Expression of Bisecting Type and Lewis\(^x\)/Lewis\(^y\) Terminated N-Glycans on Human Sperm\(^*\)\(^\text{[1]}\)

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Human sperm lack major histocompatibility class I molecules, making them susceptible to lysis by natural killer (NK) cells. Major histocompatibility class I negative tumor cells block NK cell lysis by expressing sufficient amounts of bisecting type N-glycans on their surfaces. Therefore, sperm could employ the same strategy to evade NK cell lysis. The total N-glycans derived from sperm were sequenced using ultrasensitive mass spectrometric and conventional approaches. Three major classes of N-glycans were detected, (i) high mannose, (ii) biantennary bisecting type, and (iii) biantennary and tetraantennary oligosaccharides terminated with Lewis\(^x\) and Lewis\(^y\) sequences. Immunostaining of normal sperm showed that glycoproteins bearing Lewis\(^x\) sequences are localized to the acrosome and not the plasma membrane. In contrast, defective sperm showed distinct surface labeling with anti-Lewis\(^y\) antibody. The substantial expression of high mannose and complex type N-glycans terminated with Lewis\(^x\) and Lewis\(^y\) sequences suggests that sperm glycoproteins are highly decorated with ligands for DC-SIGN. Based on previous studies, the addition of such carbohydrate signals should inhibit antigen-specific responses directed against sperm glycoproteins in both the male and female reproductive systems. Thus, the major N-glycans of human sperm are associated with the inhibition of both innate and adaptive immune responses. These results provide more support for the eutherian fetoembryonic defense system hypothesis that links the expression of carbohydrate functional groups to the protection of gametes and the developing human in utero. This study also highlights the usefulness of glycomic profiling for revealing potential physiological functions of glycans expressed in specific cell types.

Studies performed over the past four decades indicate that conditions are not optimal for human sperm in the female reproductive system (1). Women release neutrophils, monocytes, and lymphocytes from the specialized mucosal surface of their cervix during the leukocyte reaction after coitus (2, 3). This sequestration occurs specifically in response to contact with sperm but not seminal plasma (2, 3). Increased immunological awareness related to histocompatibility and its implications for reproduction in eutherians initially raised concerns about how foreign human sperm are tolerated in the female reproductive system (4). Early studies suggested that human sperm express MHC\(^3\) class I molecules that define self, suggesting the possibility that histocompatibility based responses must be suppressed in the female reproductive tract (5). However, more recent studies confirm that sperm precursors down-modulate their MHC class I molecules, yielding mature sperm that are MHC class I negative cells (5). This absence enables these gametes to evade histocompatibility based responses mediated by MHC class I-restricted cytotoxic T lymphocytes (6).

This lack of MHC class I molecules does not make sperm completely invulnerable to cell mediated responses, however. NK cells survey their potential targets for the expression of MHC class I molecules and lyse those cells that lack such markers (7). Therefore, both human sperm and eggs represent potential targets for NK cells. This issue is very relevant for human sperm because of their potential exposure to NK cells during the leukocyte reaction (2, 3).

Like sperm, human tumor cells also lose their classical MHC class I molecules, especially as they become more metastatic (8). This transformation also makes them highly resistant to MHC class I-restricted cytotoxic T lymphocytes but more sensitive to NK cell-mediated cytotoxicity (7). Unlike sperm, a pathway for escaping natural cytotoxic responses has been established in tumor cells. Previous studies indicate that K562 erythroleukemia cell targets can avoid NK cell-mediated responses by increasing their surface expression of bisecting type N-glycans (9, 10). Glycoprofiling analysis indicates that 35–92% of the

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\# The abbreviations used are: MHC, major histocompatibility; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin; LacNAc, Galβ1-4GlcNAc; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; MS, mass spectrometry; MS/MS, tandem MS; NK, natural killer; TRITC, tetramethylrhodamine isothiocyanate; MOPS, 3-(N-morpholino)propanesulfonic acid.
N-Glycans Expressed by Human Sperm

Oligosaccharides linked to polymorphic MHC class I molecules (human major histocompatibility A, B, and C) at their only glycosylation site (Asn-86) are biantennary bisecting type N-glycans (11). Tri- and tetraantennary bisecting type N-glycans are not present. These observations suggest that NK cells can also survey potential targets for biantennary bisecting type N-glycans in addition to MHC class I molecules. This type of carbohydrate-mediated recognition is not unprecedented. Previous studies suggest that Siglec7 is an NK cell inhibitory receptor that recognizes Neu5Acα2–8Neu5Acε-terminated glycans on target cells (12–14).

