Glycodelin is a human glycoprotein with suppressed IL-2 secretion of Jurkat cells and peripheral blood mononuclear cells. In contrast, no immunosuppressive effect was observed for GdS and GdC.

Glycodelin is a member of the lipocalin family. It consists of 180 amino acid residues (1) with two sites of N-linked glycosylation. There are four reported glycodelin isoforms, namely glycodelin-A (amniotic fluid isoform, GdA), glycodelin-F (folicular fluid, GdF), glycodelin-C (cumulus matrix, GdC) and glycodelin-S (semenal plasma, GdS) (2-5). Among the four glycodelin isoforms, only the N-glycan structures of GdA and GdS have been previously determined. This was achieved using fast atom bombardment mass spectrometry (6,7). The glycan structures of GdA and GdS are completely different. In GdA, the Asn-28 site carries high mannose, hybrid and complex-type structures, whereas the second Asn-63 site is exclusively occupied by complex-type glycans (6). The major non-reducing epitopes characterised in the complex-type glycans are Galβ1-4GlcNAc (lacdiNAc), GalNAcβ1-4GlcNAc (lactdiNAc), Galβ1-4(Fucα1-3)GalNAc (sialylated lacdiNAc), and Galβ1-4(Fucα1-3)GlcNAc (Lewis-x) and GalNAcβ1-4(Fucα1-3)GalNAc (lactdiNAc) analog of the blood group substance Lewis-x (6). Many of these oligosaccharides are rare in other human glycoproteins. GdS glycans are unusually fucose rich, and the major complex type glycan structures are bi-antennary glycans with Lewis-x and Lewis-y antennae. Glycosylation of GdS is highly site-specific. Asn-28 contains only high mannose antennae. Glycodelin is a member of the lipocalin family.
structures, while Asn-63 contains only complex type glycans. More than 80% of the complex glycans have 3-5 fucose residues/glycan, and none of the glycans is sialylated, which is unusual for a secreted human glycoprotein. The glycan structures of GdA and GdC are not known, though they differ in lectin binding properties and isoelectric point from GdF. The other two glycodelin isoforms exhibit different glycosylation patterns.

Glycans are involved in various intracellular and intercellular cell-matrix recognition events. Glycosylation determines the biological activities of GdA and GdC. For example, both GdA and GdF inhibit the spermatid acrosome reaction, thus preventing a premature acrosome reaction of the spermatozoon. There is evidence that cumulus cells can convert exogenous glycans. GdA and -F to GdC, the physicochemical properties of which suggest that it is differently glycosylated compared with GdA/F. Moreover, GdC stimulated spermatid acrosome reaction binding in a dose-dependent manner and it effectively displaced sperm-bound GdA and -F (4,5). GdS suppresses cholesterol efflux from spermatozoa (13). Except for the effects on fertilization, GdA inhibits apoptosis of T-cells (2) and also inhibits natural killer cell and B-cell activities (15). Glycosylation is involved in the binding of GdA to receptors on T-cells (16). The sialic acid of GdA contributes to the apoptotic activity in T-cells (17,18) and binding to CD45, a potential GdA receptor (16,92). The importance of glycosylation in glycodelin is further shown by the absence of immunosuppressive activities in GdS with different glycosylation patterns. The immuno-modulating activities of GdF and GdGdC are unknown.

Our previous work showed that glycans are indispensable for the different glycodelins to exhibit their bioactivities in GdS with different glycosylation patterns. The immuno-modulating activities of GdF and GdGdC are unknown.

**Experimental Procedures**

**Preparation of peripheral blood mononuclear cells (PBMCs)-** Human peripheral blood from healthy female donors was obtained from the Hong Kong West Cluster. Red blood cell transfusion service. PBMCs were isolated from the buffy coat by Ficoll-Paque density gradient centrifugation (GE Healthcare, Uppsala, Sweden). In brief, diluted buffy coat (1:1 with phosphate buffer saline, PBS, pH 7.2) was layered on the Ficoll and centrifuged at 400 g for 40 minutes at room temperature. The remaining red blood cells were removed using red blood cell lysing buffer (150 mM NH4Cl, 10 mM NaHCO3, 0.1 mM EDTA). The PBMCs were then washed twice with PBS and resuspended in RPMI medium containing 10% fetal bovine serum (FBS), 50 units/ml of penicillin and 50 μg/ml of streptomycin. Monocytes were removed by cell adhesion in plastic culture flask for 2 hours.

