HUMAN SPERM CARBOHYDRATE ANTIGENS DEFINED BY AN ANTISPERM HUMAN MONOCLONAL ANTIBODY DERIVED FROM AN INFERTILE WOMAN BEARING ANTISPERM ANTIBODIES IN HER SERUM

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Although female sterility was induced in experimental animals by active immunization with sperm (1, 2), and antibodies that react with human sperm were found to be present in sera of infertile women (3), no strong correlation has been found between the presence of such antibodies and naturally occurring human female infertility (4, 5). However, Isojima et al. (6) reported the novel finding that sera of infertile women inactivate human sperm motility in the presence of guinea pig complement. Approximately 17% of infertile women with unknown cause had antibodies in their sera that inactivated human sperm motility in the presence of complement. No such antibody was detectable in sera of parous women. These findings led to a generally accepted hypothesis that the presence of such antibodies in sera of infertile women can be regarded as the cause of femal infertility (7). Thus, the specificities and immunobiological properties of antibodies present in sera of infertile women showing complement-dependent inactivation of sperm motility are of crucial importance for understanding the biological mechanism of female infertility. However, the activity of such antibodies in sera of infertile women is generally lower than a sperm immobilization titer of 1:200 (200 SI_{50}) (8), and certain serum components of normal subjects showed nonspecific binding with human sperm. Therefore, immunobiological studies on infertility have been difficult to perform in further detail. To overcome this difficulty, a human mAb H6–3C4, inducing complement-dependent sperm immotility, was established after fusion of lymphocytes from an infertile patient (patient MI), showing high-titer antisperm antibodies, with mouse myeloma NS1 (9). The antibody is an IgM with λ light chain, and reacts with ejaculated sperm, but not with testicular sperm. The antibody showed a strong inactivation of human sperm motility in the presence of complement. The binding of H6–3C4 antibody to sperm was inhibited by the serum antibodies of patient MI; thus the serum antibodies of MI and antibody H6–3C4 must be directed to the same epitope structure present at the surface of ejaculated human sperm (9). It is essential, therefore, to elucidate the chemical properties of antigen defined by mAb H6–3C4. This paper

describes the carbohydrate epitope structure defined by this mAb, as well as the chemical properties of carbohydrate antigens expressed at the surface of human sperm.

Materials and Methods

Monoclonal Antibodies. The mAb H6-3C4 (9) was prepared from hybridoma supernatant after precipitation with 50% saturated ammonium sulfate, followed by gel filtration through Sephacryl S300. Other mAbs used in this study were: (*a*) IgM antibody 1B9 defining NeuAcα2→6Galβ1→4GlcNAc (10); (*b*) IgM antibody M2590 defining NeuAcα2→3-Galβ1→4Glc (or -GlcNAc) (11); (*c*) IgM antibody 1B2 defining Galβ1→4GlcNAc (12); (*d*) IgM antibody NUH2 defining binary NeuAcα2→3Galβ1→4GlcNAc (IV^{6}NeuAcα2→3Galβ1→4 GlcNAcV^{13}NeuAcnLc$_6$; sialyl-I) (Nudelman, E., U. Mandel, H. Clausen, and S. Hakomori, unpublished data); (*e*) IgM antibody C6 defining binary Galβ1→4GlcNAc (IV^{6}Galβ1→4GlcNAcnLc$_6$; I) (13); (*f*) anti-SSEA-3 defining extended globo structure Galβ1→3GalNAcβ1→3Galα1→4-Galβ1→4Glc (14); and (*g*) anti-SSEA-4 defining sialyl galactosylgloboside (NeuAcα2→3Galβ1→3GalNAcβ1→3Galα1→4Galβ1→4Glc) (15). These antibodies were prepared, or their epitope structures were established, in this laboratory (The Biomembrane Institute). Two anti-i antisera, Bostrum and Dench, were provided by Mrs. Osaki of Puget Sound Blood Bank, Seattle, WA.

Sperm Preparation. For determination of antibody binding or mobility of sperm, a sufficient quantity of freshly ejaculated semen was diluted with PBS, and sperm cells were collected on centrifugation (3,000 rpm), washed with PBS containing 1% FCS, and suspended (10^6/ml) for the assays. For extraction of glycolipid antigens, pooled ejaculated semen was collected and frozen until a sufficiently large quantity was accumulated. ∼700 ml of semen was centrifuged at 3,000 rpm at room temperature for 20 min, and the sperm pellet was suspended in the same volume of PBS and centrifuged again. The sperm pellet, weighing 6.2 g, was extracted with 150 ml of isopropanol/hexane/water (55:20:25 vol/vol/vol) (see below).

