A Series of Disialogangliosides with Binary 2→3 Sialosyllectosamine Structure, Defined by Monoclonal Antibody NUH2, Are Oncodevelopmentally Regulated Antigens*

(Received for publication, May 24, 1989)

Edward D. Nudelman, Ulla Mandel, Steven B. Levery, Tokio Kaizu, and Sen-itiroh Hakomori

From the The Biomembrane Institute and University of Washington, Seattle, Washington 98119

A mouse IgM monoclonal antibody, NUH2, was raised after immunization of mice with the disialoganglioside fraction of human colonic adenocarcinoma. This antibody reacts specifically with disialogangliosides having the Structure 1 shown below.

$$\text{NeuAc}_2\text{Galβ1\text{→4GlcNAcβ1}}$$

$$\begin{align*}
\text{Galβ1\text{→4GlcNAcβ1}} & \rightarrow R \\
3 & \\
6 & \\
\text{NeuAc}_2\text{Galβ1\text{→4GlcNAcβ1}}
\end{align*}$$

**Structure 1**

NUH2 does not react with structures lacking the sialic acid at either the β1→3 or β1→6 side chain (Table I, structure GS), nor with a binary structure having unequal chain lengths (Structure 2 below), nor with a binary type 2 chain structure having a trimannosyl core as found in the side chain of N-linked complex type oligosaccharides (Structure 3 below).

$$\begin{align*}
\text{NeuAc}_2\text{Galβ1\text{→4GlcNAcβ1}} & \rightarrow R \\
3 & \\
6 & \\
\text{NeuAc}_2\text{Galβ1\text{→4GlcNAcβ1}}
\end{align*}$$

**Structure 2**

In humans, the disialoganglioside antigens defined by antibody NUH2 are present in low quantity in normal cells (e.g., erythrocytes) and tissues, but are expressed highly in some colonic cancers, placenta, trophoblast, and sperm, and can be regarded as oncodevelopmentally regulated antigens.

The structural concept of oncodevelopmentally regulated antigens has been well established since the MoAb* approach was introduced in developmental biology and tumor immunology. Essentially all types of tumor-associated carbohydrate antigens defined by specific MoAbs have been found to be expressed at defined stages of embryogenesis and differentiation (1). A typical feature of oncodevelopmental carbohydrate antigens is an unbranched type 2 chain polylactosamine which is α1→2 terminally fucosylated, α1→3 internally fucosylated, α2→3 or α2→6 terminally sialylated, or some combination of these modifications. These structures are defined by a panel of MoAbs such as FH4 and FH5 (2), FH6 (3), KH1 (4), CC1...
TABLE I

NUH2-defined structures

| Name | Structure | Reactivity with NUH2 | Structure with C6 |
|------|-----------|---------------------|------------------|
| I    | Galβ1→4GlcNAcβ1 | - +                | Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer |
| G8   | Galβ1→4GlcNAcβ1 | - -                | Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer |
|     | NeuAcα2→3Galβ1→4GlcNAcβ1 | + -            | 3/6Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer |
| G10 (DSI) | NeuAcα2→3Galβ1→4GlcNAcβ1 | - -            | 3/6Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer |
| G11 (72-77) | NeuAcα2→3Galβ1→4GlcNAcβ1 | - -            | 3/6Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer |
| G12 (90-102) | NeuAcα2→3Galβ1→4GlcNAcβ1 | + -            | 3/6Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer |
|     | NeuAcα2→3Galβ1→4GlcNAcβ1 | + -            | 3/6Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer |

and CC2 (5), and ACFH18 (6). Accumulation of these glycolipids in human cancer, particularly adenocarcinoma, is due to a predominance in synthesis of β1→3GlcNAc transferase over that of β1→6GlcNAc transferase, thus leading to an unbranched core structure (7). In contrast to the major trends of aberrant glycosylation as described above, we have found an additional change in type 2 chain glycolipids of human colonic adenocarcinoma, defined by the new MoAb NUH2. This antibody was originally raised after immunization of mice with the disialoganglioside fraction of human colonic adenocarcinoma. NUHZ stained glycolipid fractions of various colonic cancers and was directed to an antigen characterized as having two NeuAcα2+3Galβ1+3 and β1→6 linked together to the terminal Gal of polylactosamine. A series of glycolipids reacting with NUH2 was also isolated from human placenta. The structures of these highly complex sialyl type 2 chain glycolipids, and their reactivity with NUH2, have defined the specificity of this new MoAb.

MATERIALS AND METHODS

RESULTS

Establishment of MoAb NUH2 after Immunization of Mice with Disialoganglioside Fraction of Human Colonic Adenocarcinoma—A mouse hybridoma secreting IgM MoAb NUH2 was established after immunization of BALB/c mice with disialoganglioside fraction of colonic adenocarcinoma. The antibody showed preferential reactivity with colonic cancer tissues and derived gangliosides over normal colonic epithelia and derived gangliosides. The antibody NUH2 did not react with previously known disialogangliosides of human colonic cancer (8), i.e. GD3 (II13NeuAcα2+8NeuAcβCer), GD2 (II13NeuAcα2+8NeuAcGg3Cer), GDlb (II13NeuAcα2+8NeuAc-Gg,Cer), GD1 (II13NeuAcIV3NeuAcIV3NeuAcGg4Cer), disialyl Leα (IV3NeuAcII13NeuAcIII13NeuAcIII13FucIC4Cer) (8), disialolactosylgloboside (IV3NeuAcIV3NeuAcGalβGh,Cer) (9), disialolparagloboside (IV3NeuAcα2+8NeuAcLc4Cer) (10, 11), or disialolactotetraosylceramide (III13NeuAcII13NeuAcIII13FucIV3NeuAcLc4Cer) (8). On the other hand, NUH2 did react with a major ganglioside component of human colonic cancer and a series of gangliosides present in human placenta. These gangliosides were structurally identified as G10 (DSI) or G12 (fractions 90-102) (Table I). NUH2 did not react with gangliosides G8 or G11 (Table I). Characterization of these structures is described in subsequent sections and in part in the Miniprint.

Isolation and Characterization of the NUH2-reactive Epitope—The major NUH2-reactive disialoganglioside in colonic cancer tissue was isolated by HPLC in IHW system from the elution fractions 67-84, and further purified on HPTLC. The same disialoganglioside was isolated from placenta extract by HPLC of elution fractions 72-77 (for detail, see Miniprint). The NUH2-nonreactive ganglioside (G11) was isolated, and
Monoclonal Antibody, NUH2, Defining Tumor Antigen, Disialo-I

18721

structure (12) (Fig. 2C, lane 2). TLC mobility was increased following further degradation by jackbean β-galactosidase, indicating loss of one sugar residue (Fig. 2A, lane 3). This component was further degraded by β-N-acetylgalactosaminidase from bovine epididymis; the resulting compound showed the same TLC mobility as paragloboside (nLc4) (Fig. 2A, lane 4), and was stained by 1B2 antibody (Fig. 2D, lane 4), specific for nLc4, nLc6, etc. Methylation analysis produced a pattern essentially identical to that of G10 (13) or “GD-6” (9) of human erythrocytes (Table II, Miniprint).

Further characterization of disialoganglioside A by FAB-MS of permethylated derivatives and 1H NMR spectroscopy is described in the Miniprint. All available data indicate that the structure is VI*NeuAcIV*NNeuAcα2→3Galβ1→4GlcNAc-nLc6 (Table I, structure G10).