**Cell cultures-** PBMCs and human cell lines including oviductal cells (OE E6/E7), natural killer cells (NK92mi), cells from a chronic myelogenous leukaemia line (K562), T-lymphoma cells (Jurkat), hepatocarcinoma cells (Hela), trophoblast choriocarcinoma cells (BeWo) and endometrial cells (RL95) were cultured in RPMI 1640 (K562, Jurkat, PBMCs), DMEM (Hela, OE E6/E7, BeWo and RL95) or MEM (NK92mi) supplemented with 10% fetal bovine serum, 50 units/ml of penicillin and 50 μg/ml of streptomycin at 37°C in an atmosphere of 5% CO2 in air.

**Purification of glycodelins-** The study protocol was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster. GdA, -S, -F and -C were purified as described from first trimester amniotic fluid, seminal plasma, follicular fluid, and cumulus matrix of human, respectively (5,12,19) at Queen Mary Hospital, Hong Kong. The collected samples were diluted with tris-buffered saline (TBS, pH 7.4) and 0.1% Triton X-100 in a ratio of 1:3-1:5 was added. GdA, -S and -C were purified by monoclonal anti-glycodelin (clone F43-7F9) chromatography. In brief, the dilute amniotic fluid, seminal plasma or cumulus matrix were loaded onto anti-glycodelin column, which was then washed successively by TBS, 1M NaCl with 1% isopropanol, 10 mM ammonium acetate with 0.1% isopropanol, pH 5 and TBS. Glycodelin was eluted by 20 mM CaCl2 with 0.1% trifluoroacetic acid. The eluted GdS and -C were further purified with anion-exchange Mono-Q (GE Healthcare) column by AKTA purifier 10 (GE Healthcare). The purification of GdF involved the successively use of several chromatographic columns including Hi-trap blue, protein-G, lectin affinity, Mono-Q and gel filtration as described (21).
Tris-HCl, pH 7.5. Desialylation of GdA and GdF was performed using sialidase coated agarose beads in 1M Tris-HCl (pH 7) at 37°C for 18 hours. The free sialic acid was removed by dialysis with 2 mM Tris-HCl, pH 7.5, at 4°C overnight. The success of desialylation was verified by the decreased binding of the treated glycodelin to the lectin, wheat germ agglutinin which binds strongly to sialylated glycans and weakly to other glycoconjugates (22). The concentrations of the purified glycodelins were determined by absorption of glycodelin for cell death analysis. PI (1 μl) in 0.5 ml of PBS was added into the cell suspension. After 15 minutes of incubation, the cells were washed twice with PBS. YoStain was added and the cells were incubated with 100 μl of culture medium overnight at 4°C. Then 100 μl of biotinylated detection antibody and avidin horseradish peroxidase conjugate mixture was added and incubated for 1 hour. Color development was achieved by enzymatic reaction using 3,3′,5,5′-tetramethylbenzidine as substrate. The reaction was terminated by the addition of 50 μl of 2M H2SO4. The absorbance was measured at 450 nm with λ correction of 595 nm. The wells were washed five times with 0.05% Tween-20 in PBS between each step. Cell death analysis by flow cytometry was done at room temperature. The control wells contained BSA and glycodelin instead of glycodelin.