In this study the N-glycome of human sperm was characterized using high sensitivity mass spectrometric strategies. These experiments showed that sperm express substantial amounts of biantennary bisecting type N-glycans that are similar to those linked to classical MHC class I molecules (11). Remarkably, sperm also produce major amounts of biantennary, triantennary, and tetraantennary N-glycans terminated with Lewisx and Lewisy sequences. They include a novel tetraantennary N-glycan capped on each antenna with a Lewisy sequence. Lewisx and Lewisy sequences have previously been implicated in the suppression of antigen-driven responses (15, 16). Lectin and antibody binding studies suggest that the highly fucosylated oligosaccharides are primarily localized to the acrosome rather than the plasma membrane in normal sperm. However, defective sperm show high levels of Lewisy expression on their surface.

**EXPERIMENTAL PROCEDURES**

**Materials**—This study was approved by the Health Sciences Institutional Review Board, University of Missouri–Columbia. All research procedures involving materials originating from patients were conducted following the approved study protocol. Sperm was obtained from healthy patients being tested for male-factor infertility of unknown origin. Specimens used for this study had normal semen parameters according to World Health Organization guidelines (17). Anti-Lewisy monoclonal antibody and all glycosidases except bovine testes β-galactosidase were obtained from Calbiochem. Reagents were prepared in Dulbecco’s PBS (Invitrogen) except as noted. All other reagents were obtained from Sigma except as noted.

**Isolation of Human Sperm for Structural Analyses and Lectin/Antibody Binding Studies**—Motile sperm were isolated from semen by swim-up. Sperm were washed in Dulbecco’s phosphate-buffered saline, and the pellet was overlaid with protein-free modified Ham’s F-10 basal medium-HEPES (Irvine Scientific, Santa Ana, CA). Tubes were incubated at a 30° angle for 60 min at 37 °C. After a 1-h migration, the overlying swim-up sperm were collected, washed twice in Dulbecco’s phosphate-buffered saline, and stored at −20 °C. Sperm from a fertile sperm bank donor (Fairfax Cryobank, Fairfax, VA) were washed and used for lectin and antibody binding studies as previously described (18).

**Processing of the Human Sperm N-Glycans**—Fourteen swim-up sperm preparations from individual donors were subjected to sonication, reduction, carboxymethylation, and tryptic digestion as described previously (19). Peptide N-glycosidase F digestion of the tryptic glycopeptides was carried out in 50 mM ammonium bicarbonate, pH 8.5, for 20 h at 37 °C with 3 units of enzyme (Roche Applied Science). The released N-glycans were purified by using a Sep-Pak C18 cartridge (Waters Corp.) as described (20). The purified native N-glycans were either subsequently derivatized or subjected to modifications before derivatization.

To define structures with bisected GlcNAc, galactosylation using β-1,4 galactosyltransferase was carried out. A solution of 50 mM MOPS containing 45 μM UDP-galactose was prepared, and the pH of the solution was adjusted to 7.4 using ammonia before the addition of 20 mM manganese (II) chloride 4-hydrate. Lyophilized purified native N-glycans was dissolved in 150 μl of this solution, and 10 μl of bovine milk β-1,4 galactosyltransferase (Calbiochem) was added. This enzymatic reaction was incubated for 20 h at 37 °C and lyophilized.

Hydrofluoric acid (HF) hydrolysis was performed to remove α1–2- and α1–3-linked fucose as previously described (21). Lyophilized purified native N-glycans were transferred from the glass tube to a microcentrifuge tube by re-dissolving them in 5% acetic acid and re-lyophilizing after transfer. Dryness of the sample was ensured before the careful addition of 50 μl of the 48% HF solution (Sigma-Aldrich). The tube containing the reaction was carefully sealed with Parafilm and then incubated on ice at 4 °C for 20 h. The reaction was terminated by drying the HF under a gentle stream of nitrogen. The sample was then redissolved in 5% acetic acid, transferred to a glass tube, and lyophilized before derivatization.

Before mass spectrometric analyses, the native N-glycans were derivatized using the sodium hydroxide permethylation procedure as described previously (20). Partially methylated alditol acetates were prepared from permethylated samples for gas chromatography-MS linkage analysis also as described (19).

**MS and MS/MS Analyses of Permethylated Human Sperma-tozoa N-Glycans**—MALDI-TOF MS data on permethylated samples were acquired in positive ion mode ([M+Na]+) using a Perseptive Biosystems Voyager DE-STR mass spectrometer in the reflector mode with delayed extraction. Collision-activated decomposition-MS/MS data were acquired using a 4800 MALDI-TOF/TOF (Applied Biosystems) mass spectrometer. The collision energy was set to 1 kV, and argon was used as collision gas. Samples were dissolved in 10 μl of methanol, and 1 μl was mixed at a 1:1 ratio (v/v) with 2,5-dihydroxybenzoic acid (20 mg/ml in 70% methanol in water) as matrix.

