

LIPOOLIGOSACCHARIDES (LOS) OF *NEISSERIA*
GONORRHOEAE AND *NEISSERIA MENINGITIDIS* HAVE
COMPONENTS THAT ARE IMMUNOCHEMICALLY SIMILAR
TO PRECURSORS OF HUMAN BLOOD GROUP ANTIGENS
Carbohydrate Sequence Specificity of the Mouse Monoclonal
Antibodies that Recognize Crossreacting Antigens on
LOS and Human Erythrocytes

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Lipooligosaccharides (LOS)¹ of *Neisseria* are heterogeneous mixtures of glycolipids of M_r 3,200–7,000 as estimated by migration in SDS-PAGE (1, 2). LOS components have epitopes that have been defined by various mAbs (1–3). Apicella et al. (3) found that mAb 3F11 recognized a common epitope on gonococcal LOS that was also found on some meningococcal LOS. A second mAb, 06B4, was shown to have a specificity very similar to that of 3F11 (2). Both mAbs bind to an epitope on a 4.8-kD LOS (1, 2) that is made by all but three of over 90 gonococcal strains that we have examined (one of the negative strains is the pyocin-resistant mutant, JW31R [2, 4, 5]). The epitope defined by 06B4 is also expressed on the LOS of many strains of *Neisseria meningitidis*, whereas the 3F11-defined epitope is expressed only occasionally on meningococcal LOS (6). Apicella et al. (3) used monosaccharide and disaccharide ELISA inhibition data to suggest that the 3F11-defined epitope was composed of GalNAc→Gal1→4Glc, a structure similar to the terminal oligosaccharide found on some meningococcal LOS. The chemical specificity of 06B4 has not been defined.

Jennings and colleagues (7–9) have reported the structures of the carbohydrate portions of partially degraded LOS of three meningococcal strains of different LOS serotypes (an L2 strain, L3,7,9 strain and an L5 strain). The same terminal tetrasaccharide occupied the nonreducing end of the LOS molecules of each of these strains. This terminal structure (Galβ1→4GlcNAcβ1→3Galβ1→4Glc) is identical to the ter-

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¹ Abbreviations used in this paper: LOS, lipooligosaccharide; SPRIA, solid-phase radioimmunoassay.

minal tetrasaccharide (lacto-*N*-neotetraose, LNnT) of paragloboside (nLc₄Cer), a glycosphingolipid precursor of the major human blood group antigens (10), and terminates in the disaccharide *N*-acetylglucosamine (LacNAc). Although complete chemical structures have not been reported for the oligosaccharides of any gonococcal LOS, several laboratories have reported that the LOS of various strains were composed of different ratios of the same monosaccharides (Gal, Glc, GlcNAc, Hep, dOclA) as meningococcal LOSs (11-16).

The chemical identity of the terminal LNnT of meningococcal LOS and of nLc₄Cer suggested that the two glycolipids might also share epitopes and that human and neisserial cells might express the same antigenic structures. Since mAbs 06B4 and 3F11 bind to the LOS of some meningococcal and most gonococcal strains, and the latter is specific for a LacNAc-containing structure that could be represented on nLc₄Cer, we decided to use them as probes to look for the presence of structures on human cell surfaces that are immunochemically similar to those within neisserial LOS.

Since the development of immunochemical procedures for detecting antibody binding to glycosphingolipids (17, 18), these compounds have proven useful in determining the oligosaccharide binding specificities of mAbs. TLC immunostaining and solid-phase binding assays used in conjunction with a variety of purified glycosphingolipids of known structure have made it possible to determine the fine differences in the oligosaccharide binding specificities of mAbs. This report describes the presence of an immunochemically similar structure on the gonococcal and human erythrocyte cell surfaces and describes the use of glycosphingolipids of known structure to determine the fine specificity of mAbs 3F11 and 06B4 that identify this conserved epitope.

Materials and Methods

Bacterial Strains. *Neisseria gonorrhoeae* strains 56 and 220 were kindly provided by Dr. Herman Schneider (Walter Reed Army Institute of Research, Washington, DC) and have been extensively characterized (1, 2, 13). Strain 56 is sensitive to lysis by normal human sera (ser⁻); 220 is serum resistant (ser⁺). The *Neisseria meningitidis* prototype strains for LOS serotypes have also been described previously (19-22). The meningococcal strains used in this study are of the following serotype classifications (19, 23): 126E (C:3:P1.2:L1,8), 35E (C:20:P1.1:L2,5), 138I (C:2a:P1.2:L3), 89I (C:11:L4), 118V (C:?:L2,4), 6155 (B:2a:P1.2:L7,3). Culture procedures for each *Neisseria* were described [1, 19].

Lipooligosaccharides. LOS were extracted from saline-washed or acetone-dried organisms by the hot phenol-water method (13, 24). Stock solutions of LOS used in solid-phase RIA (SPRIA) were prepared as described previously (2). Briefly, LOS was dissolved in 50 mM NaOH, heated for 1 h at 37°C, then carefully neutralized with 50 mM HCl and stored at 4°C.

Monoclonal Antibodies. mAbs were kindly provided by Drs. Michael A. Apicella (06B4, 3f11) and Julie Westerink (1F10), State University of New York, Buffalo; and William W. Young, Jr. (1B2), University of Virginia Medical School, Charlottesville, VA. The preparation and characteristics of mAbs 06B4 (2), 3F11 (1-4), and 1B2 (25) have been reported. mAb 1F10 was prepared as described (3); it is specific for meningococcal group A polysaccharide. mAbs 06B4 and 3F11 were prepared from cells of mice immunized with a gonococcal strain and mAb 1F10 was prepared from cells of mice immunized with a group A meningococcal strain. mAb 1B2 was prepared from immunocytes of mice that had been inoculated with the *N*-acetylglucosamine glycosphingolipid, lacto-*N*-norhexaosylceramide, nLc₆Cer, (Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc-Ceramide), which had been hydrophobically complexed to *Salmonella minnesota* (25). mAb 1B2 agglutinates human adult, but not fetal,

erythrocytes when incubated with them at 4°C (25); this hemagglutinating activity can be inhibited by *N*-acetyllactosamine glycosphingolipids including some that represent various I blood group antigens.