Processing of the glycodelin N-glycans- Preparations of GdA, GdF and GdC (25-50 μg each) were used for all MALDI experiments and GC-MS analyses. Each of the 50 μg of GdA preparations was subjected to reduction, carboxymethylation, and PNGase F treatment the cells were labeled with YoPro®-1 and YoPro®-1 dye (1 μl) and washed twice with PBS. Yo-Pro®-1 dye (1 μl) and 0.5 ml of PBS was added into the cell suspension. After 15 minutes of incubation, the cells were washed twice with 0.05% Tween 20 and incubated overnight at 4°C. After washing the wells twice with 0.05% Tween-20, monoclonal anti-glycodelin antibody (clone F43-7F9, 2.5 μg in 200 μl) was added and the mixture was incubated for 2 hours. The wells were washed 4 times and 100 μl of HRP conjugated anti-mouse IgG at a dilution of 1:5000 (Sigma, St. Louis, MO, USA) was added. After 2 hours of incubation, the wells were washed and incubated with 100 μl of HRP conjugated anti-mouse IgG at a dilution of 1:5000 and 100 μl per well of o-phenylenediamine (Sigma) was added. The OD490 was determined with a microplate reader (MR5000, Dynatech, Embrach, Switzerland). The assay was done at room temperature. The control wells contained BSA instead of glycodelin.

Levels of IL-2 was measured from the culture supernatant of the treated cells according to the manufacturer protocol (BD Biosciences Pharmingen, San Diego, USA). In brief, capture antibody in 0.1 M sodium carbonate buffer (pH 9.5) was coated on a 96-well ELISA plate overnight at 4°C. The wells were blocked with 200 μl of 10% FBS in PBS for 1 hour and were incubated with 100 μl of culture medium overnight at 4°C. Then 100 μl of biotinylated detection antibody and avidin horseradish peroxidase conjugate mixture was added and incubated for 1 hour. Color development was achieved by enzymatic reaction using 3,3′,5,5′-tetramethylbenzidine as substrate. The reaction was terminated by the addition of 50 μl of 2M H2SO4. The absorbance was measured at 450 nm with λ correction of 595 nm. The wells were washed five times with 0.05% Tween-20 in PBS between each step. The OD450 was determined with a microplate reader (MR5000, Dynatech, Embrach, Switzerland).
Determination of sialidase activity on cumulus cells - The Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster approved the protocol for collection and use of cumulus oophorus in this study. Cumulus-oocyte complexes were obtained from women who underwent treatment with intracytoplasmic sperm injection for male infertility. Human menopausal gonadotropin (Serono, Geneva, Switzerland) was used for ovarian stimulation after down-regulation with buserelin (26). The cumulus-oocyte complexes were dispersed in 1 ml of 0.1% hyaluronidase in human serum albumin supplemented G-MOPS medium (Vitrolife, Kungsbacka, Sweden) at 37°C. After digestion, cumulus cells were pelleted at 300 g for 5 minutes. The dispersed cumulus cells were then washed twice in TC-199 medium (Sigma) and resuspended to a final concentration of 4×10^6 viable cells/ml. Trypan blue exclusion test was employed to determine the viability of the cells. They were then cultured in 2 ml of TC-199 medium supplemented with 20% fetal bovine serum as described (27).

After 48 hours, sialidase activity on the cell surface of cumulus cells was determined using 4-methylumbelliferonyl N-acetylneuraminic acid (4MU-NANA; Sigma-Aldrich, St. Louis, MO, USA) as a substrate, according to previously described method (28). Briefly, after washing with fresh medium, the cells were incubated with 50 μmol/l of 4MU-NANA in 1 ml of culture medium for 2-8 hours at 37°C. The conditioned medium was then collected, centrifuged at 500 g for 15 minutes, and the liberated 4-methylumbelliferone in the supernatant was measured using a fluorometer with excitation at 340 nm and emission at 505 nm (Infinite F200, Tecan, Männedorf, Switzerland).

Data analyses - All values were expressed as mean ± S.E.M. (standard error mean). The data were compared by student’s t-test or ANOVA followed by a post-hoc test (Tukey’s test) to discern differences between individual groups. The data were analysed using SigmaStat 2.03 (Jandel Scientific, San Rafael, CA, USA). A P value below 0.05 was taken as significant.

