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TWO MOUSE HYBRIDOMA ANTIBODIES AGAINST HUMAN MILK-FAT GLOBULES RECOGNISE THE I(MA) ANTIGENIC DETERMINANT β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)*

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ABSTRACT

Two mouse hybridoma antibodies (LICR-LON-M39 and LICR-LON-M18) against the human-milk-fat globules were found to resemble human autoantibodies of anti-I type in their cold agglutinating property and their preferential reactions with erythrocytes of I- rather than i-type. From inhibition of binding assays with glycoproteins having known A, B, H, Le[a], Le[b], I, and i activities, and oligosaccharides of the Type 1 and Type 2 *lacto-N*-glycosyl series, it was established that these antibodies are directed at Type 2 structures, and that the I(Ma) determinant, β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6), which is usually found on branched oligosaccharides, is the preferred sequence. The hybridoma antibodies as well as anti-I Ma were shown to react well with the β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-D-Gal or -D-Man sequence. Studies of the reactions of these antibodies with glycolipids on thin-layer plates showed that the two hybridoma antibodies differ from anti-I Ma in reacting weakly with the unbranched i-type sequence β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4) as found on *lacto-N-nor*hexaosylceramide. Furthermore, they differ from anti-I Ma but resemble anti-I Woj and Sti, and a hybridoma antibody 1B2 in their failure to react with their determinant in the presence of α-D-(1→3)-linked galactosyl groups. From their lack of reactions with blood-group-A and -H active glycoproteins, and their reactions with neuraminidase-treated erythrocytes, it was deduced that the determinants recognised by the two hybridoma antibodies are also masked in the presence of α-L-(1→2)-linked fucosyl groups and sialic acid.

*Dedicated to Professor Elvin A. Kabat.

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INTRODUCTION

Natural monoclonal autoantibodies of man known as anti-I and anti-i cold agglutinins are among the best characterised monoclonal antibodies[1]. In particular, the reactivity of the anti-I antibody Ma with analogues of the oligosaccharide sequence βGal→4βGlcNAc→6* has been studied in considerable detail[2–6]. This antibody, as well as other types of monoclonal anti-I and -i antibodies which recognise various domains[1,7,8] on branched and linear oligosaccharides, respectively, of the *lacto-neo* series, have served as valuable reagents in providing structural information on the glycoconjugates of cell surfaces and secretions. There would be obvious advantages to having hybridoma-derived anti-I and -i antibodies in unlimited supplies. In the course of screening culture supernatants of hybridomas produced against milk-fat globules of human milk, we detected two antibodies, I ICR-LON-M39 and LICR-LON-M18, that behaved as cold agglutinins of anti-I type. We describe herein studies of the carbohydrate specificities of these antibodies.

EXPERIMENTAL

Monoclonal antibodies. — Two hybridoma antibodies, LICR-LON-M18 (Clone 3) and LICR-LON-M39 (Clone 6), raised[9] against human-milk-fat globule membranes are referred to as M18.3 and M39.6. Anti-I Ma has been described previously[1]. These monoclonal antibodies were used as ascites fluid (M39.6 and M18.3) or plasma (anti-I Ma).

Erythrocytes and haemagglutination assays. — Haemagglutination titres were assayed[10] by use of normal group O erythrocytes (OI), and group O erythrocytes from cord blood (Oi_{cord}) and from an adult of i type (Oi_{adult}).

Double-antibody radioimmunoassays. — The binding of the monoclonal antibodies to radioiodinated meconium glycoproteins (M39.6 and M18.3) or sheep-gastric mucin (anti-I Ma) was determined by a double-antibody radioimmunoassay[11,12]. Inhibition assays were performed at 20–30% level of binding of the monoclonal antibodies to the radioiodinated glycoproteins using 1:30 000 (M18.3) and 1:2500 (M39.6) dilutions of ascites and 1:4000 dilution of Ma plasma. Several glycoproteins with known blood-group A, B, H, Le^a, Le^b, I, and i activities were used as inhibitors (Table I). Glycoproteins designated Fl (gift of Dr. Winifred M. Watkins), N-1, JS, MSS, and Beach (gifts of Dr. Elvin A. Kabat), derived from ovarian cysts, and Mec (nonsec), derived from human meconium of nonsecretor type, have been described previously[3,11–14]. Mec (sec minus H) was a glycoprotein extract from human meconium of secretor type which had been depleted of I and i activities by affinity chromatography on an anti-I (Low) immunoadsorbent column[14]. The oligosaccharides used as inhibitors were chemically synthesized[15,16]