Characterization of the NUH2-nonreactive Ganglioside Component from Placenta—One component with migration slower than G10 and showing no reactivity with NUH2, was isolated from placenta and termed “G11.” This ganglioside was degraded by sequential treatment with C. perfringens sialidase, β-galactosidase, and β-N-acetylgalactosaminidase to give lactonorhexaosylceramide (Fig. 2E, lane 5), and then biotted by MoAb 1B2 (Fig. 2F, lane 5). No intermediate degradation products showed reactivity with C6 antibody. These results, together with data from methylation analysis

3G12 and G13 are, respectively, tri- and tetrasialogangliosides having three and four NeuAcα2→3Galβ1→4GlcNAc side chains linked together.
various gangliosides. Abcissa: serial dilution of ganglioside dissolved in ethanol and evaporated on 96-well plastic plate (Costar Vinyl 2595); well 1 contained 100 ng of pure ganglioside, well 2 contained 50 ng, well 3 contained 25 ng, etc. No cholesterol residue was added. O, ganglioside A (disialyl lacto-oligosaccharide; disialyl I); Δ, ganglioside B from placenta (G11); Δ, ganglioside C (G12) from placenta (see Table I for structure; isolation and characterization will be described elsewhere); ●, sialidase-treated ganglioside A; ▲, sialidase-treated ganglioside C; ■, G8 ganglioside (14) (Table I), 2→3sialyl-N-acetylgalactosamine, 2→6sialyl-N-acetylgalactosamine, 2→3sialylparagloboside, 2→6sialylparagloboside, and GM3. Ordinate, antibody binding expressed by 125I-labeled protein A.

Quantitative Reactivity of NUH2 Antibody with Various Gangliosides and Glycoproteins—In addition to G10 and G11 gangliosides (fractions 72–77), G8 ganglioside previously isolated and characterized from human erythrocytes (14) (Table I), higher trisialoganglioside G12 (fractions 90–102; see Table I), and various lacto-series and ganglio-series gangliosides were compared by solid-phase antibody binding assay. The results (Fig. 3) clearly indicate that NUH2 bound only to those gangliosides having binary NeuAc2-→3Galβ1→4GlcNAc with the same chain length and linked α1-6/α1-3 to the Gal residue of type 2 chain; i.e. it reacted only with G10 and G12, but not with G8 or G11 gangliosides. Other gangliosides so far tested were all nonreactive.

Fetuin, transferrin, and α-1 acid glycoprotein (orosomucoid), which has the binary NeuAc2-→3Galβ1→4GlcNAc structure but is linked to the trimannose core structure of the complex-type N-linked carbohydrate chain of glycoprotein, were all nonreactive (Fig. 4).

Glycolipid Profiles of Human Cancer, Placenta, and Normal Tissue Reacting with MoAb NUH2—TLC immunostaining of ganglioside fractions prepared from various human cancer cases showed the presence of a common doublet, which was
identified as disialosyl I (G10 ganglioside) (Table I). 56 cases of various types of cancer showed the presence of this ganglioside and other higher gangliosides through positive staining with NUH2 (Fig. 5). Likewise, human placenta showed a series of bands with extremely strong reactivity. In contrast, ganglioside fractions from normal kidney, brain, and large intestine showed no reactivity, while normal liver gave a weak doublet band (Fig. 5).

**DISCUSSION**

Human gastrointestinal and bronchopulmonary tumors have been characterized by a preponderance of unbranched type 2 chain (i type) having extensive α1→3 fucosylation at the penultimate as well as internal GlcNAc. The fucosylation is coupled with terminal α2→3 sialylation or α1→2 fucosylation, resulting in a variety of tumor-associated epitopes defined by MoAbs showing preferential reactivity with tumors over corresponding normal tissues (1). A branched type 2 chain based on a β1→6GlcNAc linkage has been shown to be a relatively minor component in various tumors, and the branching process is regarded as developmentally regulated (15, 16). However, this assumption is based on lack of branched type 2 chain ABH and I antigens in gastrointestinal tumors and fetal tissues. The results of the present study indicate that the majority of terminal Gal residues of essentially all branched type 2 chain structures in tumors could be α2→3 sialylated, thus resulting in accumulation of NUH2-positive antigens in a large variety of human cancers. Therefore, branched type 2 chain appears to be a relatively major component in human tumors, yet its fully sialylated derivative may be a tumor-associated antigen. This situation could result from greatly enhanced α2→3 sialyltransferase activity. Fucosylation, a prerequisite to formation of branched ABH determinant, could be competitively inhibited.