Glycolipid Preparation. The following glycolipids were prepared from the upper neutral or ganglioside fraction of human blood group O erythrocytes (16), human placenta (17), or human sperm (this paper): 2→3 sialyl paragloboside (IV^{3}NeuAcnLc$_4$) (18); 2→6 sialyl paragloboside (IV^{6}NeuAcnLc$_4$); 2→3 sialyl lacto*nor*hexaosylceramide (VI^{3}NeuAcnLc$_6$) (16); 2→6 sialyl lacto*nor*hexaosylceramide (VI^{6}NeuAcnLc$_6$) (16); lacto*nor*octaosylceramide and sialyllacto*nor*octaosylceramide (VIII^{3}NeuAcnLc$_8$) (19); Le^x nonasaccharide (VII^{3}FucnLc$_8$; Z$_1$ glycolipid) (20); lactoisooctaosylceramide (IV^{6}Galβ1→4-GlcNAcnLc$_6$; C6 antigen or I-antigen) (21, 22); 2→3 disialyl lactoisooctaosylceramide (VI^{3}NeuAcIV^{6}NeuAc2→3Galβ1→4Glc-NAcnLc$_6$; disialyl I-antigen) (22, 23); and GM$_3$ ganglioside. Structures of these glycolipids are shown in Table I. The method of preparation was described previously (24). Briefly, tissues were extracted by isopropanol/hexane/water 55:30:25, followed by Folch's partition repeated three times, DEAE cellulose chromatography, HPLC on porous silica gel column (Iatrobeads 6RS8010), and finally, purification on high-performance thin-layer chromatography (HPTLC)[1] as free state or as acetate. Each glycolipid preparation was homogeneous on HPTLC, and some of their structures were identified by nuclear magnetic resonance (NMR) and fast-atom bombardment mass spectrometry (FAB-MS).

Immunostaining and Solid-Phase RIA for Glycolipids. TLC immunostaining of glycolipids was performed on HPTLC (Baker Chemical Co., Phillipsburg, NJ; or Whatman Co., Kent, England), according to a modified method (14, 20) of a procedure originally described by Magnani et al. (25). Briefly, the HPTLC plate, after development, was treated with 3% BSA in PBS, followed by reaction with the primary antibody at 4°C overnight. Subsequently, the plate was treated with the secondary antibody at room temperature for 1 h, and ^{125}I-protein A for 0.5 h, then washed three times with PBS, dried, and subjected to autoradiography. Solid-phase RIA of various glycolipids was performed on 96-well flexible vinyl plastic plate (Falcon 3000, Becton Dickinson & Co., Oxnard, CA) as previously described (26).

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[1] *Abbreviations used in this paper:* FAB-MS, fast-atom bombardment mass spectrometry; HPTLC, high-performance thin-layer chromatography; NMR, nuclear magnetic resonance.

TABLE I

Trivial Names and Structures of Glycolipids Used in this Study

Name	Structure
GM₃	NeuAc2→3Galβ1→4Glc→Cer
Paragloboside	Galβ1→4GlcNAcβ1→3Galβ1→4Glc→Cer
2→3 Sialylparagloboside	NeuAc2→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc→Cer
2→6 Sialylparagloboside	NeuAc2→6Galβ1→4GlcNAcβ1→3Galβ1→4Glc→Cer
Lactonorhexaosylceramide	Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc→Cer
2→3 Sialyllactonorhexaosylceramide	NeuAc2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc→Cer
2→6 Sialyllactonorhexaosylceramide	NeuAc2→6Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc→Cer
Lactonoroctaosylceramide	Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc→Cer
2→3 Sialyllactonoroctaosylceramide	NeuAc2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc→Cer
Le^x Nonasaccharide (Z₁; VII³FucnLc₈)	Galβ1→4[Fucα1→3]GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc→Cer
Lactoisooctaosylceramide; I antigen	Galβ1→4GlcNAcβ1→6 　　　　　　　　　＼Galβ1→4GlcNAcβ1→3Galβ1→4Glc→Cer Galβ1→4GlcNAcβ1→3／
Disialyllactoisooctaosylceramide; G10 ganglioside	NeuAc2→3Galβ1→4GlcNAcβ1→6 　　　　　　　　　　　　　＼Galβ1→4GlcNAcβ1→3Galβ1→4Glc→Cer NeuAc2→3Galβ1→4GlcNAcβ1→3／

Determination of Antibody Binding on Sperm Cells by ELISA Assay or RIA. A suspension of sperm cells in PBS containing 2×10^6/ml was prepared. 50-μl aliquots of sperm cell suspension were placed in each well of a flexible microplate (Falcon 3004, Becton Dickinson & Co.) and the plate was placed on a heating stage and dried at 45°C for 30 min. Each well of the microplate then had 3% BSA in PBS added and was incubated for 1 h, followed by incubation with undiluted supernatant of hybridoma or 300 times diluted anti-i serum (Dench or Bostrum) with PBS containing 0.1% BSA as the primary antibody for 1 h. For ELISA, each well was treated with 1,000 times diluted peroxidase-conjugated anti–mouse or anti–human gamma globulin, followed by reaction with O-phenylenediamine–hydrogen peroxide to develop color, and if necessary, the intensity of the color was measured by ELISA colorimeter fitted for 96-well plate. For RIA, the secondary antibody was added instead of peroxidase-conjugated antibody, washed, subsequently combined with ^{125}I-labeled protein A, and counted by gamma counter after excision of each well.