*All sugars in abbreviated form are assumed to have the D configuration, to be in the pyranose form, and to be linked at O-1 in oligosaccharides.

TABLE I

INHIBITION OF BINDING OF MONOCLONAL ANTIBODIES M39.6, M18.3, AND ANTI-I Ma TO ^{125}I GLYCOPROTEINS OF HUMAN MECONIUM

Glycoprotein inhibitors (blood-group activities)	*Antibodies (μg/mL giving 50% inhibition)*		
	M39.6	*M18.3*	*Anti-I Ma*
FI (I,i)	0.046	0.032	60
N-1 (Le^aI)	0.07	0.06	45
Mec nonsec (Le^a,I,i)	0.4	0.2	>100
Mec sec (Le^b minus Ii)	>10	>10	>1000
JS (HLe^b)	>115	>115	>1000
MSS (A)	>100	>100	>100
Beach (B)	34	38	>400

(oligosaccharides **1**, **2**, and **5**–**7**, gifts of Dr. Serge David and Dr. Alain Veyrières), or isolated from human milk (oligosaccharides **3** and **4**, gifts of Dr. W. M. Watkins).

Thin-layer chromatography, antibody binding, and autoradiography. — Binding of antibodies to glycolipids was detected by t.l.c. and autoradiography as described previously[17,18]. In brief, glycolipids were chromatographed on HP-TLC plates (aluminium sheets, Silica gel 60, E. Merck) in 55:45:12 (v/v) chloroform–methanol–water. The chromatograms were incubated for 4 h at 4° with 1:100 dilution of M39.6 or M18.3 (ascites fluid), or with 1:300 dilution of anti-I Ma plasma, followed by ^{125}I-labelled, rabbit anti-human IgM or anti-mouse immunoglobulin antibodies (4×10^6 c.p.m./mL) for 16 h at 4°. Autoradiograms were obtained with Singul-X RP X-ray films (Ceaverken Ab, Sweden) after 40- to 64-h exposure. *Lactoneo*tetraosylceramide (paragloboside) was prepared by neuraminidase treatment of sialyl*lactoneo*tetraosylceramide from bovine erythrocytes[19]. *Lacto-N-nor*hexaosylceramide from bovine erythrocytes was a gift of Dr. Sen-itiroh Hakomori. Ceramide decasaccharide was isolated from rabbit erythrocytes and ceramide octasaccharide obtained by α-D-galactosidase treatment of the ceramide decasaccharide[20] (gifts of Dr. Peter Hanfland). Bovine erythrocyte gangliosides were treated with mild acid (50mM HCl, 30 min, 80°) as described previously[21], and used as the mixture of several glycolipids of the *lactoneo* series.

RESULTS

Hybridoma antibodies M39.6 and M18.3 behave as anti-I cold agglutinins. — Both antibodies agglutinated group OI erythrocytes at 4° but not at 37°. They resembled human anti-I cold agglutinins rather than anti-i antibodies in their preferential reaction with group OI erythrocytes, rather than group Oi_{cord} and Oi_{adult} cells (Table II). Neuraminidase treatment of the i erythrocytes rendered these cells