All four mAbs are of the IgM class. The concentrations of mAbs 06B4, 3F11, and 1B2 were 3.65, 0.47, and 0.16 mg/ml, respectively, as determined by a modification (2) of a quantitative SPRIA (26).

Human Erythrocytes and Hemagglutination Assay. Adult human blood specimens were obtained from the hematology laboratory of the San Francisco VA Medical Center and from colleagues in our laboratory. Infant blood samples were obtained from unused specimens from the hematology laboratory of the Moffitt-Long Hospital/UCSF Medical Center, San Francisco, CA. We determined the blood type of each human erythrocyte specimen with mouse monoclonal anti-A and anti-B, and rabbit polyclonal anti-D grouping reagents (Ortho Diagnostics, Raritan, NJ).

A small aliquot of cells was washed four times with PBS, pH 7.2, and then suspended to 2% cells in PBS. The 2% cell suspension was then added to an equal volume of twofold dilutions of mAbs in PBS, incubated for 2 h at the designated temperature, and then examined for agglutination. We scored agglutination as follows: complete agglutination, pellet with crinkled edges = 3+; complete agglutination, absence of settled cells = 2+; partial agglutination, presence of partially clumped cells = 1+; no agglutination, settled cells with no visible clumping after remixing pellet = 0. The data are presented as the concentration of mAb at the highest dilution producing 1+ agglutination or as the reciprocal of that dilution of mAb.

Enzymatic Treatment of Erythrocytes. To identify specificity for cryptic antigens, mAbs were tested in parallel with cells that had been treated with either trypsin or neuraminidase. A 150- μ l aliquot of whole blood was washed four times with PBS and the cells resuspended in 150 μ l of PBS. After dividing the cell suspension into three equal aliquots, an equal volume of either PBS (pH 6.0), trypsin (2.5 mg/ml in PBS, pH 7.2; type IIIS, Sigma Chemical Co., St. Louis, MO), or neuraminidase (25 mg/ml in PBS, pH 6.0; type V, Sigma Chemical Co.) was added. The mixtures were incubated for 1 h at 37°C and then washed three times with PBS to remove enzyme and degradation products. The cells were further treated as described above.

Affinity Purification of mAb 3F11 with Enzyme-treated Human Erythrocytes (RBC-3F11). We used differential binding at 4°C and 37°C of mAb 3F11 to adult erythrocytes to purify mAb 3F11 from ascites. A 300- μ l aliquot of mAb 3F11 was incubated with 300 μ l of packed trypsin-treated adult human erythrocytes for 1 h at 4°C. The erythrocytes were pelleted and then washed extensively with PBS at 4°C to remove unbound ascites. A 500- μ l aliquot of PBS was added to the pelleted cells and the mixture was incubated in a 37°C water bath for 30 min to elute the bound antibodies. We pelleted the cells and used the SPRIA and SDS-PAGE/immunoblot analysis to test whether the supernatant contained eluted antibody (RBC-3F11) to LOS from various meningococcal and gonococcal strains.

SDS-PAGE and Immunoblot Analysis. LOS were separated by SDS-PAGE by the method of Laemmli (27), as slightly modified (2). LOS were separated by electrophoresis through duplicate slab gels. The LOS in one slab was stained with silver (28) and the LOS in the other slab was electroblotted to nitrocellulose paper. The immunoblot analysis has been described (29). The paper was cut, incubated in a "filler" buffer consisting of 1% casein in 10 mM Tris-hydrochloride/150mM NaCl/5mM MgCl₂/30 mM NaN₃ (pH 7.4), and was incubated for 1 h with the appropriate mAb diluted to ~5 μ g/ml in filler buffer. The antibody-treated nitrocellulose papers were washed and then incubated with an alkaline phosphatase-conjugated goat anti-mouse IgM (Sigma Chemical Co., St. Louis, MO) diluted 1:200 in filler buffer. The papers were washed three times with PBS and once with 50 mM Tris/HCl, pH 8.0. A solution consisting of 0.2% Fast Red TR salt (Sigma Chemical Co.) and 0.1% naphthol AS MX phosphoric acid (Sigma Chemical Co.) dissolved in 50 mM Tris-HCl, pH 8.0 was added to the paper. The color reaction was stopped when no further color development was noted (usually 5–7 min). Photographs were made of the dried papers. All antibody steps and washes were done at room temperature on a rotating shaker. We identified the components that bound mAbs by reference to component profiles from previous mAb studies with these mAbs and LOS from gonococcal strains (1, 2).

SPRIA with mAb 3F11, 06B4, and 1B2. The binding of mAbs to neisserial LOSs was assessed by a previously described SPRIA (20, 26). Briefly, plastic microtiter wells were sensitized with 25 $\mu\text{g/ml}$ concentrations of meningococcal or gonococcal LOS, followed by the addition of fourfold dilutions of each of the mAbs. LOS were diluted in Dulbecco's PBS, pH 7.4 (containing 20 mM MgCl_2); mAb and secondary antibodies were diluted in filler buffer (see above). After incubating the plates overnight, we removed antigen and washed the wells. Bound mAb was quantified with use of ^{125}I -goat anti-mouse IgM (Kirkegaard and Perry, Gaithersburg, MD). The wells were washed and cut, after they had incubated with secondary antibody for 6 h. Bound radioactivity was measured with a gamma counter.

Different LOS bound different amounts of each mAb at saturation, as judged by the binding of secondary antibody. To compare the relative expression of each epitope among different LOS, the LOS that bound the most mAb at saturation was assigned a value of 100% and the amount of mAb that bound to each of the other LOS was expressed as a percentage of the highest binding. Comparisons among different mAbs that bound a particular LOS were limited to negative or positive because of differences among mAb affinities and the goat antibodies against the different mouse Igs. Comparisons among different LOSs that bound a particular mAb could be made quantitatively.

Glycosphingolipids. Total neutral glycosphingolipids from human erythrocytes were purified by standard procedures (30). Preparation of other glycosphingolipids has been described previously (30, 31).

TLC Immunostaining. The immunostaining procedure for the TLC plates has been described in detail (32). The TLC conditions used in each experiment are presented in the figure legends.

Binding Assays with Glycosphingolipids. Solid-phase binding assays and the ELISA procedure with purified glycosphingolipids have recently been described (33).