Complement-dependent Immobilization Test. A complement-dependent sperm immobilization test was performed as originally described by Isojima et al. (7) with minimal modification. Briefly, 50 μl of hybridoma supernatant or anti-i serum adequately diluted with PBS containing 1% heat-inactivated FCS was placed in each well of a 96-well Falcon 3040 microtissue culture plate, to which 50 μl of 10^6/ml live sperm suspended in PBS containing 1% FCS was added, and, subsequently, 10 μl of guinea pig serum was added. After 1 h of incubation at room temperature, live sperm showing active motility were counted. To evaluate sperm immobilization value of the antibody, motility rates of the sperm in the experimental wells were compared with those of sperm in control wells to which 10 μl of heat-inactivated guinea pig serum was added. The sperm immobilization value of the antibody was calculated as C/T, where C and T were the motility rates of sperm in the control and experimental wells, respectively. A C/T value >50 was considered positive.

The complement used was from guinea pig serum purchased from ICN Pharmaceuticals Inc., Irvine, CA (vendor code no. 642831). This complement did not show a cytotoxic effect on fresh human sperm; however, it did show a cytotoxic effect on sialidase-treated sperm. Therefore, the test was performed with complement absorbed on sialidase-treated sperm. The pooled sperm were mixed with 0.5 U/ml sialidase (*Clostridium perfringens* sialidase, type X; Sigma Chemical Co., St. Louis, MO; see below), centrifuged, and washed three times with PBS. One part of sialidase-treated sperm was mixed with one part of complement, followed by centrifugation. The supernatant was treated twice with one part sperm. Such complement showed full activity, but had no cytotoxic effect on sialidase-treated sperm.

Hemagglutination Assay. Blood group O erythrocytes from umbilical cord and adult peripheral blood were used for study of hemagglutination by various antibodies. A suspension of erythrocytes with an approximate concentration of 0.2% in PBS containing 0.1% BSA was prepared; 25-μl aliquots were mixed well with 25 μl of serially diluted antibody in each well of a 96-well U-bottomed plate, and allowed to stand for 2 h at room temperature and at 4°C.

Glycosidase Treatment of Sperm Cells. Sperm cells fixed on plastic plates, as previously described, were treated with *Clostridium perfringens* sialidase, jackbean β-galactosidase (27) (Sigma type X, Sigma Chemical Co.), or by both enzymes. Aliquots of 25 μl of sialidase (0.1 U/ml) in 20 mM acetate buffer, pH 5.5, were added to each well and incubated for 16 h at 37°C. To observe the effect of sialidase on the sperm immobilization test, a suspension of 4×10^7 sperm in 0.5 ml of PBS containing 1% heat-inactivated FCS was mixed with 1 U/ml of *C. perfringens* sialidase and incubated for 1 h at 37°C. Sperm motility was unchanged after this treatment, compared with the same treatment without sialidase. Endo-β-galactosidase of *Escherichia freundii*, which preferentially cleaves unbranched type 2 chain or its sialylated derivatives (28), was donated by Dr. Minoru Fukuda (La Jolla Cancer Research Foundation, La Jolla, CA). The enzyme solution containing 0.1 U/ml in 100 mM acetate buffer, pH 5.0, was prepared and 50 μl was added to fixed sperm on plastic wells after sialidase treatment as described above.

Immunofluorescence Assay of Fresh Sperm Cells. Fresh sperm were mixed with culture supernatant of hybridoma, or with PBS containing 100 times diluted anti-i antiserum (either Dench or Bostrum). These sperm suspensions, mixed with antibody, were allowed to stand for 30 min at 4°C, centrifuged, and washed once with PBS. The sperm were then treated with FITC-

labeled anti-mouse or anti-human Ig, incubated for 30 min at 4°C, washed with PBS, centrifuged, suspended in PBS, and observed by immunofluorescence microscopy.

Results

Reactivity and Specificity of mAb H6-3C4 with Glycolipid Antigens by TLC Immunostaining and by Solid-Phase RIA. Several glycolipids characterized as lacto-series type 2 chain, separated on HPTLC from erythrocytes and placenta, were immunostained with H6-3C4 antibody (Fig. 1, *A* and *B*). Of the major positive bands identified, lacto-*nor*hexaosylceramide (nLc6) and lacto*nor*octaosylceramide (nLc8) were characterized as neutral glycolipids, and 2→3sialyl *nor*hexaosylceramide (VI³NeuAcnLc6), 2→6-sialyl *nor*hexaosylceramide (VI⁶NeuAcnLc6), 2→3sialyl *nor*octaosylceramide (VIII³NeuAcnLc8), and 2→6sialyl *nor*octaosylceramide (VIII⁶NeuAcnLc8) as gangliosides. In addition, Le^x nonaosylceramide (Z₁ glycolipid; VII³FucnLc8) was found to be reactive. The antibody did not react with lactoisooctaosylceramide (IV⁶Galβ1→4GlcNAcnLc6; I antigen) defined by C6 antibody (13), its disialyl derivative (VI³NeuAcIV⁶NeuAc2→3Galβ1→4GlcNAcnLc6), 2→3sialyl paragloboside (IV³NeuAcnLc4), 2→6sialyl paragloboside (IV⁶NeuAcnLc4), or GM3 ganglioside. The reactivity demonstrated on TLC immunostaining was reproduced on solid-phase RIA, as shown in Fig. 2. Thus, H6-3C4 reacted with glycolipids having a long unbranched type 2 chain, regardless of substitution at the terminal region, but did not react with those having a branched or shorter type 2 chain. This specificity is similar to that of anti-i antibodies. However, anti-i antibody Bostrum showed a much more restricted reactivity with sialylated or fucosylated i-structures (Fig. 1 *C*, lanes *5-11* and *14*). Symbolized structures of these antigens, and their reactivities with H6-3C4 in comparison with other antibodies, are summarized in Table II.