TABLE II

AGGLUTINATION OF I, i_{cord}, AND i_{adult} ERYTHROCYTES AT 4°

Antibody	*Erythrocyte-agglutination titre*[a]					
	I		i_{cord}		i_{adult}	
	N−	*N*+	*N*−	*N*+	*N*−	*N*+
M18.3	6 400	204 800	<20	2 560	<20	5 120
M39.6	6 400	51 200	<20	5 120	40	5 210
anti-I Ma	32 000	128 000	4 000	16 000	160	8 000
anti-I Step	64 000	128 000	32 000	128 000	16 000	32 000
anti-i Den	32 000	256 000	1 024 000	>2 048 000	512 000	1 024 000

[a]Titres are expressed as reciprocal of dilution ascites fluid (M18.3 and M39.6) or plasma (anti IMa). Abbreviations: N−, untreated; and N+, neuraminidase treated

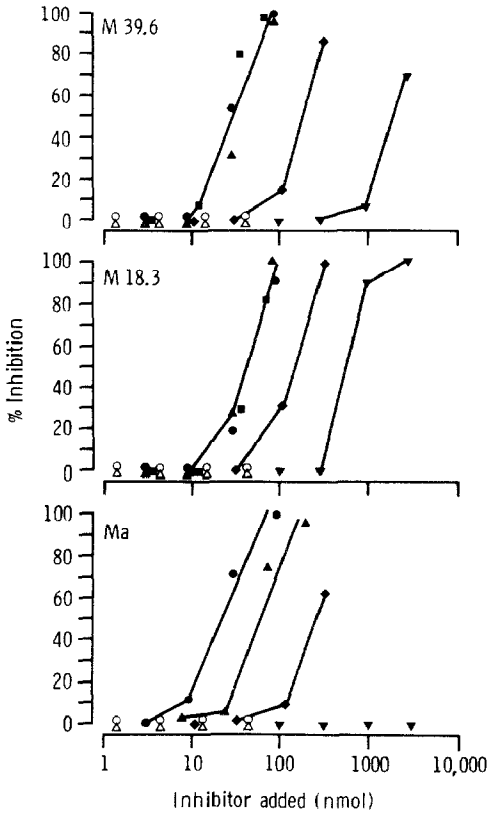


Fig. 1 Inhibition of binding of monoclonal antibodies M39.6, M18.3, and anti-I Ma to ^{125}I-glycoproteins of meconium by oligosaccharides. (▼) **1**, (◆) **2**, (○) **3**, (△) **4**, (●) **5**, (▲) **6**, and (■) **7**. Oligosaccharide **7** was shown previously[22] to have the same inhibitory activity as oligosaccharide **5** with anti-I Ma.

agglutinable by the two hybridoma antibodies. However, as with the human anti-I antibodies, the agglutination titres reached were 10- to 20-fold lower than those with similarly treated I cells.

Strong reactions with blood-group precursor substances. — In double-antibody radioimmunoassays, antibodies M39.6 and M18.3 gave binding curves with a radioiodinated glycoprotein extract from human meconium (results not shown). The I-active glycoproteins Fl and N-1 were potent inhibitors of their binding (Table I). These two glycoproteins were ~600–2000 times more potent as inhibitors of the two hybridoma antibodies than anti-I Ma. Glycoproteins lacking in I-antigen activities were not inhibitory or they were ~300–800 times less active per unit weight than glycoproteins Fl and N-1, which are known to be rich in blood-group precursor-like structures[2,13].

Inhibition with oligosaccharides of the Type 2 (lacto-neo) *series.* — The hybridoma antibodies resembled anti-I antibodies of Ma-type in their reactions with Type 2 rather than Type 1 oligosaccharide sequences (Fig. 1). Anti-I Ma has previously been shown not to react with the chemically synthesized oligosaccharide[3] β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→6)-D-Gal and in experiments performed after this paper was first submitted, this trisaccharide was found not to inhibit the binding of the monoclonal antibodies, M18.3 and M39.6, to radiolabelled meconium. With all three antibodies, oligosaccharides **5** and **7** having the β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-D-Gal sequence were more potent than *N*-acetyllactosamine [β-D-Gal*p*-(1→4)-D-GlcNAc]. Lactose gave some inhibition with M39.6 and M18.3 (being 10 and 6 times less active, respectively, than *N*-acetyllactosamine), but not with anti-I Ma.