Results

Structural Similarities between LOS and Blood Group Antigens. Jennings and colleagues (7-9) have reported the structure of three meningococcal strains of different LOS serotype (Table I). LNnT ($\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{Glc}$) is the nonreducing terminus of the oligosaccharides of each of these strains (7-9). Since LNnT is also the terminal tetrasaccharide of the major blood group antigen precursor, nLc₄Cer (Table I), this structural similarity led us to test for immunochemical similarity between human cells and LOS.

Binding of mAbs to Meningococcal LOS. Because of the LNnT structure present in meningococcal LOS (7-9), we tested mAbs for binding to multiple serologically diverse meningococcal LOS. Table II compares the SPRIA binding activity with LOS of eight prototype meningococcal LOS serotype strains by mAbs (3F11 and 06B4) that identify conserved and similar epitopes on gonococcal LOS. In contrast to studies with gonococcal LOS, the 3F11 and 06B4 epitopes were expressed on a lower percentage of meningococcal LOS and were not always coexpressed on the meningococcal LOS. mAb 3F11 bound to the LOS of 3/8 strains; mAb 06B4 bound 5/8 LOS. The mAb-defined epitopes were coexpressed only on strains 6155 and 120M. Strains 35E, 6275, 89I, and M981 expressed one or the other, but not both, epitopes. We then tested human adult erythrocytes with mAbs 3F11 and 06B4.

Binding of mAbs 3F11 and 06B4 to Adult Human Erythrocytes. Table III compares the binding of mAbs 3F11 and 06B4 with adult human erythrocytes, as judged by agglutination. mAb 3F11 (23.5 $\mu\text{g/ml}$) agglutinated all 47 specimens at 4°C, regardless of their blood type. mAb 06B4 agglutinated 38 (80%) of these same erythrocyte specimens; higher concentrations (>175 $\mu\text{g/ml}$) of mAb 06B4 failed to agglutinate

TABLE I
Structures of Three Different Meningococcal LOS and Erythrocyte Glycosphingolipids I, i, and Paragloboside

Sample	Structure	Reference
Strain 1 LOS	Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→4Hepα1→5KDO 3 α† 1 GlcNAcα1→2Hep	7
Strain 2 LOS	Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→4Hepα1→5KDO 3 α† 1 GlcNAcα1→2Hep3←1αGlc	8, 9
Strain 3 LOS	Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→4Hepα1→5KDO 3 α† 1 GlcNAcα1→2Hep3←1αGlc	8, 9
paragloboside (nLc ₄ Cer [‡])	Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer	62
i antigen (nLc ₆ Cer)	Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAβ1→3Galβ1→4Glcβ1→1Cer	63
I antigen (nLc ₈ Cer) [§]	Galβ1→4GlcNAcβ1→6 Galβ1→4GlcNAβ1→3Galβ1→4Glcβ1→1Cer Galβ1→4GlcNAcβ1→3 [†]	63

* The oligosaccharide portion of the LOS molecule is shown.

† Cer, ceramide.

§ For I antigen, the branched form of nLc₈Cer is most active with various human cold agglutinins (10).

TABLE II
SPRIA Binding* of mAbs to LOS from Meningococcal Prototype LOS Serotype Strains

Strain	L type [†]	mAb 06B4	mAb 3F11
126E	L1,8	<1	<1
35E	L2	15	<1
6275	L3	20	<1
89I	L4	100	<1
M981	L5	1	6
6155	L7,3	96	100
M978	L8,3,(4,7)	<1	2
120M	L9,7,(4,6)	81	100

* To compare the relative expression of each epitope among different LOS, we assigned a value of 100% to the LOS that bound the most mAb at saturation, and expressed the amount of the same concentration of mAb that bound to each other LOS as a percentage of the first LOS. 5% was arbitrarily selected as the positive cutoff value. As each LOS is a mixture of different LOS components, relative binding reflects the relative proportion of LOS bearing the mAb-recognized epitope among all the LOS made by the population of that strain from which the LOS was extracted (64, 65).

† Numbers in parentheses indicate minor serotypes (<50% inhibition of typing serum) (20).

TABLE III
*Agglutination of Adult Human Erythrocytes by mAbs Specific for
 Neisserial LOS*

RBC	N	Agglutinated* by:	
		3F11	06B4
A +	15	15 (100)	14 (93)
B +	5	5 (100)	4 (80)
AB +	1	1 (100)	1 (100)
O +	21	21 (100)	17 (81)
A -	3	3 (100)	1 (33)
B -	1	1 (100)	0 (0)
O -	1	1 (100)	1 (100)
Total	47	47 (100)	38 (81)

* Number (%) of samples of each ABO type that agglutinated with the mAb. MAbs were tested at a concentration of 23.5 µg/ml and 182 µg/ml for 3F11 and 06B4, respectively. Neither BALB/c nor New Zealand White rabbit erythrocytes were agglutinated by either mAb at these concentrations.

the remaining nine specimens. Agglutination with 3F11 was always greater than with 06B4, even though 06B4 was used at nearly a sevenfold higher antibody concentration (182 µg/ml) compared with 3F11 (23.5 µg/ml). On the basis of the limited number of samples analyzed there was no obvious correlation between agglutinability by mAb 06B4 and ABO type of erythrocytes. Neither mAb agglutinated erythrocytes from BALB/c mice nor New Zealand White rabbits (data not shown).

Effect of Temperature on Agglutination of Adult Human Erythrocytes. Agglutination by mAb 3F11 was optimal at incubation temperatures of 4°C. A 2-3 log decrease in agglutination titer occurred at temperatures of 22°C and 37°C (Table IV). Cells that agglutinated with a 3+ reaction at 4°C were easily disrupted and resuspended when the cells reequilibrated to room temperature. mAb 06B4 also agglutinated erythrocytes better at 4°C than 37°C.

Binding of RBC-3F11 and 1B2 to Meningococcal and Gonococcal LOS. The contrasting expression of the mAb-defined epitopes on erythrocytes at 4°C and 37°C, proved useful as a method for purifying the erythrocyte (RBC) binding antibody (RBC-3F11) from ascites. Erythrocyte-purified mAb 3F11 (RBC-3F11) bound to the LOS

TABLE IV
*Effect of Temperature on Agglutination of Adult Human Erythrocytes
 by mAb 3F11*

RBC sample	Agglutination titer*		
	4°C	22°C	37°C
1	>1,000	10	10
2	>1,000	10	1
3	>1,000	10	1
4	>1,000	10	10

* Reciprocal of the dilution of mAb 3F11 that resulted in 1+ agglutination; RBC samples 2 and 3 were not agglutinated at 37°C by undiluted mAb. See Materials and Methods for criteria for scoring agglutination reactions.