**Gas Chromatography-MS Linkage Analysis**—Partially methylated alditol acetates were analyzed using a PerkinElmer Clarus 500 instrument fitted with a RTX-5 column (30 m × 0.25-mm internal diameter, Restek Corp.) The sample was dissolved in hexanes and injected onto the column at 65 °C. The column was maintained at this temperature for 1 min and then heated to 290 °C at a rate of 8 °C per min.

**Lectin and Antibody Binding Studies**—Sperm were settled on the lysine-coated microscopy coverslips, fixed in 2% formaldehyde, and processed with or without permeabilization with 0.1% Triton-X-100 as described (22). Fixed sperm were incubated overnight at 4 °C with anti-Lewisy monoclonal antibody diluted 1:50, washed, and incubated for 40 min at room temperature with a mixture containing the TRITC-conjugated goat anti-mouse IgM (Zymed Laboratories Inc., San Francisco, CA) diluted 1/100. Acrosomal integrity was determined by the
addition of fluorescein isothiocyanate-conjugated Arachis hypogea (peanut) lectin (Sigma) diluted 1/100 into TRITC-conjugated goat anti-mouse IgM/4',6-diamidino-2-phenylindole mixture as described previously (18). Coverslips with the processed sperm were mounted on microscopy slides in VectaShield medium (Vector Laboratories, Burlingame, CA) and examined under a Nikon Eclipse 800 microscope (Nikon Inc., Melville, NY) with epifluorescence and differential interference contrast optics and a CoolSnap HQ CCD camera (Roper Scientific, Tucson, AZ) operated by MetaMorph software (Universal Imaging Corp., Downingtown, PA). The figure plate was assembled in Adobe Photoshop.

RESULTS

Strategy for Characterizing Human Sperm N-Glycans—Scheme 1 summarizes the structural analysis strategy employed. Briefly, sperm samples were homogenized in the presence of detergent, and the resulting extracts were reduced, carboxymethylated, and digested with trypsin. N-Glycans were released by peptide N-glycosidase F and permethylated. After Sep-Pak purification the methylated derivatives were characterized by MALDI-TOF profiling, MALDI-TOF/TOF sequencing, and gas chromatography-MS linkage analyses. These experiments were supplemented by MALDI profiling of the products of enzymatic galactosylation and hydrofluoric acid hydrolysis.

MS Profiling of Human Sperm N-Glycans—MALDI-TOF MS analysis of permethylated human spermatozoa N-glycans was carried out on 14 purified sperm cell preparations from individual donors. A representative MS profile obtained from donor 001 is shown in Fig. 1. The structures shown in the annotations were assigned from compositional information provided by these MALDI data together with the results of the additional experiments described in the following sections. The most notable features of the data include (i) the presence of high mannose type N-glycans, (ii) the presence of biantennary bisected glycans, and (iii) substantial amounts of heavily fucosylated complex type N-glycans terminated with Lewisα and/or Lewisβ sequences, the most abundant of which are biantennary. The bisected N-glycans have both non-fucosylated (m/z 2315) and fucosylated (m/z 2489) chitobiose core sequences but lack fucosylation on their antenna. Although the bisected glycans are only a very minor portion of the total glycan profile, they nevertheless make a significant contribution to the complex glycans that are expected to be on the sperm surface (see “Discussion” for acrosomal location of fucosylated glycans).

All of the glycans carrying Lewisα and/or Lewisβ antennae are core-fucosylated. Thus, biantennary core-fucosylated N-glycans with up to 4 antennal fucose residues are indicated by signals at m/z 2418, 2592, 2766, and 2940. Signals at m/z 3216, 3390, 3564, and 3738 are consistent with triantennary N-glycans with 3–6 fucose residues on their antennae. Remarkably, tetraantennary N-glycans are substituted with up to 8 fucose residues on their antenna (m/z 4013, 4187, 4361, and 4535). The following glycans were also observed: (i) several truncated structures lacking terminal galactose on one or more antenna (m/z 1591, 1836, 1907, 2040, 2081, 2111, and 2285); (ii) a core-fucosylated biantennary N-glycan (m/z 2244); (iii) triantennary N-glycans with or without core fucose (m/z 2519 and 2693); (iv) tetraantennary N-glycans with or without core fucose (m/z 2968 and 3142); (v) high mannose-type glycans from Man9GlcNAc2 to Man9GlcNAc2 (m/z 1580, 1784, 1988, 2192, and 2396).