The lack of inhibitory activity of *lacto-N-neo*tetraose (structure **4**) at the highest level tested (40 nmol per assay) clearly indicates that the β-D-Glc*p*NAc-(1→6)-D-Gal linkage found on branched structures is more complementary than the β-D-Glc*p*NAc-(1→3)-D-Gal linkage (see also next section).

Oligosaccharide **6** with the (1→4, 1→6) sequence having a D-mannose rather than D-galactose unit at the reducing end was a strong inhibitor of the three antibodies. With the two hybridoma antibodies, the inhibitory activity was equal to those of oligosaccharides **5** and **7**, but it was 3 times less active with anti-I Ma.

βGal→4Glc
1

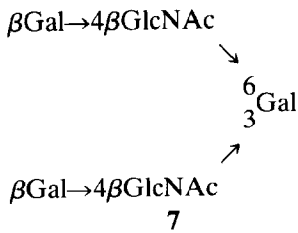
βGal→4GlcNAc
2

βGal→3βGlcNAc→3βGal→4Glc
3

βGal→4βGlcNAc→3βGal→4Glc
4

βGal→4βGlcNAc→6βGal
5

βGal→4βGlcNAc→6Man
6



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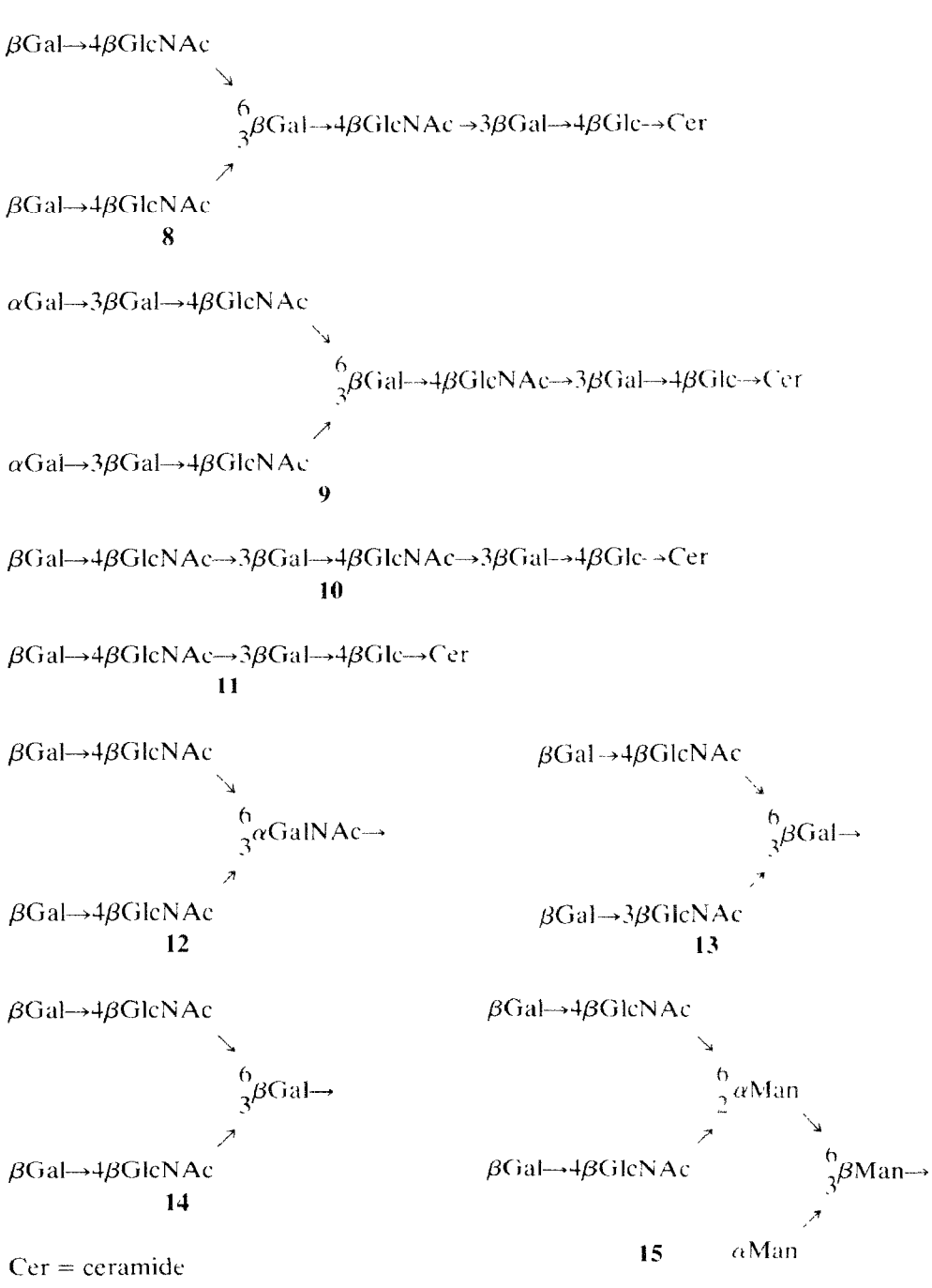