TABLE V
Comparison of Binding to Neisserial LOS by
mAbs 1B2, 3F11, RBC-3F11, and 06B4

mAb	LOS of strain*				
	126E	89I	6155	220	56
1B2†	<1	<1	43	100	58
3F11	<1	<1	27	100	70
RBC-3F11	<1	<1	33	100	71
06B4	<1	35	50	100	31

* mAbs were tested in a SPRIA; binding activity was designated as a percentage of the maximum binding value for that mAb, as described in Materials and Methods.

† mAb 1B2 is specific to glycolipids having a nonreducing terminal *N*-acetyl-lactosamine (Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow R) (25).

of gonococcal strains 220 and 56 and meningococcal strain 6155, but not to meningococcal strains 89I and 126E (Table V). The similar binding activity of RBC-3F11 and unpurified 3F11 suggests that the LOS and erythrocyte epitopes are defined by the mAb and not by a contaminating polyclonal antibody. This was further confirmed by the binding to neisserial LOS by mAb 1B2, which was produced in a mouse immunized with a glycosphingolipid found on human erythrocytes and specific for terminal *N*-acetyl-lactosamine (Gal β 1 \rightarrow 4GlcNAc). The LOS-binding pattern of mAb 1B2 was the same as that of mAb 3F11 (Table V). mAb 06B4 differed from those of 3F11, 1B2, and RBC-3F11 in that it bound 89I LOS.

Immunoblot Analysis of Neisserial LOS with mAbs. Each of the mAbs that bound a particular LOS in SPRIA also bound at least one component of that LOS after separation of the components by SDS-PAGE. Fig. 1 shows the silver-stained profile of each of the five LOS tested for binding of 1B2 in the SPRIA (Table V) and their

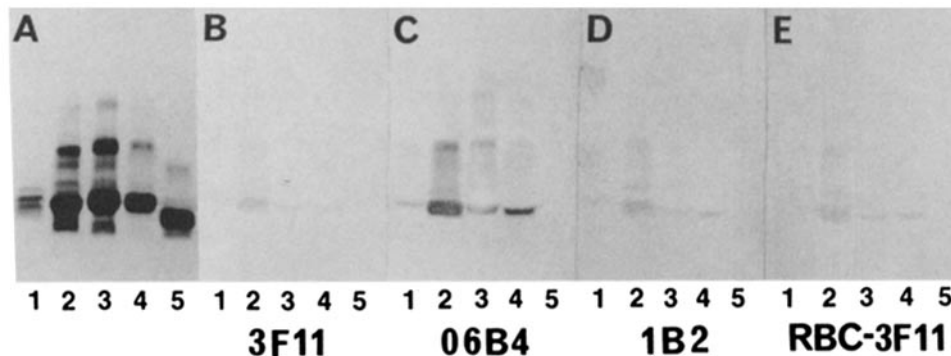


FIGURE 1. SDS-PAGE/immunoblot analysis of mAbs 3F11, 06B4, 1B2, and RBC-3F11 with gonococcal (220 and 56) and meningococcal (126E, 89I, 6155) LOS. Lane 1, 56 LOS; lane 2, 220 LOS; lane 3, 6155 LOS; lane 4, 89I LOS; lane 5, 126E LOS. (A) Silver-stained gel; (B) immunoblot with 3F11; (C) immunoblot with RBC-3F11; (D) immunoblot with 1B2; (E) immunoblot with 06B4. The binding of mAbs 3F11, 1B2, and RBC-3F11 are very weak to 56 LOS, but can be seen on the original blot. Electroblooded LOS incubated with each of the reagents, except mAb, showed no color development.

TABLE VI
Agglutination of Human Adult and Infant Erythrocytes by mAb 3F11

Erythrocyte source	Age*	ABO type	mAb concentration†
Infant 8	1 d	O -	>23.5
Infant 13	2 d	O +	>23.5
Infant 10	2 d	O +	>23.5
Infant 1	4 d	A +	>23.5
Infant 7	4 d	A +	5.9
Infant 5	5 d	O +	>23.5
Infant 12	8 d	A +	>23.5
Infant 4	9 d	O +	>23.5
Infant 9	18 d	A +	2.9
Infant 3	28 d	O -	>23.5
Infant 11	30 d	O +	>23.5
Infant 2	85 d	A +	1.5
Infant 6	200 d	B -	>23.5
Child 1	1 yr	O -	0.4
Child 2	4 yr	AB +	0.4
Adult 2	VA	B +	<0.4
Adult 4	VA	O +	<0.4
Adult 1	17 yr	O +	0.4
Adult 3	VA	A -	0.7
Adult 6	VA	A +	1.5
Adult 10	VA	A +	1.5
Adult 13	VA	O +	1.5
Adult 5	VA	B +	2.9
Adult 7	VA	B +	2.9
Adult 8	VA	B +	2.9
Adult 12	VA	O +	2.9
Adult 16	VA	A +	2.9
Adult 9	VA	B +	5.9
Adult 11	VA	O +	5.9
Adult 14	VA	A +	5.9
Adult 15	VA	O +	5.9

* VA, adult specimens obtained from Veterans Administration Hematology Laboratory.

† Concentration ($\mu\text{g/ml}$) of mAbs at highest dilution producing 1+ agglutination. See Materials and Methods for criteria for scoring agglutination reaction.

immunoblot profile with each of the mAbs. RBC-3F11, 1B2, 3F11, and 06B4 all bound to a component of 220, 6155, and 89I LOS of $\sim 4,800 M_r$ and to a component of 56 LOS of $\sim 5,000 M_r$. None of the mAbs bound to 126E LOS which lacks the $4,800 M_r$ component. In addition, each of the three mAbs, although negative for 89I LOS in the SPRIA binding assay, were positive for the LOS after separation of the components by SDS-PAGE (Fig. 1, lane 4). A "negative-control" paper with electroblotted LOS treated with all reagents except mAb, was completely negative, even after >10 min of color development.

