, the homogenate was centrifuged at 2000 rpm for 10 min, and the supernatant was separated and centrifuged at 35,000 rpm for 1 h. The pellet was suspended in 10 ml of distilled water containing aprotinin, and the protein concentration was adjusted to 2.5 mg/ml.

An aliquot of 0.5 ml containing 1.25 mg of protein was injected 4 times intra-peritoneally on every 4th day. Fusion of the host spleen cells with SP/2 was performed on the 3rd day after the last injection. In one experiment, only one intravenous injection with the membrane preparation was made, followed by fusion with SP/2 after 3 days. Cloning of hybridomas was performed on plates coated with purified \( \text{II}^{13}\text{V}_{3}\text{FuC}_{2}\text{nLc}_{6} \), \( \text{II}^{13}\text{Fuc}_{n}\text{Lc}_{8} \), and \( \text{V}_{3}\text{Fuc}_{n}\text{Lce} \).

In another series of experiments, a pure glycolipid was used as immunogen with \( S. \text{minnesota} \) as the carrier (10, 13). An ethanol solution (50 \( \mu \)l) containing 20 \( \mu \)g of a purified glycolipid, \( \text{II}^{13}\text{V}_{3}\text{Fuc}_{n}\text{nLc}_{8} \), was mixed with 800 \( \mu \)l of phosphate-buffered saline (pH 7.4). The solution was further mixed with 260 \( \mu \)g of acid-treated \( S. \text{minnesota} \) suspended in 250 \( \mu \)l of phosphate-buffered saline. The whole mixture was thoroughly mixed at 40°C. The suspension containing 5 \( \mu \)g of glycolipid was intravenously injected on the 1st day; subsequently, an aliquot containing 2 \( \mu \)g of glycolipid was injected 3 times on every 4th day. The fusion of the host spleen cells with SP/2 was performed on the 3rd day after the last injection. The hybridoma was cloned on 96-well plates (Dynatech Immunolon, Dynatech Laboratories, Inc., Alexandria, VA) coated with purified glycolipid (10 ng/well) and cholesterol (30 ng/well) and lecithin (50 ng/well). Cloning was performed repeatedly.

Gastric cancer cell lines MKN series (16), were donated by Dr. Toshimitsu Suzuki (Department of Pathology, Niigata University, Japan); lung cancer cell lines, QG-56, QG-90, PC-1, 3, 7, 9, and 10 (17) were donated by Mr. S. Adachi (Immunoresearch Laboratory, Takasaki, Japan); epidermoid tumor cells RT-4 and ovari-al adenosarcoma SK-OV3 (18) were donated by Dr. Jorgen Fogh (Sloan Kettering Institute, New York, NY); monocytic leukemia cell line THP-1 (19) was donated by Dr. Shigeru Tsuchiya (Biochemical Oncology, Fred Hutchinson Cancer Research Center, Seattle, WA); mouse myeloma SP-2, myelocytic leukemia KG-1, erythroleukemia WI-38 and L-5 were donated by Dr. Cecile Berglund (Pediatric Oncology, Fred Hutchinson Cancer Research Center, Seattle, WA); human lung cancer cell line LX-1 and human bladder carcinoma cell line K562, B cell lines Prenl, Crow, and Kasner, and human fibroblasts EJ-23 were donated by Dr. Geoffrey M. Cooper (Sidney Farber Cancer Institute, Harvard Medical School, Boston, MA); PC-7, PC-9, KG-1, HL-60, K562, THP-1, and B cell lines were cultured in suspension in RPMI 1640 medium supplemented with 10% fetal calf serum. All other cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Trypsinized cells were washed and resuspended in phosphate-buffered saline, and 5 \( \times \) 10^5 cells/well were seeded in Linbro plates which were precoated with 0.5 mg/ml of polylysine. Plates were centrifuged, and cells were fixed with 0.1% glutaraldehyde and used for antibody-binding assay.