All 14 sperm preparations expressed Lewis sequences as well as low levels of bisected glycans (data not shown). Sialylated glycans were also present; see for example, m/z 2431 and/or 2605 in Figs. 1, 3, and 4. The full MALDI profiles for all preparations are shown in supplemental Fig. S1 and Table S1. The abundance of sialylated components varied from a few percent in six of the preparations up to about 25% in a few preparations. Further analysis of these sialylated components was not pursued because their m/z signals were consistent with the N-glycans expressed by CD52, a sperm-associated glycosphatidylinositol-linked N-glycopeptide that has already been rigorously characterized (23). Future glycomic analyses of many more samples will be required to assess whether the differences in sialylation or fucosylation correlate with fertility. In addition, some minor glycans carrying poly-Galβ1–4GlcNAc antennae were observed.

MALDI-TOF/TOF MS/MS Defines Antenna Sequences and Core Fucosylation Status—Collision-activated decomposition MALDI-TOF/TOF tandem MS of molecular ions observed in the MALDI experiment yielded fragment ions that defined core fucosylation status as well as the sequences of individual antennae. Representative data from analysis of m/z 2285, 2592, and 4535 are shown in Fig. 2, A, B, and C, respectively. Assignments of key signals are given in the insets.

As shown in Fig. 2A, MS/MS analysis of m/z 2285, which has the composition Fuc2HexHexNAc3, gave a spectrum dominated by a fragment ion at m/z 2026 that corresponds to the loss of non-reducing terminal HexNAc. The signals at m/z 1822 (loss of a LacNAc antenna) and 1835 (loss of reducing-end fucosylated HexNAc) indicate that the glycan is core-fucosylated.


N-Glycans Expressed by Human Sperm

FIGURE 1. MALDI-TOF mass spectrum of permethylated N-glycans derived from human sperm reveals different classes of these oligosaccharides. Cell preparations containing human sperm with normal morphological and motion parameters were prepared using the swim-up technique. The N-glycans from 14 different donors were released by peptide N-glycosidase F, permethylated, and subsequently subjected to Sep-Pak cleanup (“Experimental Procedures”). Data were acquired in positive ion mode (M+Na)+. Peak assignments are based on molecular mass composition of the 12C isotope together with the knowledge of the biosynthetic pathways, and structures were confirmed by various rigorous structural analyses. The sugar symbols are those employed by the Consortium for Functional Glycomics for the representation of glycan structures. Structures that show sugars outside a bracket have not been unequivocally defined. For convenience, specific linkages are not assigned in this mass spectrum, and only one branching pattern for tri-antennary structures is shown. Therefore, the position of an antenna in a schematic does not imply designation of a specific arm. A representative spectrum obtained from donor 001 is shown. Note the high level of fucosylation and the presence of biantennary bisected N-glycans.

rather than capped with a Lewisy/a antenna. Fragment ions derived from multiple cleavages (m/z 1563, 1372, and 1113) and the lack of a fragment ion corresponding to the loss of a Lewisy/a antenna provide additional evidence for core fucosylation. Moreover, the MS/MS data show that there are two non-reducing GlcNAc residues as opposed to a lacdiNAc sequence (GalNAcβ1–4GlcNAc), which would be expected to give a fragment ion at m/z 1781 arising from loss of the two HexNAc residues as a single moiety. The MS/MS data cannot distinguish between the alternative possibilities of a bisected biantennary glycan or a non-bisected triantennary N-glycan (see the inset to Fig. 2A).

MS/MS data for m/z 2592, which has the composition Fuc3Hex3HexNAc5, are consistent with a mixture of core-fucosylated biantennary glycans having either two Lewisy/a antennae or one Lewisy/a and one lacNAc antenna (Fig. 2B). Thus, fragment ions at m/z 660, 1318, 1505, 1751, and 1955 very clearly establish the presence of terminal Lewisy/a sequences, whereas signals at m/z 1781 and 2129 indicate that some glycans carry Lewisy/b together with an undecorated lacNAc antenna (see the inset). The relative abundance of the fragment ions indicates that the glycan with two Lewisy/a antennae is the major constituent. The signal at m/z 2386 corresponds to the loss of fucose from the 3-position of GlcNAc via β-elimination (20), thus confirming the Lewisy, rather than the isomeric Lewisi structure.