Binding of mAb 3F11 to Infant Human Erythrocytes. Because of the similar specificity of mAbs 3F11 and 1B2 and the reported activity of 1B2 (Table V and Fig. 1) with

TABLE VII
*Effect on Agglutination by mAb 3F11 of Neuraminidase and
 Trypsin Treatment of Erythrocytes*

Source of erythrocytes	ABO type	Concentration of mAb resulting in agglutination of erythrocytes treated with:*		
		Untreated	Neuraminidase	Trypsin
Infant 1	A +	>23.5	<0.2	<0.2
Infant 3	O -	>23.5	<0.2	<0.2
Infant 5	O +	>23.5	<0.2	<0.2
Adult 2	B +	<0.2	<0.2	<0.2
Adult 4	O +	0.4	<0.2	<0.2
Adult 6	A +	1.5	<0.2	<0.2
Adult 13	O +	0.7	<0.2	<0.2

* Concentration ($\mu\text{g/ml}$) of mAb 3F11 at highest dilution producing 1+ agglutination (see Materials and Methods).

human adult and cord erythrocytes, we tested for the ability of mAb 3F11 to agglutinate infant cells. Only 3 of 13 erythrocyte specimens from infants <7 mo old were agglutinated by the mAb, even at concentrations of 23.5 $\mu\text{g/ml}$ (Table VI). This was in contrast to adult cells, all of which were agglutinated by <6 $\mu\text{g/ml}$ of mAb 3F11. Erythrocytes from 1- and 4-yr-old children had a similar reactivity as adult cells in that they were agglutinated by only 0.4 $\mu\text{g/ml}$ of 3F11.

Effect of Enzymatic Treatment on Binding of mAb 3F11 to Human Erythrocytes. The failure of 3F11 to agglutinate most of the infant erythrocyte specimens could reflect blocking of the epitope by other sugars such as sialic acid and/or by large glycoproteins in the erythrocyte membrane. A second possibility is the delayed expression of the appropriate glycosyltransferases involved in the developmental changes in the glycoproteins and glycolipids in the erythrocyte membranes (10). We addressed these two possibilities, by investigating the effect of treatment of erythrocytes with neuraminidase or trypsin on their agglutinability with mAb 3F11 (Table VII). A representative group of three infant and four adult erythrocyte samples were each treated separately with the two enzymes. None of the infants' cells were agglutinated by 23.5 $\mu\text{g/ml}$ of mAb 3F11; the adults' cells were all agglutinated by <2 $\mu\text{g/ml}$. Treatment of both adult and infant cells with either enzyme markedly increased their sensitivity to agglutination. After treatment all seven samples were agglutinated by <0.2 $\mu\text{g/ml}$ of 3F11. Enzymatic treatment of adult cells increased the expression of the 3F11-defined epitope at all assay temperatures; however, epitope expression at room temperature or 37°C remained 1 and 2 logs less, respectively, than that at 4°C. In a control experiment, high concentrations (40–50 $\mu\text{g/ml}$) of a mouse IgM mAb specific for group A polysaccharide, mAb 1F10, did not agglutinate either untreated or treated infant or adult cells. These data suggest that the 3F11-defined epitope is present, but blocked on infant erythrocytes. mAb 06B4 also agglutinated infant erythrocytes treated with neuraminidase, but not untreated erythrocytes. In a preliminary experiment with a small number of specimens of infant erythrocytes, neuraminidase-treated infant erythrocytes were agglutinated better by 06B4 than by 3F11. mAb 06B4 aggluti-

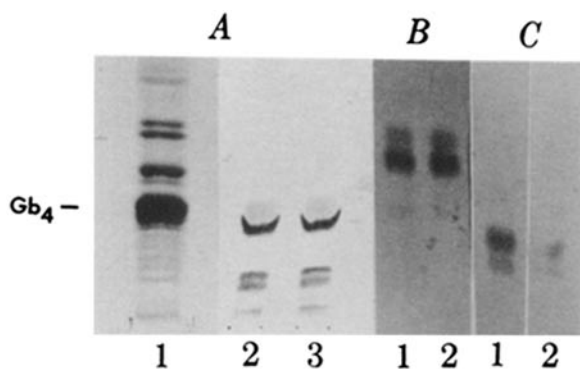


FIGURE 2. Thin-layer chromatogram of glycosphingolipids immunostained with mAbs 3F11 and 06B4. (A) Human erythrocyte neutral glycosphingolipids stained with orcinol reagent (lane 1), and immunostained with 3F11 (lane 2); 06B4 (lane 3). (B) nLc₄Cer purified from human myeloid cells immunostained with 3F11 (lane 1) and 06B4 (lane 2). (C) Rabbit erythrocyte branched-chain nLc₆Cer (see Fig. 3 for structure) immunostained with 3F11 (lane 1); 06B4 (lane 2). TLC plates were developed in a standard solvent tank (A) or in a short bed continuous development tank (B and C) (32) in chloroform/methanol/water, 60/35.8, vol/vol.

nated-treated infant erythrocytes at a concentration of mAb two- to fourfold lower than the concentration of 3F11 necessary for the same activity (data not shown).

Determination of Carbohydrate Sequence Specificity of mAbs. To determine the carbohydrate sequence specificity recognized by the mAbs, the antibodies were tested with purified glycosphingolipids of known carbohydrate structure. Fig. 2 shows a TLC of human erythrocyte neutral glycosphingolipids and other purified glycosphingolipids immunostained with 3F11 and 06B4. Panel A shows that both 3F11 and 06B4 are able to bind a series of neutral glycosphingolipids isolated from human erythrocytes, and give qualitatively similar patterns. Lane 1 of Fig. 2 A shows a chemically stained chromatogram of the erythrocyte neutral glycosphingolipids. As shown by the immunostaining patterns in lanes 2 (3F11) and 3 (06B4), the glycosphingolipids that these antibodies bind are minor components, accounting for only a small percentage of the orcinol-stained compounds. Neither antibody bound to the major red cell glycosphingolipid Gb₄Cer (see Fig. 3 for structure). All of the immunostained glycosphingolipids are more polar than Gb₄Cer, and thus contain four or more saccharides. Panel B demonstrates that both antibodies are able to bind to the purified glycosphingolipid nLc₄Cer (Fig. 3) and appear to have qualitatively similar affinity for this compound. Panel C shows the binding of these two antibodies to a branched biantennary glycosphingolipid (Fig. 3, rabbit erythrocyte branched chain nLc₆Cer), which was prepared by treatment of the rabbit erythrocyte glycosphingolipid C-deca (Fig. 3) with α -galactosidase. Fig. 2 clearly shows that 3F11 (panel C, lane 1) binds more strongly to the biantennary branched-chain glycosphingolipid than does 06B4 (panel C, lane 2).