The epitope of MoAb NUH2 is clearly identified as having two identical NeuAcα2→3Galβ1→4GlcNAc structures, β1→3 and β1→6 linked to Galβ1→4GlcNAc, i.e. disialosyl I. Any carbohydrate chain containing this structure (e.g. ganglioside G12) is reactive with NUH2. However, G11 ganglioside, in which the unequal side chains (NeuAcα2→3Galβ1→4GlcNAc and NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAc) are β1→3 and β1→6 linked together at the Gal residue of Galβ1→4GlcNAcβ1→R, was completely nonreactive. The presence of two sialic acids is another prerequisite for NUH2 reactivity, since G8 ganglioside (13, 14) (Table I) was also nonreactive. G11 ganglioside was previously isolated and characterized from human whole blood cell membrane as a minor component (9, 13). An accumulation of this glycolipid together with G11 and G12 in various human cancers indicates a new trend of aberrant glycosylation expressed in some human cancers, i.e. extensive sialylation of all branched type 2 chain.

Distribution of the NUH2-defined antigen in normal tissue is limited. It is virtually absent in various parenchymatous organs (brain, kidney, lung, liver, or spleen) as well as in normal gastrointestinal tissues. In contrast, high quantities of the antigen were detected in human placenta (as shown in this paper), human sperm, myelocytic leukemia cells, megakaryocytes, and trophoblast. NUH2 reacts strongly with ejaculated sperm and induced immobilization of sperm in the presence of complement, as we described (17). In colonic tissues, it is completely absent in normal mucosa regardless of location (distal or proximal regions). A strong focal expres-