The predicted composition of m/z 4535 (Fuc3Hex3HexNAc5) suggests the presence of a core-fucosylated glycan carrying four Lewisy/b antennae. MS/MS analysis confirmed this assignment (Fig. 2C). Thus, a dominant fragment ion is observed at m/z 3724 that would be obtained by loss of any of the Lewisy/b antennae. The signal at m/z 2913 corresponds to separate losses of two Lewisy/b antennae, whereas the m/z 834 signal is the Lewisy/b ion itself. The m/z 4085 fragment ion confirms that the glycan is core-fucosylated. Similar to the m/z 2592 experiment, a significant signal corresponding to the loss of fucose by elimination from the 3-position of GlcNAc is observed (m/z 4329), thus confirming the Lewisy, rather than the isomeric Lewisi structure. The data are, thus, fully consistent with the proposed tetraantennary Lewisy structure. The experiment also clearly shows the power of the MS/MS methodology for defining antennae sequences in low abundance high mass components. Based on molecular ion abundances, the m/z 4535 component is only about 0.2% of the overall N-glycan repertoire.
Confirmation of Bisecting-type N-Glycans—The ions at \( m/z \) 1907, 2081, 2111, 2285, 2315, and 2489 in the MALDI MS profiles have compositions consistent with either biantennary bisected structures or truncated triantennary N-glycans. The data obtained from MALDI MS/MS could not discriminate between these two possibilities. Therefore, to address this issue, an aliquot of the purified native N-glycans was incubated with \( \beta \)-galactosyltransferase and UDP-galactose before permethylation. The \( \beta \)-galactosyltransferase is able to catalyze the attachment of \( \beta \)-linked galactose onto GlcNAc on truncated antenna(e) but not onto bisecting GlcNAc (24). Therefore, components with truncated antenna(e) will be galactosylated resulting in a mass shift, whereas signals for the bisected components should be unchanged. Simple biantennary truncated glycans such as the \( m/z \) 1836, 1866, and 2040 components (see Fig. 1) were used as internal controls for assessing the efficiency of the reaction. Fig. 3 (upper panel) shows part of the spectrum for an aliquot of the N-glycans from donor 007 after direct MALDI MS profiling, and Fig. 3 (lower panel) shows the equivalent spectrum of this sample after treatment with \( \beta \)-galactosyltransferase and UDP-galactose. Note that \( m/z \) 1836 and 2040 have converted fully to \( m/z \) 2244, whereas \( m/z \) 1866 has shifted to \( m/z \) 2070 indicating that the reaction has been successful. In principle, if the signals at \( m/z \) 1907, 2111, and 2315 are non-bisected structures, they should all have shifted to \( m/z \) 2519. However, the signal at the \( m/z \) 2315 has not only remained but has increased slightly in abundance, thus providing evidence for the presence of bisected biantennary structures. The same principle applies to components at \( m/z \) 2081, 2285, and 2489. If non-bisected, they would have been converted to \( m/z \) 2693, but the signal at \( m/z \) 2489 is retained and has increased slightly in abundance.

**Linkage Analysis Confirms Bisected Structures**—Preliminary experiments indicated that the glycan content of individual samples was insufficient for obtaining good quality linkage analysis data. Therefore, we pooled permethylated glycans from six donors for these studies. The linkage data (summarized in Table 1) are in accord with the MALDI data suggesting that high mannose and biantennary complex type structures are the major constituents. Key features of these data are as follows: (i) 3,4,6-linked Man confirms the presence of the minor bisected glycans; (ii) the high abundance of 2-linked mannose compared with 2,4- and 2,6-linked mannose is consistent with biantennary structures being the major complex glycans; (iii) 2,4- and 2,6-linked mannose are indicative of tri- and tetraantennary glycans; (iv) 3,4-GlcNAc is derived from the Lewis sequences, whereas the presence of 2-Gal supports the Lewis\(^\circ\) assignments; (v) 4,6-GlcNAc is derived from the reducing end of the core-fucosylated glycans; (vi) 6-Gal is likely to be derived from sialylated components.

**HF Treatment Defines Core-fucosylated Structures**—Incubation of fucosylated glycans with HF results in the rapid hydrolysis of Fuca1–3GlcNAc linkages and slower release of \( \alpha \)-2-linked fucose, whereas the \( \alpha \)-6-linked fucose residues linked to the N-glycan cores are relatively resistant (21). This differential susceptibility to HF was exploited to define the location of fucose in N-glycans by comparing MALDI profiles before and after HF treatment. Fig. 4A shows part of the MALDI MS profile for the N-glycans from donor 004, and Fig. 4B shows the equivalent spectrum of this sample after HF defucosylation. Fucosylated components in this region are indicated by signals at the \( m/z \) 2081, 2244, 2285, 2418, 2489, 2592, 2766, and 2940. Note in Fig. 4B that after HF treatment, monofucosylated components at \( m/z \) 2081, 2244, 2285, and 2489 show very little alteration in their abundance, whereas signals corresponding to components which have more than one fucose residue (\( m/z \) 2418, 2592, 2766, and 2940) are significantly reduced in abundance. This observation strongly supports the MS/MS data, suggesting that all of the fucosylated N-glycans carry a fucose on the core.

**Major Sperm N-Glycan Sequences**—Taking into consideration the MS, collision-activated decomposition-MS/MS, and linkage data together with biosynthetic knowledge and the results of HF and \( \beta \)-galactosyltransferase treatments, we conclude that three major families of N-glycans are expressed in human sperm, namely high mannose bisected and highly fucosylated glycans (Fig. 5). The latter include a novel tetraantennary structure carrying four Lewis\(^\circ\) antennae.