Reactions of glycolipids on thin-layer plates. — The subtle differences in the specificities of the three antibodies were further shown by their reactions with glycolipids on thin-layer plates (Fig. 2). All three antibodies gave strong immuno-

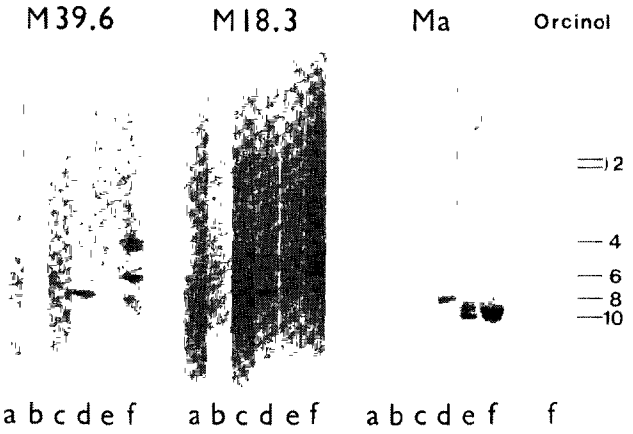

Fig 2. Different binding patterns of monoclonal antibodies, M39 6, M18.3, and anti-I Ma to glycolipids on thin-layer chromatograms in 55:45:12 (v/v) chloroform–methanol–water: Lane a, 0.1 μg of *lactoneo*tetraosylceramide (paragloboside, **11**); lane b, 0.5 μg of *lactoneo*tetraosylceramide; lane c, 0.1 μg of *lacto-N-nor*hexaosylceramide (**10**), lane d, 0.1 μg of ceramide octasaccharide (*lacto-N-iso*octaosylceramide, **8**); lane e, 0.1 μg of ceramide decasaccharide (**9**); and lane f, a mixture of glycolipids obtained by mild acid hydrolysis of bovine erythrocyte ganglioside subfraction (containing approximately 4 μg of paragloboside and 1 μg of *lacto-N-nor*hexaosylceramide with more complex glycolipids). The bars and numbers adjacent to the strip stained with orcinol indicate the position and number of sugars in the glycolipids as follows: 2, lactosylceramide; 4, *lactoneo*tetraosylceramide; 6, *lacto-N-nor*hexaosylceramide; 8, ceramide octasaccharide; and 10, ceramide decasaccharide.

staining of 0.1 μg of the reference I-active ceramide octasaccharide **8**. However, only anti-I Ma reacted with decasaccharide **9**, which has two α-D-(1→3)-linked galactosyl groups at the nonreducing end. In addition, the two hybridoma antibodies reacted weakly with the ceramide hexasaccharide *lacto-N-nor*hexaosylceramide (**10**), and M39.6 reacted with paragloboside (**11**). These reactions were not detected when 0.1 μg of **10** and **11** were applied, but were detected when 1 and 0.5 μg, respectively, were applied.