---

**REFERENCES**

1. Hakomori, S., and Kannagi, R. (1983) *J. Natl. Cancer Inst.* 71, 231-251.
2. Fukushi, Y., Hakomori, S., Nudelman, E., and Cochran, N. (1984) *J. Biol. Chem.* 259, 4681-4685.
3. Fukushi, Y., Nudelman, E., Levery, S. B., Hakomori, S., and Rauvala, H. (1984) *J. Biol. Chem.* 259, 10511-10517.
4. Kaizu, T., Levery, S. B., Nudelman, E., Stenkamp, R. E., and Hakomori, S. (1986) *J. Biol. Chem.* 261, 11254-11258.
5. Kim, Y. S., Yuan, M., Itzkowitz, S. H., Sun, Q., Kaizu, T., Palekar, A., Trump, B. F., and Hakomori, S. (1986) *Cancer Res.* 46, 5985-5992.
6. Nudelman, E., Levery, S. B., Stroud, M. R., Salyan, M. E. K., Abe, K., and Hakomori, S. (1988) *J. Biol. Chem.* 263, 13942-13951.
7. Holmes, E. H., Hakomori, S., and Ostrander, G. K. (1987) *J. Biol. Chem.* 262, 15649-15658.
8. Nudelman, E., Fukushi, Y., Levery, S. B., Higuchi, T., and Hakomori, S. (1986) *J. Biol. Chem.* 261, 5487-5495.
9. Kundu, S. K., Samuelsson, B. E., Pascher, I., and Marcus, D. M. (1983) *J. Biol. Chem.* 258, 13587-13586.
10. Rauvala, H. (1976) *J. Biol. Chem.* 251, 7517-7520.
11. Hansson, G. C., Karlsson, K.-A., Larson, G., Stromberg, N., Teneberg, S., Thunin, J., Brodin, T., Spjogren, H.-O., Hellstrom, L., and Hellstrom, K.-E. (1983) in Proc. 7th Intl. Symp. on Glycoconjugates (Chester, M.A., Heinegard, D., Lundblad, A., and Svensson, S., eds), pp. 854-855, Lund-Ronnesby, Sweden.
12. Fenderson, B. A., Nicholas, E. J., Clausen, H., and Hakomori, S. (1986) *Mod. Immunol.* 23, 747-775.
13. Okada, Y., Kannagi, R., Levery, S. B., and Hakomori, S. (1984) *J. Immunol.* 133, 835-842.
14. Watanabe, K., Powell, M. E., and Hakomori, S. (1979) *J. Biol. Chem.* 254, 8223-8229.
15. Watanabe, K., and Hakomori, S. (1976) *J. Exp. Med.* 144, 644-653.
16. Fukuda, M., Fukuda, M. N., and Hakomori, S. (1979) *J. Biol. Chem.* 254, 3700-3703.
17. Teuji, Y., Clausen, H., Nudelman, E., Kaizu, T., Hakomori, S., and Isoguma, S. (1986) *J. Exp. Med.* 168, 343-356.
18. Young, W. W., Jr., Portoukalian, J., and Hakomori, S. (1981) *J. Biol. Chem.* 256, 10967-10972.
19. Hakomori, S., Patterson, C. M., Nudelman, E., and Sekiguchi, K. (1983) *J. Biol. Chem.* 258, 11819-11822.
20. Abe, K., McKeibh, J. M., and Hakomori, S. (1983) *J. Biol. Chem.* 258, 11793-11797.
21. Kannagi, R., Stroup, R., Cochran, N. A., Urdal, D. L., Young, W. W., Jr., and Hakomori, S. (1983) *Cancer Res.* 43, 4997-5006.
22. Magnani, J. L., Smith, D. F., and Ginsburg, V. (1980) *Anat. Biochem.* 109, 399-407.
23. Kannagi, R., Fukuda, M. N., and Hakomori, S. (1982) *J. Biol. Chem.* 257, 4438-4442.
24. Kannagi, R., Nudelman, E., Levery, S. B., and Hakomori, S. (1982) *J. Biol. Chem.* 257, 14865-14874.
25. Kannagi, R., Levery, S. B., Iihigami, F., Hakomori, S., Shievinsky, L. H., Knowles, B. B., and Solter, D. (1983) *J. Biol. Chem.* 258, 8934-8942.
26. Taki, T., Matsuo, K., Yamamoto, K., Matsubara, T., Hayashi, A., Abe, T., and Matsumoto, M. (1988) *Lipids* 23, 192-198.
27. Dabrowski, J., Hanfland, P., and Egg, H. (1980) *Biochemistry* 19, 5652-5656.
28. Levery, S. B., Nudelman, E., Kannagi, R., Symington, F. W., Andersen, N. H., Clausen, H., Baldwin, M., and Hakomori, S.

---

*T. Kaizu, Y. Teuji, and S. Hakomori, unpublished data.
H. Nojiri, and S. Hakomori, unpublished data.
Y. Teuji, and S. Hakomori, unpublished data.
T. Orntoft, and U. Mandel, unpublished data.
Monoclonal Antibody, NUH2, Defining Tumor Antigen, Disiulo-I

18724

Levery, S. B., and Hakomori, S. (1987) Methods Enzymol. 138, 13-25

29. Bjorndal, H., Hellervqvist, C. G., Lindberg, B., and Svensson, S. (1970) Angew. Chemie Int. Ed. 9, 610-619

30. Jansson, P.-E., Kenne, L., Liedgren, H., Lindberg, B., and Longmo, J. E. (1984) Biochim. Biophys. Acta 781, 351-393

31. Stoffel, K., Saito, H., and Hakomori, S. (1973) Arch. Biochem. Biophys. 55, 464-472

32. Kool, M. E., and Hakomori, S. (1989) Biochemistry 28, 7772-7781
Monoclonal Antibody, NUH2, Defining Tumor Antigen, Disialo-I