RESULTS

Differential lectin binding properties of glycoselins - Glycodelin-A, -F, -S and -C were purified from amniotic fluid, follicular fluid, seminal plasma and cumulus matrix, respectively (Figure 1), and were analysed for lectin binding. The lectin binding properties of glycoselins are shown in Table 1. Consistent with our previous data (5), GdF was characterized by significantly lower affinity to UAEI (P<0.05) and higher affinity to S-WGA (P<0.05), while GdS was characterized by a low affinity to...
WFA (P<0.05) when compared with other isoforms.58
GdA and GdF had significantly higher affinities to9
WGA and SNA than Gdc and Gds (P<0.05). N60
significantly differences were found in ConA, PNA61
and RCA120 affinity between the glycofet62
isoforms. 63
Characterisation of the N-glycomes of GdA, Gd64
and GdF - Glycomic profiling was carried out using65
derived methodologies (29,30). Briefly,66
samples were reduced, carboxymethylated, digested67
with trypsin and the N-glycans were released by88
peptide N-glycosidase F digestion and permethylated99
prior to MALDI-TOF profiling. When sample100
amounts permitted, MALDI-TOF/TOF sequencing101
and gas chromatography-MS linkage analyses were102
additionally carried out. Analyses were done on two103
preparations of each of the purified glycofetins to4
allow assessment of reproducibility. Th55
MALDI-TOF profiles for one of each of the GdA76
GdC and GdF samples are shown in Figures 2 to 477
respectively. Spectra from the replicate batches are18
presented in Supplementary Figures S1 to S379
respectively. For clarity, not all components are18
presented in Supplementary Figures S1 to S3,80
given their m/z values and annotations in Figures 281
to 4; complete assignments are presented in the82
expanded Supplementary Figures. The structure83
shown in the annotations were assigned from84
compositional information provided by the MALD85
MS data, complemented by MALDI MS/MS and66
linkage data where available, taking into account87
biosynthetic considerations plus results of the earli88
GdA study (6). Because of the limited amount of89
material, only a selection of the molecular ions90
observed in the MALDI-TOF spectra could be91
subjected to MS/MS analyses. Molecular ion92
analysed by MS/MS are flagged in the93
Supplementary Figures. 94

Important characteristics of these data are: (i)57
All of the glycofetin glycomes are immensely196
complex with many components of the same m/z97
value being mixtures of more than one type of88
structure. This trait is observed throughout the99
detectable mass range of m/z 1500-520000
5 (Supplementary Figures S1 to S3); from the low01
mass region (e.g. m/z 1836, 2040, 2070, 2244, 228802
2459, and 2592) to the middle mass region (e.g. m/m03
2646, 2663, 2775, 2861, 3095) and high mass region04
(e.g. m/z 4267, 4512); (ii) Both lactoNAc and05
lactoNAc are present as antenna backbones in all66
three glycofetins and in many cases are substituted67
with fucose or sialic acid; (iii) Some of the glycans68
have compositions consistent with bisect69
structures and this was confirmed for GdA and GdC10
by the presence of 3,4,6-linked mannos in the11
linkage analysis data (Table 2); (iv) Biantenna62
glycans are the most abundant family in all13
glycofetins but a great diversity of tri- and4
tetraantennary structures are also present; (v)14
Sialylation levels differ markedly in the three15
isoforms. GdA is the most heavily sialylated, GdF16
has a similar glycan composition as GdA but with17
much lower sialylation, whilst GdC has the smallest18
repertoire of glycans carrying sialic acid. The19
duplicate batches gave broadly similar data, albeit20
with quantitative differences in some components.21
Whether these quantitative differences are an22
experimental artefact arising during sample handling23
prior to glycomic analysis or are due to differences24
between individual women remains to be established.25
With respect to the latter, it is significant that26
quantitative differences have previously been27
observed between individual GdA samples (31).28
Also it should be noted that GdC and GdF are low29
abundance glycofetins requiring the pooling of30
follicular fluid from almost 300 women for the31
preparation of a batch of 50 μg. (vi) Consistent with32
earlier observations (6), low levels of high mannose33
structures were observed in GdA and they were also34
detected as minor components of the GdC and GdF35
glycomes; (vii) All of the molecular ions observed in36
the original Fast Atom Bombardment (FAB) data for37
GdA (6), Figure 2) are recapitulated in the current38
GdA data and abundances are comparable once the39
poor sensitivity at high mass of the FAB-MS40
methods of the 1990’s is taken into account. The41
MALDI experiments have additionally revealed a42
wealth of components above m/z 3000 which were43
not detected using the FAB ionization. Many of44
these glycans were unexpectedly found to carry the45
Sda epitope (NeuAcα2-3GalNAcβ1-4Gal) which gives46
characteristic fragment ions in MS/MS47
analysis (Figure 5 and 32, Figure 2B) and48
3,4-linked Gal in the linkage analysis (49 Supplementary
Figure S4). The structures of40
Sda-containing glycans are shown in Supplementary
Table S1; (ix) Sda containing glycans were also
found in GdC and GdF (see Table S1). Interestingly
those present in GdC are characterised by the
absence of sialylation on other antennae. Indeed,
careful scrutiny of the MALDI profiles allows us to
conclude that the sialic acid on GdC is almost
exclusively associated with the Sda epitope.
Moreover, with the exception of this epitope, the
GdC N-glycome appears to be the asialylated
counterpart of the GdA glycome (see Discussion).