The Presence of Lactosaminolipids (Lacto-Series Type 2 Chain Glycolipids) in Human Sperm Cells. In both Folch's lower and upper phase of sperm cell extracts, we found a series of glycolipids reacting with 1B2 antibody, which defines Galβ1→4GlcNAc structures, i.e., lactosaminolipids (Fig. 3 *B*). These glycolipids were identified as nLc4, nLc6, nLc8, iso-Lnc8 (IV⁶Galβ1→4GlcNAcnLc8), and other higher analogues of unbranched and branched unsubstituted lactosaminolipids (Fig. 3 *B*, lanes *6* and *7*). The Folch's upper phase of sperm also contained 2→3sialyllacto*nor*hexaosylceramide (VI³NeuAcnLc6), defined by H6-3C4 antibody as a major compound (Fig. 3 *C*, lane *10*), and sialyl-I, defined by antibody NUH2 (Fig. 3 *D*, lane *13*).

Comparative Hemagglutination with H6-3C4 and Anti-i Antibodies. Since the reactivity pattern of various glycolipids with H6-3C4 resembled that seen with anti-i antibody, which was previously established as an autohemolytic antibody associated with some hemolytic diseases and myeloma (for review see references 29, 30), we compared hemagglutination of these two antibodies with umbilical cord blood and adult erythrocytes. We used a typical anti-i antibody, Dench, which is characterized by its strong reactivity with neonatal erythrocytes at low temperature (4°C). Antibody H6-3C4 also showed stronger hemagglutination with neonatal erythrocytes at 4°C, as compared with adult erythrocytes or higher temperatures. However, H6-3C4 had a much lower hemagglutinating power than Dench antibody, as shown in Table III.