Table II. Molar proportions of partially methylated alditol acetates produced by ganglioside fractions.

| Fr. | 2,3,6-Glc* | 2,4,6-Gal | 2,4-Gal | 2,3,4,6-GNAcMe |
|-----|------------|----------|--------|-----------------|
| G10 (erythrocyte) | 1.00 (1) | 3.00 (3) | 1.00 (1) | 3.00 (3) |
| G10 (tumor) | 1.00 (1) | 2.85 (3) | 0.90 (1) | 3.16 (7) |
| G11 (placenta Fr. 72-77) | 1.00 (1) | 4.28 (4) | 1.18 (1) | 3.88 (4) |

* All results normalized to 2,3,6-Glc = 1.00

Results from erythrocyte G10 taken as standard values given, and all other values calculated using response factors of each derivative from this ganglioside.

Figure 4. HPLC elution profile of gangliosides from placenta.

Panel A: cocrystallization staining of various fractions obtained from HPLC step II, eluted from anion exchange column by gradient elution with 25% water (see Materials and Methods (Supplemental)), and developed in CMNO (50:47:14) containing 0.07% CaCl2. Disialo I was eluted in fractions 53-67, G11 in fractions 72-77, and G12 in fractions 96-102 (G13, eluted in fractions 125-126, is not shown). Panel B: cocrystallization staining of HPTLC pattern of various pooled fractions from HPLC steps I and II. Lane A, upper neutral glucosylated fraction from O erythrocytes. Lane B, upper neutral glucosylated fraction from placenta. Lane C, monosialoganglioside fraction from O erythrocytes. Lane D, disialoganglioside fraction from placenta. Lanes 1-3, pooled fractions from HPLC step I as shown in Panel A (lane 1, fractions 65-86; lane 2, fractions 85-106; lane 3, fractions 101-203). Lanes 4-14, pooled fractions from HPLC step II (lane 4, fractions 53-67; lane 5, fractions 66-71; lane 6, fractions 72-77; lane 7, fractions 78-89; lane 8, fractions 90-102; lane 9, fractions 103-117; lane 10, fractions 118-120; lane 11, fractions 121-126; lane 12, fractions 127-139; lane 13, fractions 131-140; lane 14, fractions 141-150). Panel C: Immunostaining with MoAb NuH2 of gangliosides separated on HPTLC, as described for Panel B.

Figure 5. Positive ion FAB mass spectrum of permethylation ganglioside A (G1) from human tumor with proposed fragmentation scheme.

Fragments are assigned nominal masses only. Scan range was 100-3600 a.m.u., scan slope 1 max 15 sec/decade. 5 scans were accumulated and averaged to increase signal-to-noise ratio.

Figure 6. Sections of the region-enhanced 2D-NMR spectrum of ganglioside A (G1-2) from human tumor in DMSO-d<sub>6</sub> at 300 MHz at 27°C.

Arabic numbers refer to positions of residues indicated by roman numerals in the structure drawn at the top of the figure. Resonances marked with "R" are from oligosaccharide backbone, while resonances marked "X" are from α-gliceryl and α-D-galactopyranosyl residues of unsaturated fatty acyl chains. sFAB-2 refers to a spin of nonsaturation fatty acids. "X" marks an interference spike. Assignments of III-I and IV-V may be reversed.

Figure 7. Positive ion FAB mass spectrum of permethylation ganglioside A (G1) from human tumor with proposed fragmentation scheme.

Fragments are assigned nominal masses only. Scan range was 100-3600 a.m.u., scan slope 1 max 15 sec/decade. 5 scans were accumulated and averaged to increase signal-to-noise ratio.