Comparison of Binding Affinities of 3F11, 06B4 and 1B2. To quantify the relative affinities of 3F11 and 06B4 for linear versus branched-chain lactosamine compounds, a solid-phase binding assay was done using the avidin-biotin-alkaline phosphatase ELISA assay method (33). Fig. 4 A shows a plot of the binding of 3F11 and 06B4 to different quantities of nLc₄Cer. Both antibodies bound with high affinity to this compound, showing significant binding at 250 ng and reaching a plateau at 1,000 ng. The antibody 1B2 showed a similar binding curve to those obtained with 3F11 and 06B4.

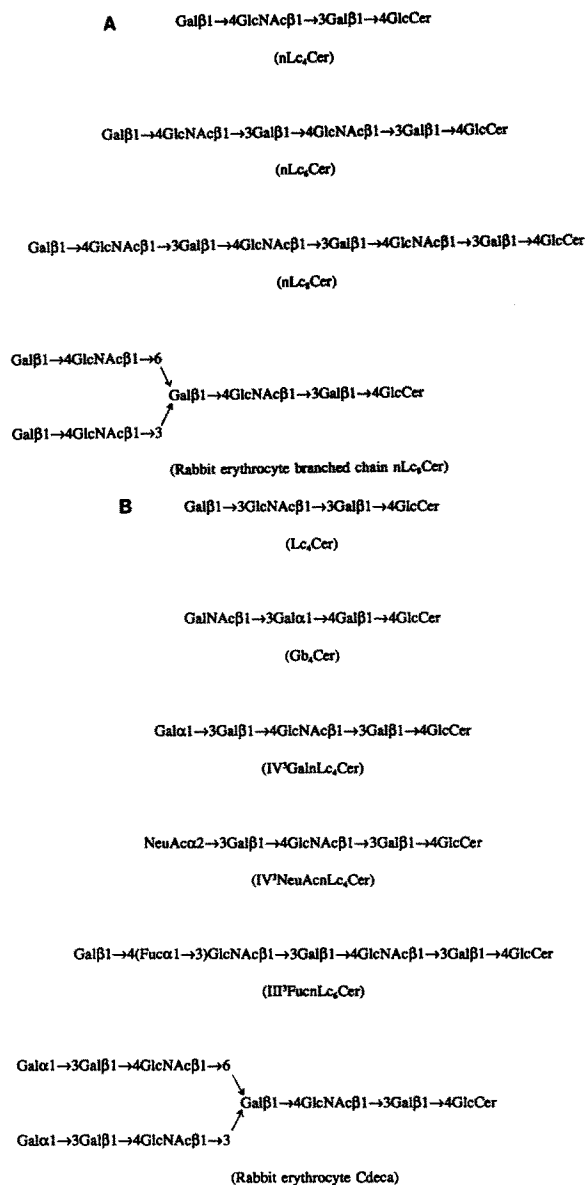


FIGURE 3. Glycosphingolipids reactive (A) and unreactive (B) with mAbs 3F11, 06B4, and 1B2.

A comparison of the binding affinity of the same three antibodies for the biantennary glycosphingolipid, nLc₈Cer, is shown in Fig. 4 B. In contrast to the results obtained with the straight-chain glycosphingolipid, nLc₄Cer, 3F11 and 06B4 gave markedly different binding curves with the branched-chain compound. The affinity of 3F11 for the branched compound was three to five times greater than that of 06B4. Interestingly, 1B2 paralleled the binding of 3F11, showing an equally high affinity for nLc₈Cer.

Binding of mAbs to Other Glycosphingolipids. To further evaluate the binding specificity of 3F11 and 06B4, TLC immunostaining assays were done with a number of purified

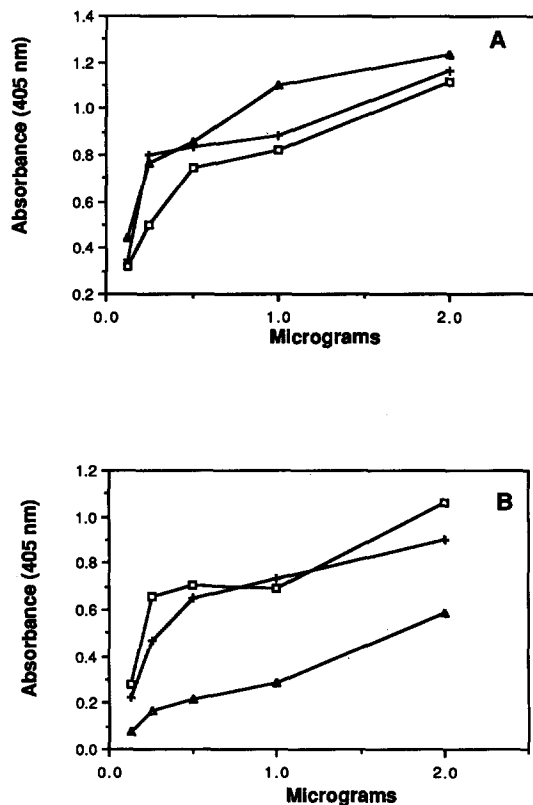


FIGURE 4. Binding of mouse mAbs 3F11 and 06B4 to linear and branched-chain *N*-acetyl-lactosamine glycosphingolipids. The solid-phase binding assay (ELISA) was used to quantify the binding of 3F11, 06B4, and 1B2 to purified glycosphingolipids. The label on the *x*-axis refers to the micrograms of mAb added to microtiter well. (A) Antibody binding to nL₄Cer. (B) Antibody binding to rabbit erythrocyte branched-chain nL₈Cer. 3F11 (□); 06B4 (Δ) 1B2 (+).

glycosphingolipids. Fig. 3 summarizes these results, and shows that both antibodies bind to glycosphingolipids of the neolacto family (nL₄Cer, nL₆Cer, linear and branched nL₈Cer), but only if the nonreducing terminal disaccharide *N*-acetyl lactosamine is unsubstituted. Addition of a terminal sialic acid (IV³NeuAcnL₄Cer) or galactose residue (IV³GalnL₄Cer), or a branching fucose at the penultimate glucosamine (III³FucnL₄Cer) blocked binding. Glycosphingolipids of other families globo (Gb₄Cer) and lacto (L₄Cer) were not recognized. Compounds with less than four sugar residues including GlcNAcβ1→3Galβ1→4GlcCer, were not recognized by either antibody (data not shown).