Comparative Inhibition of Complement-dependent Sperm Motility by Various mAbs. Besides the antibody H6-3C4, which reacts with both i and sialyl-i antigens, several mAbs defining various related structures are available. Therefore, we compared the
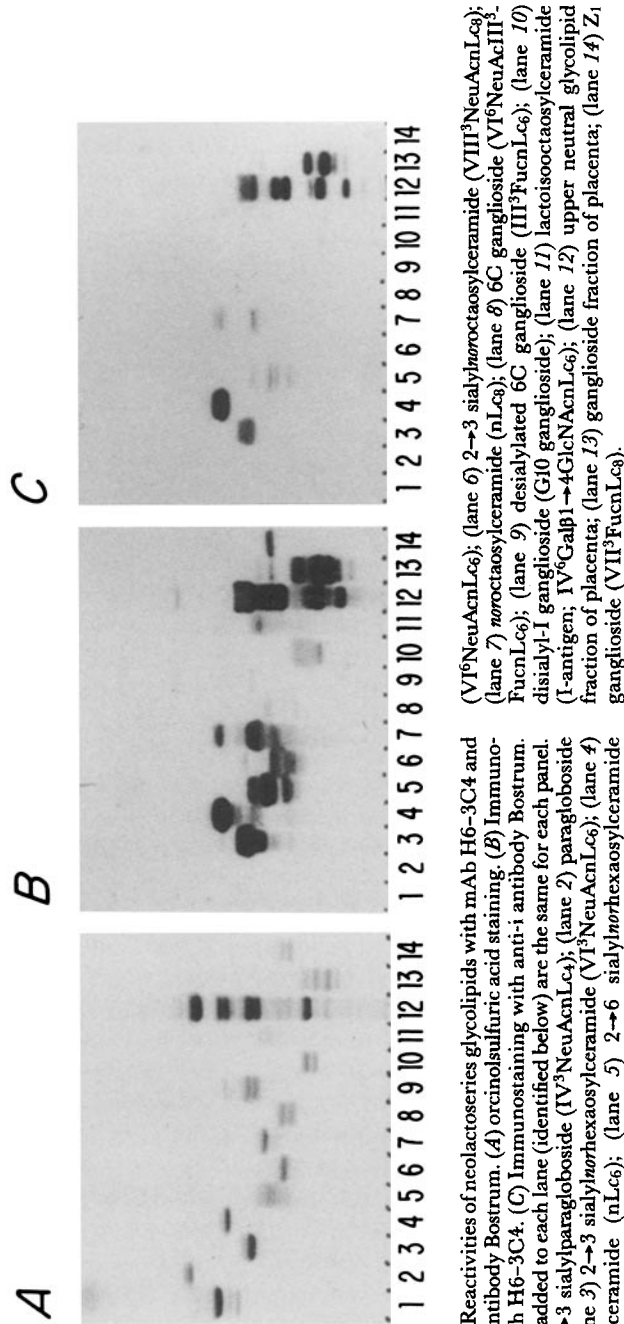
FIGURE 1. Reactivities of neolactoseries glycolipids with mAb H6–3C4 and with anti-i antibody Bostrum. (A) orcinolsulfuric acid staining. (B) Immunostaining with H6–3C4. (C) Immunostaining with anti-i antibody Bostrum. Glycolipids added to each lane (identified below) are the same for each panel. (Lane 1) 2→3 sialylparagloboside (IV^{3}NeuAcnLc$_4$); (lane 2) paragloboside (nLc$_4$). (Lane 3) 2→3 sialylnorhexaosylceramide (VI^{3}NeuAcnLc$_6$); (lane 4) norhexaosylceramide (nLc$_6$); (lane 5) 2→6 sialylnorhexaosylceramide (VI^{6}NeuAcnLc$_6$); (lane 6) 2→3 sialylnoroctaosylceramide (VIII^{3}NeuAcnLc$_8$); (lane 7) noroctaosylceramide (nLc$_8$); (lane 8) 6C ganglioside (VI^{6}NeuAcIII^3-FucnLc$_6$); (lane 9) desialylated 6C ganglioside (III^{3}FucnLc$_6$); (lane 10) disialyl-I ganglioside (Gi10 ganglioside); (lane 11) lactoisooctaosylceramide (I-antigen; IV^{6}Galβ1→4GlcNAcnLc$_6$); (lane 12) upper neutral glycolipid fraction of placenta; (lane 13) ganglioside fraction of placenta; (lane 14) Z$_1$ ganglioside (VII^{3}FucnLc$_8$).
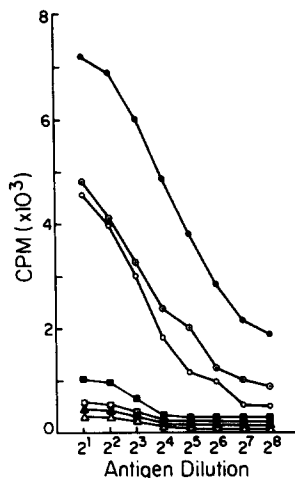
FIGURE 2. Antibody binding activity of various neolactoseries glycolipids with H6-3C4 antibody by solid-phase RIA. (●) 2→6 sialyl-*nor*hexaosylceramide; (⊙) 2→3 sialyl*nor*hexaosylceramide; (○) *nor*hexaosylceramide; (■) 2→6 sialylparagloboside; (□) paragloboside; (▲) 6B ganglioside (VI³NeuAcV³FucIII³FucnLc₆); (△) 2→3 sialyl-paragloboside.

reactivity of these antibodies on complement-dependent sperm immotility, using intact and sialidase-treated sperm. Besides H6-3C4, only the antibody NUH2, which defines disialyl I structure, inhibited the motility of intact sperm in the presence of complement. However, when sialidase-treated sperm (which still showed active motility; see Materials and Methods) were used, antibodies Dench, 1B2, and C6 also showed inhibition of the motility (Table IV). It is clear that the receptors in intact sperm that are susceptible to complement-dependent immobilization consist of sialyl-i (as defined by H6-3C4) and sialyl-I (as defined by NUH2), whereas those in sialidase-treated sperm may consist of any type 2 chain terminal structure, as defined by H6-3C4, Dench, 1B2, or C6 (see Discussion).

Reactivity of Sperm Cells with Various mAbs. The fixed sperm showed a clear reactivity with H6-3C4, anti-i (Dench, anti-N-acetyllactosamine (1B2), and anti-I (C6), as shown in Fig. 4. No reactivity was detected with 1B9, which defines 2→6sialyl paragloboside (IV⁶NeuAcnLc₄). Neither anti-SSEA-3 nor anti-SSEA-4 antibodies reacted with sperm (data not shown). The sperm cell reactivity with H6-3C4 was unchanged, whereas that with NUH2 was abolished, after sialidase treatment. In contrast, the reactivity with anti-i (Dench), anti-N-acetyllactosamine (1B2), and anti-I (C6) was enhanced after sialidase treatment, as shown in Fig. 4.

A weak reactivity, detected by either immunofluorescence or antibody binding with Dench, C6, and 1B2 as applied on sialidase untreated cells fixed on plastic surface, was observed. Such a weak reactivity should be ascribed to spontaneous hydrolysis of sialic acid during fixation of the cells. When fresh intact sperm cell suspension was used, only H6-3C4 and NUH2 showed a strong reactivity, in accordance with the complement-dependent sperm immobilization in intact sperm cells shown by these two antibodies. Only a very weak reactivity was found with antibodies 1B2, Dench, or C6 with fresh untreated sperm cells on immunofluorescence, and these reactivities were enhanced greatly by sialidase treatment (data not shown).