Glycofetin-A and -F reduced viability/proliferation
of lymphocytes - The effects of glycofetin treatment
for 48 hours on the viability/proliferation of various
cell lines are shown in Table 3. GdA and GdF at
concentrations of ≥0.1 μg/ml significantly decreased
(P<0.05) the viability of Jurkat and PBMCs, whereas
Gds, GdC and deglycosylated glycofetin had no
effect. At 1 μg/ml, GdA decreased the viability/proliferation of Jurkat cells and PBMC to
30.4±3.8% and 44.3±7.3% respectively. GdF at this concentration reduced lymphocyte viability to 69% of the control (Jurkat: 37.6±3.3%, p=0.002; PBMC: 50.7±7.5%, P<0.001). None of the glycodelin isoforms affected cell viability of Jurkat cells at tested concentrations (data not shown). GdA and GdF induced cell death of PBMCs. GdA and GdF treatment for 48 hours significantly increased the percentage of necrotic cells from 6.4±0.3% to 73.9±2.7% and GdF from 851.6±228.3 pg/ml (untreated control) to 925.9±53.1 pg/ml (P<0.01) and 857.1±117.0 pg/ml (P<0.01), respectively. Other glycodelin isoforms had no significant effects on apoptosis and necrosis of Jurkat cells and PBMCs.

Sialylation is important for activity of glycodelin-A. GdA and GdF suppressed IL-2 secretion by Jurkat cells and PBMCs. The effects of glycodelin treatment on cell death of lymphocytes - Sialidase treatment decreased the sialic acid content of GdA and GdF as shown by a decrease in binding to wheat germ agglutinin (Supplementary Table S2). Desialylation abolished the ability of GdA and GdF in inducing cell death (Table 4).

Glycodelin-A and -F suppress IL-2 secretion by lymphocytes- The effects of glycodelin treatment on cell death of lymphocytes. GdA and GdF treatment for 48 hours significantly increased the percentage of necrotic cells from 6.4±0.3% to 73.9±2.7% and GdF from 851.6±228.3 pg/ml (untreated control) to 925.9±53.1 pg/ml (P<0.01) and 857.1±117.0 pg/ml (P<0.01), respectively. Other glycodelin isoforms had no significant effects on apoptosis and necrosis of Jurkat cells and PBMCs. The corresponding medium control after 4 hours of incubation.

**DISCUSSION**

This is the first study to investigate the immunosuppressive activity of the two recently discovered glycodelin family members, GdF and GdC. Like GdA, GdF reduced cell viability/proliferation, induced cell death and reduced PHA-induced production of IL-2 from lymphocytes. The reduction in IL-2 production may be partly responsible for the decrease in cell viability/proliferation as IL-2 modulates proliferation of stimulated T cells (33). Our data on the immunosuppressive effects of GdA agree with previous reports that GdA inhibits lymphocyte growth by induction of cell death and reduction of IL-2 synthesis (18,34,35). Importantly, the immunosuppressive activity of GdA and GdF was glycosylation dependent and was abolished after deglycosylation and desialylation.