**RESULTS**

Of many hybridomas produced, six clones have been established which produce ascites with high antibody titer. The monoclonal antibodies that are capable of distinguishing among various fucosylated type 2 chain glycolipids and their specificities and immunoglobulin class are shown in Table I. The reactivities of the antibodies with various types of glycolipids having fucosylated type 2 chain structure are shown in Figs. 1 and 2. The reactivity of varying concentrations of glycolipid coating on a vinyl strip with cholesterol and lecithin was inhibited by liposomes containing \( \text{II}^{13}\text{V}_{3}\text{Fuc}_{n}\text{nLc}_{8} \) or \( \text{V}_{3}\text{Fuc}_{n}\text{Lce} \). The initial concentration of inhibitory glycolipid in liposomes was 1 \( \mu \)g/well. The glycolipid concentration on vinyl strips was 10 ng with 50 ng of lecithin and 30 ng of cholesterol/well. The concentration of antibody was a 1:50 dilution of the culture supernatant. A, inhibition of the reactivity of antibody FH3 to \( \text{II}^{13}\text{V}_{3}\text{Fuc}_{n}\text{nLc}_{8} \) by liposomes containing \( \text{II}^{13}\text{V}_{3}\text{Fuc}_{n}\text{nLc}_{8} \) (b), inhibition of the reactivity of antibody FH4 to \( \text{II}^{13}\text{V}_{3}\text{Fuc}_{n}\text{nLc}_{8} \) by liposomes containing \( \text{II}^{13}\text{V}_{3}\text{Fuc}_{n}\text{nLc}_{8} \) (○) and by liposomes containing \( \text{V}_{3}\text{Fuc}_{n}\text{Lce} \) (△).
the antibodies with constant quantities of glycolipids is shown in Fig. 1, and the reactivity of the antibodies with varying quantities of glycolipid antigens is shown in Fig. 2. FH1 antibody reacted with tri-, di-, and monofucosylated type 2 chain equally well, although it did not react with II\(^2\)FucnLc\(_4\) (Fig. 1A and Fig. 2A). In contrast, antibodies FH2 and FH3 reacted with all fucosylated type 2 chain glycolipids, including lactofucopentaosyl(II\(^1\))ceramide, although a subtle difference in the order of reactivity was observed between FH2 and FH3 (Fig. 1, B and C and Fig. 2, B and C). A remarkable selective reactivity with trifucosylated and difucosylated type 2 chain was observed for the antibody FH4 which did not react with monofucosylated type 2 chain \(\text{V}\,\text{FucnLc}_2\), \(\text{VIF}\,\text{FucnLc}_3\), or II\(^2\)FucnLc\(_4\). This particular antibody was obtained by immunization with a pure difucosyl neolactonorhexaosylceramide and selection by various purified glycolipids. The antibody, however, did not discriminate between di- and trifucosylated type 2 chain glycolipids (Fig. 1D and Fig. 2D). The antibody FH5 showed a preferential reactivity with trifucosylated neolactonorhexaosylceramide (Fig. 1E and Fig. 2E), but \(x\) cross-reaction with di- and monofucosyl neolactonorhexaosylceramide was observed. The antibody did not cross-react with lactofucopentaosyl(II\(^1\))ceramide. A similar, but more obvious, preferential reactivity with trifucosyl neolactonorhexaosylceramide was observed for the antibody clone ACDFH18 (Fig. 2F), which did not react with monofucosyl neolactonorhexaosyl- or noroctaosylceramide (\(\gamma_2\); \(\kappa_2\)) nor with lactofucopentaosyl(II\(^1\))ceramide. The antibody, however, cross-reacted minimally with difucosyl neolactonorhexaosylceramide. None of these monoclonal antibodies reacted with a glycolipid having an internal fucosyl residue (II\(^2\)FucnLc\(_4\)) (solid line with solid triangle in Fig. 1, A–E) which was obtained by desialylation of a unique ganglioside (14).