Discussion

The specificity of mAb H6-3C4, which was established after fusion of lymphocytes of an infertile woman with mouse myeloma NS-1, may represent a part of the

TABLE II

mAbs Defining Various Neolacto Series Structures and their Antisperm Motility Activity

Anti-sperm motility activity		1B2	1B9	Dench	H6-3C4	C6	NUH2	M2590	Bostrum
▽-O-●	GM₃	–	–	–	+	–	+	–	–
O-●-●	PG	+	–	–	–	–	–	+	–
O-●-●-●	2,3SPG	–	–	–	–	–	–	–	–
▽-O-●-●	2,6SPG	–	–	–	–	–	–	–	–
△-O-●-●	i (nHex)	+	–	+	+	–	–	–	+
O-●-O-●	2,3S-i (nHex)	–	–	–	+	–	–	–	–
▽-O-●-O-●	2,6S-i (nHex)	–	+	–	+	–	–	–	–
△-O-●-O-●	i (nOct)	+	+	+	+	–	–	–	+
O-●-O-●-●	2,3S-i (nOct)	–	–	–	+	–	–	–	+
▽-O-●-O-●-●	Leˣ (Z₁)	–	–	–	+	–	–	–	–
O-●-O-●—▲									
O-●-O-●	I	+	–	–	–	+	–	–	–
▽-O-●									
▽-O-●-O-●	2,3S-I	–	–	–	–	–	+	–	–

▽, 2,3-linked sialic acid; △, 2,6-linked sialic acid; O, galactose; ●, glucose or N-acetylglucosamine; ▲, fucose.
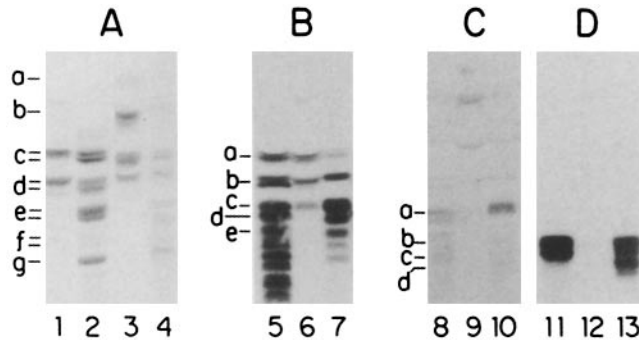
A B C D

Figure 3. Glycolipids of human sperm cells and their immunostaining patterns with various antibodies. (Lane *1*) Monosialogangliosides from human O blood cells; (lane *2*) monosialogangliosides from human placenta; (lanes *3, 6, 9, 12*) total glycolipids from Folch's lower-phase (nonpolar) fraction of human sperm cells purified by C18-silica gel column; (lanes *4, 7, 10, 13*) total glycolipids from Folch's upper-phase (polar) fraction of human sperm cells purified by C18-silica gel column. (lane *5*) upper neutral glycolipids from blood group O erythrocytes; (lane *11*) standard sialyl-I glycolipid (G10 ganglioside). (*A*, lanes *1-4*) Stained by 2% orcinol in 2 M sulfuric acid; (*B*, lanes *5-7*) stained by mAb 1B2, which defines terminal Galβ1→4GlcNAc; (*C*, lanes *8-10*) stained by mAb H6-3C4; (*D*, lanes *11-13*) stained by mAb NUH2, which defines sialyl-I. Bands in *A* identified in the left margin as *a, b, c, d, e, f,* and *g* were identified as ceramide monohexoside (CMH), ceramide dihexoside (CDH), GM$_3$, IV^{3}NeuAcnLc$_4$, IV^{3}NeuAcn-Lc$_6$, IV^{6}NeuAcnLc$_6$, and disialyl-I (IV^{6}NeuAc2→3Galβ1→ 4GlcNAcVI^{3}NeuAcnLc$_6$), respectively. Bands immunostained by 1B2 in *B*, marked in the left margin as *a, b, c, d,* and *e*, were identified as nLc$_4$, nLc$_6$, nLc$_8$, iso-nLc$_8$ (I antigen), and nLc$_{10}$, respectively. Slower migrating bands were not identified. Bands immunostained by H6-3C4 in *C*, marked in the left margin as *a, b, c,* and *d*, were identified as IV^{3}NeuAcnLc$_6$, IV^{6}NeuAcn-Lc$_6$, VIII^{3}NeuAcnLc$_8$, and VIII^{6}NeuAcnLc$_8$, respectively. (*D*) Only total upper-phase glycolipids from sperm cells were immunostained, corresponding to sialyl-I. Slower migrating bands were not identified.

specificity detectable in antibodies present in infertile women, which causes complement-dependent sperm immobilization. The specificity is now identified as being directed to internally located, repetitive, unbranched *N*-acetyllactosamine structure, regardless of terminal substitutions at Gal, i.e., sialyl-i, i, as well as fucosyl-i structure. Anti-i antibodies, showing a preferential cold agglutination of fetal erythrocytes over adult erythrocytes, are of low affinity and were found originally in sera of patients with reticulosis, hemolytic anemia, or various hematopoietic malignancies (30–32). The antibody H6-3C4 also showed a weak but clear preferential hemagglutination of neonatal erythrocytes at low temperature; this property again resembles that of anti-i antibodies. However, i-antigen expressed on fetal erythrocytes and on a few normal adult tissues is qualitatively different in its fine specificity and binding affinity from the i-like antigenicity highly expressed at the sperm cell surface, defined by H6-3C4 antibody (see below).