**Localization of Lewis\(^\circ\) Expression on Human Sperm**—Sperm are unlike other somatic cells in that they are heavily glycosylated on both their plasma membranes and the acrosome, a large, lysosome-like organelle that lies just beneath the plasma membrane on the anterior 60% of the human sperm head (1). Sperm initially bind to the surface of the specialized extracellular matrix, coating the egg known as the zona pellucida. This binding triggers the acrosome reaction encompassing the following sequence of events; (i) fusion of the plasma membrane with the outer acrosomal membrane; (ii) loss of this fused membrane remnant from the head of the sperm; (iii) exposure of the inner acrosomal membrane that enables the sperm to undergo secondary binding to the zona pellucida; (iv) modification of the plasma membrane adjacent to the equatorial fusion point, the equatorial segment, that enables the sperm to fuse with the oolemma after zona penetration (1).

The acrosomal and plasma membranes are differentially glycosylated based on lectin binding studies. The lectin from *Lotus tetragonolobus* displays preferential binding to the acrosomal membrane of human sperm rather than the plasma membranes (25). It is well established that this lectin binds to both Lewis\(^x\) and Lewis\(^y\) sequences (26, 27). This specificity combined with the current results implies that glycoproteins terminated with Lewis\(^x\) and Lewis\(^y\) sequences are localized to the acrosome and not the plasma membrane in normal human sperm. Consistent with this result, antibodies directed against Lewis\(^x\) sequences also bind to the acrosomal membranes rather than plasma membranes (28).

Studies were undertaken to establish whether there is colocalization of *Lotus* lectin binding sites and Lewis\(^y\) expression on human sperm. The latter was explored using a monoclonal antibody directed against the Lewis\(^y\) sequence. This antibody displayed only weak binding to intact human sperm (Fig. 6A). However, the acrosomal contents displayed substantial reaction with this anti-Lewis\(^y\) antibody in direct relationship to the progression of acrosomal loss or exocytosis (Fig. 6B and C, respectively). Like the interaction with *Lotus* lectin, the anti-Lewis\(^y\) antibody binding first appeared on the equatorial seg-
ment during acrosomal exocytosis (Fig. 6C, arrow). Intriguingly, malformed spermatozoa were labeled with anti-Lewis\(^y\) antibody all over their surface (Fig. 6D and E). Detergent permeabilization of intact normal sperm also resulted in the acrosomal staining of the head region with anti-Lewis\(^y\) antibody (Fig. 6F). However, acrosome-reacted normal human sperm incubated with non-immune mouse serum followed by a fluorescent anti-mouse IgM showed no binding (Fig. 6G). In summary, the anti-Lewis\(^y\) antibody reacted primarily with the acrosomal contents of normal human sperm but not the plasma membranes (25). By contrast, defective sperm showed distinct surface labeling with anti-Lewis\(^y\) antibody, indicating differential expression of these fucosylated sequences on their surfaces.

**DISCUSSION**

In this report the major N-glycans associated with human sperm were analyzed using glycomic methodologies (20) involving detergent solubilization and enzymatic digestions to obtain the total N-glycan fraction. Biophysical analyses were carried out using ultrasensitive MALDI-TOF/TOF instrumentation that enabled data to be collected from the N-glycans from swim-up sperm isolated from only a single donor (12–157 million sperm).

The results of our detailed analyses have confirmed the following structural characteristics of the major sperm N-glycans: (i) high mannose type oligosaccharides range in size from Man\(_3\)GlcNAc\(_2\) to Man\(_9\)GlcNAc\(_2\); (ii) biantennary bisecting type N-glycans are expressed at substantial levels on this cell type, consistent with previous lectin binding studies (29–31); (iii) unusual biantennary, triantennary, and tetraantennary N-glycans terminated with Lewis\(^x\) and Lewis\(^y\) sequences are also present. The tetraantennary N-glycan terminated on each antenna with the Lewis\(^y\) sequence is a novel structure.

The current findings confirm that human sperm present biantennary
bisecting type N-glycans that are very similar to those associated with the majority of classical MHC class I molecules (11). Erythroagglutinating phytohemagglutinin profusely binds to intact sperm, indicating that these biantennary bisecting type glycans are expressed on the plasma membrane (29–31). Trio- or tetraantennary bisecting type N-glycans were not detected on this cell type. The expression of bisecting type N-glycans has previously been associated with the suppression of NK cell-mediated responses glycans (9, 10). Thus, it is possible that human tumor cells engage the same system employed to protect normal MHC class I negative cells including sperm from NK cell-mediated responses.