Discussion

These data show that the glycolipids in the membranes of gonococci and human red blood cells share a highly conserved and exposed epitope. In addition, the epitope defined by mAb 3F11 is also expressed on some meningococcal LOS (Table II) (34). The epitope is expressed more effectively at 4°C incubation temperatures and on adult erythrocytes better than on infant erythrocytes (Tables III, IV, and VI). A comparison of 3F11 hemagglutinating activity with erythrocytes from human adults and infants showed a stronger expression of the 3F11 antigen on adult cells (Table VI), but increased expression on infant cells after enzymatic treatment of the cells. The increased expression of the 3F11 epitope on infant cells after enzy-

matic treatment with trypsin and neuraminidase (Table VII) is probably due to enzymatic stripping of the glycoprotein molecules blocking epitopes recognized by 3F11 and to hydrolysis of sialic acids capping terminal lactosamine structures, respectively. Agglutination of untreated adult erythrocytes at 4°C likely is a result of a conformation shift at low temperatures of the position of large membrane glycoproteins that are blocking epitopes.

The expression of the 3F11 epitope on LOS was not affected by temperature. Immunoblot assays performed at room temperature and SPRIA performed at either room temperature or 37°C resulted in equivalent or slightly increased expression of the LOS 3F11 epitope (data not shown). These results are consistent with those of human cold agglutinin-binding activity, at 4°C and 37°C, with erythrocytes or antigens removed from erythrocytes (35).

The fact that mAb 3F11: (a) had a differential binding activity with human adult and infant erythrocytes (Table VI), (b) bound to infant erythrocytes after enzymatic treatment of the cells (Table VII) and (c) agglutinated cells better at 4°C compared with higher temperatures (Table IV), suggests that mAb 3F11 may have activity similar to human cold agglutinins (36–38). The existence of human cold agglutinins has been known for many years (39–41). They are paraprotein molecules, usually of the IgM subclass, and usually specific for one or more of the molecules that compose the Ii blood group antigen system (10). The human myeloma paraprotein, Ma, has a human adult/cord cell activity very similar to 3F11 (42), while a second myeloma paraprotein, McC, has an inverse binding activity to Ma and 3F11 in that at 4°C it agglutinates infant cells better than adult cells (38). The McC cold agglutinin was shown to be specific for nLc₄Cer (38). Two other human cold agglutinins, Woj and Sti (43), are similar in specificity to mouse mAb 1B2. These antibodies all are specific for *N*-acetylactosamine carbohydrate structures.

mAb 1B2 was made in a mouse immunized with nLc₆Cer and was shown to be specific for glycosphingolipids with *N*-acetylactosamine structures and to bind to adult human erythrocytes, but not to umbilical cord erythrocytes (25). We have shown in this study that 1B2 and 3F11 also have a similar reactivity pattern with gonococcal and meningococcal LOS, and this pattern differed from that of 06B4 in SPRIA (Table V), but not in SDS-PAGE/immunoblot analysis (Fig. 1).

Although mAbs 3F11 and 06B4 appeared quite similar with a variety of LOS (2) (Table V), other LOS from group B and C meningococcal strains bound 06B4, but not 3F11 (Table II; Dr. Janice J. Kim, personal communication). This may be a reflection of differences in the presence of the LOS components bearing the mAb-recognized epitope. For example, the expression of the 3F11/1B2- and 06B4-defined epitopes on 89I was markedly different. mAbs 3F11 and 1B2 did not bind to 89I in any of several SPRIAs (Tables II and V), but bound weakly to 89I after separation of the LOS by SDS-PAGE (Fig. 1). A difference in epitope expression in these binding assays suggests a conformational difference in the 3F11/1B2 epitope in LOS on a plastic surface in the SPRIA and LOS after separation by SDS-PAGE and transfer to nitrocellulose. Although the basis for the subtle expression differences of the epitopes defined by these antibodies is not yet clear, binding assays using 3F11, 06B4, and 1B2 with purified human erythrocyte and neutrophil glycosphingolipids that have linear and branching *N*-acetylactosamine structures provided information relevant to this expression difference.

Young et al. (25) demonstrated that 1B2 has a higher affinity for nLc₄Cer than

nLc₆Cer in some assays (complement-mediated liposome release assay, solid-phase binding assay), whereas in others (complement fixation and hemagglutination inhibition assays), the affinity was the same for both compounds. Thus, all three antibodies have a similar affinity for nLc₄Cer.

The results with purified glycosphingolipid-binding assays complement and extend the results of the binding assays with purified LOS (Tables II and V) and hemagglutination assay (Table III), which demonstrated that 3F11 and 06B4 had similar, but not identical, binding specificities. The finding that 06B4 preferentially binds to linear type 2 glycosphingolipids is in good agreement with the earlier observation that human fetal erythrocytes largely contain glycosphingolipids and glycoproteins with linear type 2 *N*-acetylactosamine chains (10, 44–46) and with our preliminary results showing that 06B4 agglutinates neuraminidase-treated human infant erythrocytes at least as well as does 3F11. The fact that 3F11 has a high affinity for both linear and branched type 2 glycosphingolipids also agrees well with the fact that adult erythrocytes have both linear and branched chain components (10, 44–46).

The binding properties of 3F11 and 06B4 are similar to those of two other antilactosamine mouse mAbs (M39.6 and M18.3) characterized by Gooi et al. (43). However, there appear to be important subtle differences in the fine specificities of these antibodies. For example, M18.3 does not bind to nLc₄Cer and M39.6 is only reactive with relatively large amounts (1 μg) of this glycosphingolipid (43), whereas both 3F11 and 06B4 bind to much lower amounts of nLc₄Cer. Finally, 3F11 and 06B4 appear to have similar binding properties to those of another mouse mAb, My28 (47). My28 was generated by immunizing mice with human myeloid cells. These cells are known to be rich in linear lactosamine glycosphingolipids. My28 binds to nLc₄cer and longer-chain, linear lactosamine glycosphingolipids. However, its relative affinity for branched-chain structures has not been reported.