Table III

Hemagglutination of Human Adult and Neonatal Erythrocytes by H6-3C4 and Anti-i (Dench)

Antibody	Temperature	Hemagglutination Assay	
		Adult erythrocytes	Neonatal erythrocytes
H6-3C4 (1 mg/ml)	25°C	—	×16
	4°C	×1	×128
Dench (serum)	25°C	×10,000	×40,000
	4°C	×40,000	×80,000

TABLE IV

Complement-dependent Sperm Immobilizing Test of Various mAbs

Antibody	Nontreated sperm	Sialidase-treated sperm
H6-3C4 (sup)*	×2,048	×4,056
NUH2 (sup)	×1,024	—
C6 (sup)	—§	×512
1B2 (sup)	—	×2,048
1B9 (sup)	—	—
Dench (serum)‡	—	×4,056
Bostrum (serum)‡	—	×4,056

* sup, culture supernatant of hybridoma containing 12 μg IgM/ml for H6-3C4; 18 μg IgM/ml for NUH2; 25 μg IgM/ml for C6; 8 μg IgM/ml for 1B2; and 1.9 μg IgM/ml for 1B9.
‡ Serum of myeloma patients, Dench and Bostrum, containing anti-i myeloma protein.
§ No activity even at 100 times dilution.

The antigen i structure has been characterized as an unsubstituted, unbranched N-acetyllactosamine, i.e., the structure included in nLc6, nLc8, but not terminally sialylated derivatives (21, 22, 33; see reference 29 for review). In the longer type 2 chains, such as nLc8 and nLc10, substitution at the terminal Gal may not strongly affect the i reactivity (22), but substitution at the penultimate GlcNAc abolishes i activity. In striking contrast, H6-3C4 reacts equally well with 2→3 or 2→6 sialylated or nonsialylated i having two or three repeating N-acetyllactosaminyl structures. Furthermore, it reacts with nLc8 with fucosyl substitution at VII GlcNAc, which does not react with anti-i. It is clear, therefore, that the reactivity of H6-3C4 is based entirely on internally located, repetitive N-acetyllactosamine, while the majority of anti-i specificity depends on an unsubstituted terminal structure. Another important distinction between H6-3C4 and anti-i antibodies, and between the i-like antigen in sperm and the i-antigen in various normal cells, is that H6-3C4 may be capable of recognizing polylactosamine chain organized in high density at the sperm cell surface, which may form a high-affinity binding site whose reactivity is indepen-
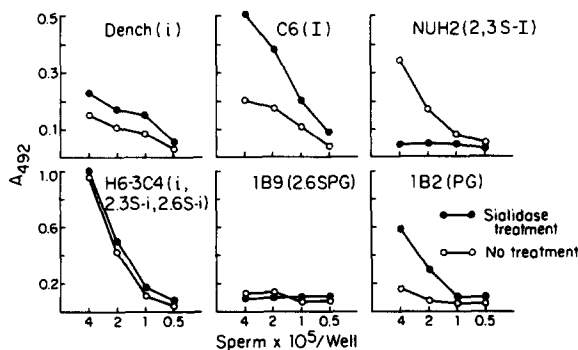

FIGURE 4. Reactivities of human sperm cells with various mAbs antibodies directed to neolactoseries structures, before and after sialidase treatment. Sperm cells were treated with sialidase and fixed on plastic surface in each well. Reactivities with each antibody were tested before and after treatment with Clostridium perfringens sialidase as described in the text. Reactivities are identified in each panel. Dench defines i (polylactosamine); 6C defines I-antigen, i.e., lactoisooctaosylceramide; NUH2 reacts with 2→3 sialyl-I; 1B9 defines 2→6 sialylparagloboside; 1B2 specifies terminal N-acetyllactosamine as in nLc4, nLc6, and nLc8.

dent of temperature (at either 4°C or at 37°C). Indeed, the reactivity of H6–3C4 with sperm cell antigen occurred at 37°C as well as 4°C (6–8). In contrast, the majority of the anti-i antibodies may not show a clear density-dependent recognition, and the low density i antigen may form a low-affinity binding site whose reactivity is restricted at 4°C. Since i-antigen at the fetal erythrocyte surface may be present in low density, and its fine specificity is different from that present at the sperm cell surface, the antibody H6–3C4 agglutinates fetal erythrocytes only weakly at 4°C. Density-dependent recognition of GM_3 or Le^x with a clear antigen threshold value has been observed with some mAbs defining these structures (34, 35). The antibody M2590, showing a specific reaction with melanoma (10), is capable of recognizing high-density but not low-density GM_3 (34). Cell type–specific antigenicity defined by mAb is closely dependent on the unique organization of the antigen, rather than the presence of a chemically unique antigen.