Comparing the MALDI data for all three glycodelins, it is clear that there are both similarities and differences in their glycans. The similarities lie in shared antennae sequences and branching patterns, whilst the differences are associated with variations in relative glycan abundances plus the absence of α2-6 linked sialic acid in GdC. GdA is the most heavily sialylated glycodelin as exemplified by the most abundant biantennary glycans observed in the MALDI profile (m/z 2646, 3007 and 3211, Figure 2) all of which are sialylated (Figure 6C). In contrast, GdF and GdC are both relatively poorly sialylated and their most abundant biantennary glycans are non-sialylated (Figure 6C). However, it is important to note that, like GdA, GdF carries α2-6 linked sialic acid on a portion of its glycans (Supplementary Figure S3), whilst GdC is unique in lacking this type of sialylation.

The shared outer arm α2-6 sialylation of GdA and GdF could explain the comparable immunosuppressive activities found in the present study, assuming that a small amount of an active glycoform is sufficient for conferring function. Similar observations have been reported for another glycoprotein with immunomodulatory properties, α1-acid glycoprotein (36). However, these observations do not exclude the possibility that GdA and GdF may suppress the lymphocyte activities by different glycans. Whether the similar immunosuppressive activities of GdA and GdF are due to the same or different glycan chain(s) is still an open question.

In some other assays GdA and GdF have been found to behave differently. This may be a reflection of the lower degree of sialylation of GdA and/or differences in abundance of other sequences such as 4-methylumbellif erone in a time-dependent manner and was significantly (P<0.05) higher than the corresponding medium control after 4 hours of incubation.
fucosylated lacdiNAc. For example, only GdF but not GdA suppresses the progesterone-induced acrosome reaction (19). GdF also has higher sperm binding affinity compared to GdA (12,19,20).

The advancement of MS technologies has led to the interesting discovery that there is an additional family of glycan recognition in all the female glycoproteins which was not characterised earlier, namely the Sda-capped family. The molecular ions of the most abundant members of this family are well above m/z 3000, and were therefore refractory to the mass spectrometry of the 1990’s; Even though smaller Sda-containing N-glycans are observed at m/z lower than 3000, they are of low abundance and are isobaric to other, more abundant, glycans. Moreover, the MS/MS methods equivalent to the TOF-TOF technology employed in the present work were not available when GdA was first characterised. Thus, the minor biantennary Sda-containing components now observed in the MALDI spectra (Figure 2A) were impossible to detect in the earlier FAB experiments.

GdC (5). One of the important findings in this report is that the structures of the N-glycans of GdC are consistent with desialylation of the α2-6 sialylated antennae in GdA/GdF. This observation suggests that GdC may be a product of remodelling of GdA/GdF by the cumulus cells and that this process might be dominated by desialylation. Furthermore, the remodelling of GdA/GdF to GdC hypothesis is consistent with the present finding that cumulus cells possess sialidase activity. Our unpublished observation has also shown that desialylation of GdA and GdF is associated with loss of sperm- zona pellucida binding inhibitory activity thereby resulting in comparable behaviour to GdC.

The sialidase of the cumulus cells is likely to have similar activity to the sialidase from Vibrio cholera (37) because it does not remove the sialic acid on the Sda epitope. To date, not much is known about the type of sialidase expressed on the surface of cumulus cells. Plasma membrane-associated sialidase (Neu3) is localized mainly on the cell surface, and has been shown to be involved in the regulation of transmembrane signalling (38). However, since Neu3 is a glycolipid-specific sialidase which acts preferentially on gangliosides substrates, but have no activity against sialylated glycoproteins (Ha et al., 2004), glycolipids would be less likely the substrates of Neu3. Alternatively, the soluble enzyme into the cumulus matrix. In this case, it would be similar to the glycogen modifying enzymes in luminal fluid of rat epididymis that are known to be involved in sperm maturation (39). The recent discovery that a cell surface tumor suppressor called Klotho is a 2,6 specific sialidase and is known to be expressed in mouse cumulus (data accessible at NCBI GEO database (40), accession GSE4260 (41)) makes this molecule a possible candidate for our observed sialidase activity (42).