DISCUSSION

These studies have shown that the hybridoma antibodies M39 and M18 resemble human anti-I antibodies Ma, Woj, and Sti, which recognise[1,23] the I antigenic determinant β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6). Earlier studies have shown that this is the most abundantly expressed epitope of the I antigen in human milk[2,24,25]. The two hybridoma antibodies differ from anti-I Ma, but resemble[23] anti-I Woj and Sti in their failure to react with their determinant in the presence of α-D-(1→3)-linked galactosyl groups. The studies with native and neuraminidase-treated erythrocytes have shown that the presence of sialic acid also results in masking of the determinants recognised by the hybridoma antibodies. From their lack of reaction with glycoproteins having blood-group H and A activities, it can be deduced that the hybridoma antibodies M39 and M18, like anti-I antibodies, can-

not react with their determinants in the presence of the blood-group H associated α-L-(1→2)-linked fucosyl groups. A mouse hybridoma antibody (1B2) with similar haemagglutinating properties to M39 and M18 has been described previously[26]. This antibody was shown to react with paragloboside (**11**) and *lacto-N-nor*hexaosylceramide (**10**), but not with their analogues substituted with α-(2→3)-linked sialyl and α-D-(1→3)-linked galactosyl groups. It was not stated whether this antibody reacted with ceramide octasaccharide (**8**).

The present studies clearly show that anti-I Ma and the two hybridoma antibodies M39 and M18 can accommodate the β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-D-Man sequence in their combining sites. Previous studies[27] with anti-I Ma have shown that 2-acetamido-2-deoxy-D-galactosyl can also be substituted for D-galactosyl residues at the reducing end. The β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6) sequence occurs as a branch of carbohydrate chains of secreted and membranes-associated glycoproteins, and glycolipids (structures **12**–**15**, for reviews see refs. 1, 7, 28, 29). Thus, any cell or body fluid that contains these structures in sufficient proportions would react with the two monoclonal antibodies and anti-I Ma, but only those containing structure **14** would react with other types of anti-I antibodies, for these require a β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3) domain in addition to the β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6) branch.

The monoclonal antibodies M39 and M18 were raised with the aim of identifying different cell types in the normal human breast[9]. By use of the immunoperoxidase technique, the latter antibody was shown to react with the luminal aspect of a subpopulation of normal breast epithelial cells and the cytoplasm of scattered cells in certain breast tumours. This antibody reacts with a number of other epithelial cell types but not with nonepithelial cells. Less information is available on the tissue reactivities of M39. Preliminary studies have shown that it binds to a wider variety of cell types than M18; in addition to epithelial cells, this antibody reacts with a population of cells in peripheral lymphoid tissues having the morphological features of dendritic cells (C. S. Foster and J. A. Thomas, unpublished results). These observations are consistent with the differences in the reaction patterns of the two antibodies observed in the present studies. In certain nonreactive epithelial cells of the normal breast and in tumours of the breast epithelium, determinants reactive with both antibodies can be revealed by desialylation (C. S. Foster, unpublished results). Thus, the M39 and M18 determinants are further examples of differentiation antigens first detected by monoclonal antibodies, and subsequently shown to consist of carbohydrate structures[30] whose antigenicity depends on the presence or absence of additional glycosylations.

Apart from detecting heterogeneities among epithelial cells[9,31,32], these two monoclonal antibodies, together with other types of I and i antibodies, will be valuable reagents in the subcellular localization of the biosynthetic steps of Type 2-based carbohydrate chains when used in conjunction with other monoclonal antibodies, such as anti-SSEA-1 (refs. 11, 33), H11 (ref. 34), C14 (ref. 35), and anti-Gd (ref. 36, K. Uemura, J. Pennington, and T. Feizi, unpublished results), directed against their α-L-fucosylated and α-sialylated derivatives.

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