Expression of the i antigen, regardless of its density or organization, is relatively limited in adult human tissue. The i antigen defined by mAb NCC1004 was found to be associated with lung cancer and to be absent in the majority of normal adult tissues, except in the mantle zone of lymph nodes, endothelium, and a few other loci (36). It is suspected that distribution of i or $\alpha 2{\rightarrow}3$ sialyl-i organized in high density, which might be defined by the H6–3C4 antibody, must be highly limited in normal tissue. Similarly, distribution of $\alpha 2{\rightarrow}3$ sialyl-I must also be limited, even though I antigen is commonly expressed in a variety of tissues, particularly those of mesodermal origin. Native sperm cells may be unique in having a high density of $\alpha 2{\rightarrow}3$ sialyl-i as well as $\alpha 2{\rightarrow}3$ sialyl-I structure. Such a structural profile constitutes a susceptible receptor to complement-dependent inactivation of sperm motility. The molecule defined by H6–3C4 and other antibodies may be found in both glycosphingolipids and glycoproteins.

The specificity of H6–3C4 may not represent the entire class of antibodies present in sera of infertile women that are capable of inhibiting sperm motility. The surface receptor of native sperm cells susceptible to complement-dependent immobilization may consist of a series of sialylated or unsialylated type 2 chains, which are defined by other antibodies with different specificities, such as NUH2. Immobilization of desialylated sperm can be caused by antibodies recognizing the $Gal\beta 1{\rightarrow}4GlcNAc$ terminus, such as C6, 1B2, and Dench. Motility of native sperm cells was inhibited by antibodies that recognize the internal repetitive N-acetyllactosamine, including sialyl i and i (as typically shown by H6–3C4); and that recognize binary $2{\rightarrow}3$ sialyl type 2 chain (sialyl I) (as demonstrated by NUH2). In fact, examination of human sperm glycolipids revealed the presence of a series of lactosaminolipids, including those representing i, sialyl i, and sialyl I structures. Nevertheless, the quantity of glycolipids representing these structures in human sperm cells seems to be relatively low, and the isolated glycolipids may not form high-affinity binding sites. The major high-affinity receptor causing inactivation of sperm motility must be a glycoprotein characterized as having lactosaminoglycan, as was previously found in fetal erythrocyte band III glycoprotein (37), placental fibronectin (38), and "embryoglycan" of teratocarcinoma cells (39). In fact, a high-affinity glycoprotein bearing lactosaminoglycan was demonstrated in sperm cells (Kaizu, T., Y. Tsuji, S. Hakomori, S. Isojima, unpublished data). There must be other unidentified structures containing

polylactosamine that constitute a spectrum of antibody specificities essentially responsible for female infertility.

Hamilton et al. (40) established a hybridoma antibody EC1 after fusion of lymphocytes from multiparous mouse and mouse myeloma. Antibody EC1 reacts with epididymal sperm but not testicular sperm. This property is identical to that of human antibody H6-3C4. Interestingly, Hamilton et al. reported that EC1 antigen was expressed not only at the sperm cell surface, but also at two-cell stage embryo. It disappeared at four-cell stage embryo and reappeared at the morula and blastocyst stage. Since stage-specific expression of I and i antigens was previously found in preimplantation mouse embryo (41), a high-density expression of $\alpha2\rightarrow3$ sialyl-i and $\alpha2\rightarrow3$ sialyl-I in human sperm may also indicate the possibility that these could be stage-specific antigens in early human embryogenesis. The antibody C6, which defines binary N-acetyllactosamine structure, i.e., blood group I structure, was originally obtained after immunization of syngeneic mice with sperm cells (13, 42). The expression of C6-defined antigen was closely associated with mesodermal development. It is of great interest to correlate these findings with $\alpha2\rightarrow3$ sialyl-i and $\alpha2\rightarrow3$ sialyl-I expression in human sperm.

Summary

The epitope structure of the human sperm antigen reacting with antibodies present in sera of infertile women has been studied using mAb H6-3C4, which produces immobilization of human sperm in the presence of complement. Another antibody, NUH2, which also induces human sperm immobilization, was used to substantiate the presence of a receptor on sperm involved in susceptibility to immobilization. Both antibodies defined type 2 chain polylactosamine structure. H6-3C4 is directed to internally located repetitive N-acetyllactosamine, i.e., sialyl-i, i, or fucosyl-i. NUH2 defines binary $\alpha2\rightarrow3$ sialyl type 2 chain, i.e., sialyl-I. Thus, the presence of antibodies in the sera of infertile women directed to sperm lactosaminoglycan or lactosaminolipid could be the basis for infertility in these cases.

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