Previous studies in our laboratory indicate that CA125, a mucin that expresses substantial amounts of biantennary bisecting type N-glycans (32), also inhibits NK cell cytotoxicity and induces specific phenotypic shifts in this cell population that are associated with the induction of tolerance (33). Thus, tumor cells may routinely employ biantennary bisecting type N-glycan to suppress NK cell-mediated responses. However, this system likely exists in the physiological context to protect normal MHC class I negative cells like human gametes from NK cell-mediated responses.

There is also another potential immunological paradox associated with human sperm. These gametes first arise from germ cell precursors in the testis after the initiation of puberty well after the period of thymic education (34–37). Thus, many sperm proteins represent “neoantigens” that could trigger autoimmune responses. Proof that neoantigens exist is the observation in many mammalian species that the injection of testicular homogenates into homologous species induces autoimmune orchitis directed against sperm antigens (38). On the other hand, allografts and even xenografts can be transplanted into the testis without rejection (39). This dichotomy was initially explained by the presence of a blood-testis barrier that protects germ cells from immune effector cells and antibodies (40, 41). However, germ cell autoantigens are expressed in the basal compartment in spermatogonia and early spermatocytes that are not protected by a blood-testis barrier (42, 43). In addition, this barrier is incomplete in the rete testis, where many sperm traverse toward the epididymis and where autoimmune orchitis reactions are initiated in mammals immunized with syngeneic testicular homogenates (44–46). Therefore, the blood-testis barrier alone does not explain the immune privilege observed in this organ.

The potential for immune activation is even more problematic in human females. Women do not secrete substantial amounts of androgens and, thus, may not express many of the proteins produced by the male reproductive organs. Therefore, they could be sensitized by repeated exposure to foreign male proteins in their reproductive tract. However, only about 2–3% of all women will ever develop antisperm antibodies in their reproductive organs (47, 48). Such women are always subfertile and usually infertile (49). These observations suggest that a highly localized system for the induction of tolerance to androgen-dependent proteins exists in the testis and the male reproductive tract.

Exactly how immune cells avoid sensitization to sperm proteins in these scenarios is currently unknown. However, studies with Helicobacter pylori have provided valuable insights about how such a system could function based on the results of this study. Variants of H. pylori that express Lewisx and Lewisy sequences on their lipopolysaccharides modulate antigen-driven responses and promote their accommodation without the development of pathology (16). Specifically, the interaction of these lipopolysaccharides with DC-SIGN on dendritic cells induces a balancing of the Th1–Th2 response toward tolerance.
By contrast, isogenic variants of *H. pylori* lacking only Lewis sequences evoke substantial immune responses, leading to the development of gastric distress, stomach ulcers, and gastric carcinomas (15). These results suggest that the presence of Lewis type lipopolysaccharides promotes the tolerance of immune stimulatory *H. pylori* proteins in the human gastric epithelium. Moreover, this effect apparently does not require that all *H. pylori* glycoconjugates express Lewis sequences but, rather, that lipopolysaccharides terminated with these epitopes create a microenvironment that promotes tolerance. This system of immune subterfuge may not be limited to *H. pylori*. Schistosomes are helminthic parasites that express both terminal Lewis and a close structural analogue of the Lewis sequence (pseudolewis) that can also interact with DC-SIGN (50, 51). Lewis sequences are also up-regulated on the surface of human immunodeficiency virus-1-infected CD4+ T lymphocytes (52, 53) and human end stage tumor cells (54–56). Thus, other pathogens and aggressive tumor cells could employ the same pathway that *H. pylori* uses to block adaptive antigen-driven immune responses, thus insuring their survival. However, it is highly unlikely that this potential system for the induction of tolerance mediated by the expression of Lewis and Lewisy sequences exists for the benefit of pathogens and aggressive tumor cells.

Human sperm apparently express substantial amounts of high mannose type *N*-glycans on their glycoproteins. Such oligosaccharides are ligands for DC-SIGN (57, 58). The major *N*-glycans terminated with Lewis type sequences in human sperm are biantennary glycans, which are core-fucosylated and carry Lewis and/or Lewisy antennae.

### TABLE 1

| Elution time | Characteristic fragment ions | Assignment       | Relative abundance |
|--------------|-----------------------------|------------------|--------------------|
| 16.96        | 102, 115, 118, 131, 175     | Terminal fucose  | 0.33               |
| 18.45        | 102, 118, 129, 145, 161, 162, 205 | Terminal mannose | 1.00               |
| 18.72        | 102, 118, 129, 145, 161, 162, 205 | Terminal galactose | 0.17               |
| 19.62        | 129, 130, 161, 190          | 2-Linked mannose | 0.73               |
| 19.78        | 129, 130, 161, 190          | 2-Linked galactose | 0.05               |
| 20.42        | 99, 102, 118, 129, 162, 189, 233 | 6-Linked galactose | 0.06               |
| 20.78        | 130, 190, 233               | 2,4-Linked mannose | 0.05               |
| 21.22        | 129, 130, 189, 190          | 2,6-Linked mannose | 0.03               |
| 21.36        | 118, 129, 189, 234          | 3,6-Linked mannose | 0.41               |
| 21.84        | 118, 333                    | 3,4,6-Linked mannose | 0.07               |
| 22.30        | 117, 129, 143, 145, 159, 203, 205 | Terminal GlcNAc | 0.03               |
| 23.17        | 117, 159, 143, 233          | 4-Linked GlcNAc | 0.25               |
| 24.00        | 117, 159, 301, 346          | 3,4-Linked GlcNAc | 0.01               |
| 24.50        | 117, 159, 261               | 4,6-Linked GlcNAc | —                  |