The putative liberation of the sialic acid residues from GdA/GdF to form GdC may result in greater exposure of the Sda epitope. However to date, no receptor for this epitope has been identified. The Sda epitope is relatively rare in human and very little is known about its function. Nevertheless it is of interest that the Sda epitope in bovine pregnancy associated glycoproteins appears to be hormonally regulated (31).

The new structural discoveries of the present work allow refinement of the hypothesis of the roles of glycodelin isoforms in human fertilisation (43). Glycodein is synthesized in the granulosa cells of the late secondary follicle. During the periovulatory period, the sialidase present in cumulus cells uses GdA and -F in the follicular fluid as substrate for the production of the Gdc, which is then released into the extracellular matrix during cumulus expansion or shortly after ovulation. During cumulus penetration, GdC in the cumulus matrix displaces sperm-bound glycodelin isoforms and promotes the zona binding capacity of the penetrated spermatozoa.

Carbohydrate interactions induce lymphocyte differentiation, maturation, activation, migration and responsiveness (44-46). In this study, both GdF and GdA were shown to possess a diversity of sialylated glycans and are immunosuppressive, while the non-sialylated GdS as well as GdC, which has Sda-restricted sialylation, lacked equivalent immunosuppressive activity. Importantly, the ability of GdA and GdF to induce lymphocyte cell death was abolished after desialylation, consistent with the reported crucial role of sialylation in the immunosuppressive activity of glycodein (47). Sialic acid is an acidic monosaccharide that is usually the terminal sugar residue of N-glycan chains (48). The presence of sialic acid receptors on leukocytes is well recognized (49). Therefore, it is tempting to speculate that GdA and GdF might mediate their immunosuppressive effect through this type of receptor on lymphocytes.

The immunosuppressive activity of glycodein was abolished after deglycosylation in the present study. It has also been proposed that the apoptotic activity of glycodein is associated with its protein backbone (50) based on the observation that GdA glycopeptide produced by trypsin digestion did not induce apoptosis (18) and mutation of both glycosylation sites (Asn-28 and Asn-68) of glycodein yielded recombinant non-glycosylated glycodein which retained anti-proliferative activity (50). The discrepancy in the immunosuppressive activity of deglycosylated glycodein in the two
studies could be due to the deglycosylation method used; enzymatic deglycosylation in the present study and site-direct mutagenesis in the previous report. The former involved a denaturing step while the latter may change the protein configuration of the resulting molecule. Experiments using recombinant glycodegin from prokaryotes may help to solve the discrepancy. Recombinant glycodegin produced in E. coli has been shown to bind to monocytes, but not to T-cells and B-cells (51). However, glycosylation is essential for proper folding of glycoproteins (52-53), and deglycosylation strategies may therefore affect the binding and bioactivity of the deglycosylated molecules.

The glycosylation of glycodeins is also important for their influence on human sperm behaviour. Deglycosylation abolishes the actions of glycodeins on capacitation, the acrosome reaction and zona pellucida binding ability (4,10,54). It has also been demonstrated that the glycans derived from GdA can modulate hormone production from trophoblast cells (55).

In conclusion, the glycosylation of GdA, GdF and GdC show many similarities but there are some important differences, most notably in the level and type of sialylation. Evidence is presented that the glycans, particularly the sialic acid residues, are important in the immunosuppressive activities of glycodegin. Further understanding of the glycosylation of glycodein isoforms, particularly their actions on lymphocytes may assist in the rational design of novel therapeutic strategies for immune-based disorders in reproduction.
REFERENCES


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FOOTNOTES

Abbreviations used: MALDI, matrix assisted laser desorption/ionization; TOF, time of flight; MS, mass spectrometry; MS/MS, tandem mass spectrometry; GC, gas chromatography; PBMC, peripheral blood mononuclear cell; Sda epitope, (NeuAcα2-3GalNAcβ1-4)Gal; XTT, sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate; Gd, glycodelin; PHA, phytohemagglutinin;

FIGURE LEGENDS

**Figure 1.** Purity of purified glycodelins. The purity of 0.5 μg GdA, -F, -S, -C and deglycosylated glycodelin (De-Gd) were determined in a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and visualized by silver staining.