The preferential reactivity of FH4 with difucosyl neolactonorhexaosylceramide was further confirmed by inhibition of antibody binding to solid phase glycolipid-lecithin-cholesterol on vinyl strips. The reactivity of the FH4 antibody with the solid phase difucosyl neolactonorhexaosylceramide was specifically inhibited by incubation of the antibody with the same glycolipid antigen in liposome (Fig. 3). None of these antibodies reacted with glycolipids carrying the H structure (H\(_2\) glycolipid was tested) even in high concentration (2 \(\mu\)g/ well) (data not shown).

The reactivity of FH4 was also tested with various cell lines in comparison to anti-SSEA-1, which recognizes the X determinant irrespective of the internal structure. As shown in Fig. 4, the reactivity of FH4 was much more restricted than that of anti-SSEA-1. A selective reactivity of FH4 with certain types of tumor cells, including gastric cancer cell line MKN74, lung cancer cell line PC-7, and monocytic leukemia cell line THP-1, was observed, in contrast to anti-SSEA-1 which reacts with a large variety of cells, including nonmalignant B cell lines.

**DISCUSSION**

The carbohydrate structure with the X determinant, in either glycoproteins or glycolipids, is highly immunogenic in mice. Since the established Köhler-Milstein procedure depends solely on immunization of mice, a great number of monoclonal antibodies, originally claimed to be “tumor-specific,” have been found to be directed to the X determinant, i.e. Ga\(\text{gb}\)\(_1\)→4[Fuc\(_\text{a}\)Fuc\(_\text{a}\)]\(_3\)GlcnAc\(_\text{b}\)→R. The first monoclonal antibody identified as being directed to this structure was anti-SSEA-1 (3–5). With this reagent, a number of glycolipids with the X determinant have been detected in normal and
tumor tissue. However, some adenocarcinomas accumulate lactofucopentaosyl(III)ceramide, difucosyl neolactonorhexa-
osylceramide, and trifucosyl neolactonoroctaosylceram-
ide. These structures were not detected in normal erythro-
cytes, granulocytes, normal colonic mucosa, or normal liver, although various other bands with X determinant structure
are present in these normal cells and tissues. Granulocytes
are particularly rich in glycolipids with the X determinant (8,
9, 15).

In order to distinguish among various structures with fu-
cosylated type 2 chain, we have prepared hybridoma antibod-
ies that can distinguish between mono- and difucosylated type
2 chain structures. The antibody FH4 is only reactive with
di- and trifucosylated type 2 chain, but is not reactive with
neolactonorhexaosylceramide that is monofucosylated at
either the V
2 or III
2 positions. Namely, the antibody recog-
nizes two adjacent fucosyl structures at the III
2 and V
2 posi-
tions. The antibody shows a preferential reactivity with some
human tumor cell lines and shows no reaction with various B
cell lines which were highly reactive with anti-SSEA-1. The
restricted reactivity demonstrated by this antibody may indi-
cate a restricted distribution of such structures with adja-
cent fucosyl residues in the type 2 chain. It is assumed that
the structures could be synthesized by a mechanism of type 2
chain elongation coupled with α-1→3 fucosylation at every
GlcNAc residue as discussed in the accompanying paper. In
contrast to the specificity of the FH4 antibody, the FH5 and
ACFH18 monoclonals showed a preferential reactivity to tri-
fucosylated type 2 chain (trifucosyl neolactonorhexaosylcer-
amide), although a cross-reaction with difucosylated type 2
chain was also observed. Therefore, the monoclonal antibod-
ies such as FH4, FH5, and ACFH18 are specific reagents that
recognize di- or trifucosyl residues linked to type 2 chain,
excluding the terminal X-hapten structure. A possible epitope
recognized by the FH series, ACFH18, and other monoclonals
directed to the X determinant is shown in Fig. 5.

The antibodies described in this paper were selected by a
chemically well defined structure rather than by cells. This
approach is unique and will be useful in the general applica-
tion of a desired antibody directed to a defined structure. It
is predicted that these antibodies will be useful in detecting
specific types of cells such as undifferentiated cells and tumor
cells that are characterized by enrichment in di- or trifuco-
sylated type 2 chain structure of human cancer. Further
studies are needed to evaluate this possibility.

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