*Quantitation was not possible because of a co-eluting contaminant.*

### FIGURE 4

Treatment of *N*-glycans with HF selectively removes terminal fucose from *N*-glycans associated with human sperm. MALDI MS profiles of the *N*-glycans obtained from donor 004 are shown before (A) and after (B) chemical defucosylation as described under “Experimental Procedures.”

### FIGURE 5

Major *N*-glycans associated with human sperm. Three families were characterized. A, high mannose structures of compositions Man₅₋₉GlcNAc₂-B, bisected biantennary structures with and without core fucose. B, bi-, tri- and tetraantennary glycans, which are core-fucosylated and carry Lewis and/or Lewisy antennae.
glycan terminated with two Lewisx sequences binds to DC-SIGN with an affinity that is 4-fold higher than the monovalent Lewisx oligosaccharide (58). In addition, these investigators reported that the monovalent Lewisx sequence binds with considerably higher affinity to DC-SIGN than the monovalent Lewisx sequence. Therefore, it is extremely likely that the biantennary N-glycans terminated with one or two Lewisx sequences will also bind with high affinity to DC-SIGN. Further testing will be required to determine whether the triantennary and tetraantennary N-glycans terminated with Lewisx and Lewisx sequences mediate high affinity binding to DC-SIGN.

Based on the evidence presented in this report and other aspects of human reproductive immunology, it is now our current operating hypothesis that Lewis-type carbohydrate sequences act as functional groups to block adaptive antigen-driven responses directed against human sperm in both the male and female reproductive systems. The observation that mucins, glycoproteins, and free oligosaccharides terminated with Lewisx and Lewisx sequences are also abundantly expressed in human seminal plasma (59 - 63) indicates that this protective system may also extend to other organs and tissues in the male reproductive tract. Persistent human pathogens and tumor cells can integrate themselves into this system by expressing the same carbohydrate sequences or close structural analogues.

Human seminal plasma also contains a fucosyltransferase activity that greatly exceeds the level of this enzyme in other male secretions (64). Based on recent proteomic analyses, this activity is likely fucosyltransferase III (65). The activity of this fucosyltransferase declines significantly after anti-androgen therapy (66), suggesting that its expression is regulated by sex-linked hormones. The coordinated expression of Lewis type fucosyltransferases and androgen-regulated proteins could provide a very useful system for tolerizing proteins that are first produced in the male reproductive organs after the onset of puberty.

In summary, the structural data obtained in this glycoprofiling study suggest that both innate and adaptive antigen-driven responses directed against human sperm could be abrogated by the major types of N-glycans expressed in this cell type. This system could play a key role in the development of immune privilege in the male urogenital tract. These observations are completely consistent with the eutherian fetoembryonic defense system hypothesis, suggesting that carbohydrate sequences act as functional groups to suppress immune responses directed against the gametes and the developing human in utero (67).

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FIGURE 6. The acrosomal contents of normal human sperm bind to anti-Lewisx antibodies. Binding of anti-Lewisx antibodies (Lex, red) to intact human sperm (A), acrosome reacted sperm (B and C), malformed sperm (D and E), and detergent permeabilized sperm (F). The lectin from peanut agglutinin (PNA; green) was also included to detect acrosome intact sperm (66). Sperm DNA was counterstained with 4’,6-diamidino-2-phenylindole (blue). In the absence of permeabilization, Lewisx is detectable in the acrosome-reacted spermatozoa (A, left, and B, top) but not in the acrosome-intact ones (A, right). At the onset of acrosomal exocytosis, Lewisx labeling first appears on the equatorial segment (C, arrow). Sperm with large amount of superfluous cytoplasm (B, bottom), twin heads (D), and malformed heads (E, note an acrosome-intact, normal spermatozoon on top) show increased Lewisx labeling. Permeabilization exposes Lewisx sequences in the acrosome of the acrosome-intact spermatozoa (F). Binding to sperm was also analyzed in the presence of non-immune mouse serum followed by a fluorescent anti-mouse IgM as a negative control (G).
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