It is important to stress that only further LOS structural data will reveal whether analogous linear and branching LOS epitopes exist and whether they are different quantitatively in meningococcal and gonococcal LOS. The antibodies described, however, will be useful tools in further studies on glycoconjugates and neisserial LOS.

Many of the carbohydrate structures recognized by the antibodies described above represent intermediate molecules in the development of blood group antigens. nLc₄Cer is the precursor structure for *i* antigen (46) (Table I). Human fetal erythrocytes predominately express *i* antigen. The structure of *i* on infant erythrocytes is not completely defined, but it is thought to be a form of the linear *N*-acetylactosamine glycosphingolipid, nLc₆Cer (Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc-Cer) (10, 48, 49). By 4–6 mo of age the concurrent expression or derepression of a glycosyltransferase enzyme (branching enzyme) results in the attachment of *N*-acetylactosamine to the subterminal galactose residue of *i* and the synthesis of *I* (Table I) on human erythrocytes (50). The addition of other substituents (fucose, sialic acid, lactosamine) to the two branches of the nascent *I* molecule results in multiple forms of *I* that are recognized by various cold agglutinins (10, 42), including some agglutinins that have been mentioned above. *Ii* are precursors of the ABH blood group antigens (10).

Our studies have demonstrated an immunochemical similarity between neisserial LOS and antigens present on human erythrocytes. The involvement of immunochem-

ically similar structures in gonococcal pathogenicity would be dependent on the presence of the antigens on the relevant host tissues. Although we have only demonstrated similar antigens on red cells, the presence of Ii antigens and/or nLc₄Cer have been detected on human lymphocytes, monocytes, polymorphonuclear leukocytes (51), other granulocytes (31, 47, 52, 53), and in various human fluids, including milk, urine, and saliva (54). Also, in recent studies mAb 1B2 has been used to detect traces of nLc₄Cer- and nLc₆Cer-containing glycolipids in human semen (55).

How could the presence of a blood group antigen precursor structure on the gonococcus potentially provide an advantage to the bacteria in human tissue with similar structures? First, the host could not mount an effective immune response to bacterial antigens similar to self. It has been shown, for instance, that normal human sera do not have functionally efficient antibodies to nLc₄Cer, nor to other glycosphingolipids of similar structure (56). If immunochemically similar antigens were predominate on the gonococcal cell surface, these may block or alter the binding of potentially protective human antibodies and complement.

Second, LOS with a terminal *N*-acetylactosamine structure potentially could act as sites of attachment or adhesion to host cells. Human cells have been shown to possess surface lectins that bind to various polyactosaminylglycans that terminate in Galβ1→4GlcNAc and/or that contain internal Galβ1→4Glc (57, 58). Lectins with such a specificity on human epithelial cells may play a role in attachment of bacteria possessing lactosamine structures.

If similar carbohydrate structures are present on the host and parasite cell surface then these cells may also possess similar glycosyltransferase enzymes. Human host cells having surface glycosyltransferases that play a role in the completion of blood group antigens on lactosamine (Galβ1→4GlcNAc) precursors might bind to an immunochemically similar substrate on the gonococcal cell surface (LOS oligosaccharide) and remain bound in the absence of the optimal conditions required for enzymatic reactivity (optimal pH, sugar-nucleotide concentrations, metals, or other cofactors). This concept has been proposed by Roseman (59) as a potential mechanism of the involvement of multiglycosyltransferase systems in intercellular adhesion. Although most glycosyltransferases are found exclusively inside the cell in the Golgi apparatus, some evidence of cell surface galactosyltransferase on ciliated cells of human fallopian tube epithelium (60) and on the mouse sperm surface (61) has been reported.

Third, when optimal conditions do exist for enzymatic activity then host cell glycosyltransferases released from damaged cells (sperm, menstrual debris) may alter the bacterial cell surface, resulting in neoantigens to which the host is tolerant. Conversely, the release of bacterial glycosyltransferases on host tissue substrates could result in new tissue antigens to which the host is intolerant, thus leading to host cell damage.

The presence on the gonococcus of a structure immunochemically similar to the precursor of human blood group antigens (ABH) is intriguing. Further studies of the human immune response to LOS, LOS/lectin binding, LOS/glycosyltransferase binding, mAb inhibition of attachment, and production of neoantigens on LOS will be required to determine what role they play in the pathogenic mechanisms of *Neisseria gonorrhoeae* and *Neisseria meningitidis*.

Summary

We have used mouse mAbs, 3F11 and 06B4, that are specific for highly conserved epitopes of *Neisseria gonorrhoeae* lipooligosaccharides (LOS) to identify immunochemically similar structures on human erythrocytes. mAb 3F11 agglutinated erythrocytes from all randomly selected adult humans, while mAb 06B4 agglutinated only 80% of the same specimens. The antibodies had an activity with erythrocytes similar to human cold agglutinins in that the agglutination occurred at 4°C and decreased with increasing incubation temperature. Human infant erythrocytes were agglutinated less well, but enzymatic treatment of either infant or adult cells resulted in an increase in expression of the 3F11- and 06B4-defined epitopes. Both antibodies bound to a series of neutral glycosphingolipids from human erythrocytes and neutrophils that have a type 2 (Galβ1→4GlcNAc) or *N*-acetylactosamine structure. Neither antibody bound to glycosphingolipids from human meconium, which have a type 1 (Galβ1→3GlcNAc) structure. The antibodies were unable to bind to *N*-acetylactosamine glycosphingolipids with a nonreducing terminal sialic acid or a Galα1→3Gal disaccharide. Antibody binding also was blocked by the presence of fucose linked to the penultimate glucosamine residue of *N*-acetylactosamine glycosphingolipids. Although both antibodies bound to linear and branched-chain *N*-acetylactosamine glycosphingolipids, 3F11 had a higher affinity for branched structures than did 06B4. The activity of 3F11 with human adult and infant treated and untreated erythrocytes with *N*-acetylactosamine glycosphingolipids, and with LOS was very similar, if not identical, in specificity to 1B2, an mAb prepared from mice inoculated with a linear *N*-acetylactosamine glycosphingolipid.

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