**Figure 2.** MALDI-TOF mass spectrum of GdA N-glycans: Panel A, m/z 1500-3500; Panel B, m/z 3500-5200. The N-glycans from purified glycodelin preparations were released by PNGase-F and permethylated (“Experimental Procedures”). A representative spectrum of GdA N-glycans is shown [batch (i)] and an additional sample analysed yielded similar data (Supplementary Figure S1). Data were acquired in the positive ion mode [M+Na]+. The figure on each right hand axis gives the total ion count for that panel of the overall spectrum. Peak assignments are based on 12C isotopic composition together with knowledge of the biosynthetic pathways, and structures were confirmed by MS/MS and linkage analyses. The sugar symbols are those employed by the Consortium for Functional Glycomics for the representation of glycan structures. Structures shown outside a bracket have not been unequivocally defined. For simplicity, specific linkages are not assigned in the mass spectra and only one branching pattern for tri-antennary structures is shown. Therefore, the position of an antenna in a cartoon does not imply designation of a specific arm. MS/MS experiments showed that the biantennary glycans have the usual α3 and α6 arms as indicated in the cartoons. However we cannot rule out minor structures carrying both antennae on the α3 arm. Note the high level of sialylation and the presence of N-glycans with Sda antennae.

**Figure 3.** MALDI-TOF mass spectrum of GdC N-glycans: Panel A, m/z 1500-2800; Panel B, m/z 2800-4500. A representative spectrum of GdC N-glycans is shown [batch (i)] and an additional sample analysed yielded similar data (Supplementary Figure S2). Data acquisition and peak assignments were carried out as for GdA (Figure 2). Note the lack of sialylation and the presence of N-glycans with Sda antennae.

**Figure 4.** MALDI-TOF mass spectrum of GdF N-glycans: Panel A, m/z 1500-2800; Panel B, m/z 2800-4500. A representative spectrum GdF is shown [batch (i)] and an additional sample analysed yielded similar data (Supplementary Figure S3). Data acquisition and peak assignments were carried out as for GdA and GdC (Figures 2 and 3).

**Figure 5.** Representative MALDI-TOF/TOF tandem mass spectra of Sda-containing glycodelin N-glycans: Panel A, MS/MS of the m/z 3253 component of GdA; Panel B, MS/MS of the m/z 3545 component of GdC. Signals present in Figures 2 and 3 were subjected to tandem MS and the resulting MS/MS data are shown. The fragment ions are consistent with the sequences shown in the inset. Fragmentation is usually favoured on the reducing side of HexNAc residues. The peaks which are labeled with an “x” are due to contaminating molecular or fragment ions from neighbouring peaks. The horizontal arrows on the spectra indicate losses from the molecular ion of the designated glycan moieties. Note the
characteristic fragment ion for the Sda epitope at m/z 1092 and the fragment ions at m/z 2184 (in Panel A), m/z 2476, 2013 and 1823 (in Panel B) for the loss of Sda-containing antenna. Other signals with potential Sda arrangements (Supplementary Figures S1 to S3) were analysed for GdA, GdC and GdF, and they yielded similar data.

Figure 6. Major N-glycans of GdA/F and GdC: Panel A, antennae and cores of GdA and GdF; Panel B, antennae and cores of GdC; Panel C, structures of the major components corresponding to the three most abundant molecular ions in the biantennary regions of the MALDI profiles of GdA, GdC and GdF. The +/- annotations in the composite structures in A and B indicate that not all glycans carry the associated structural feature.

Figure 7. Dot plot of glycoprotein on PBMCs and Jurkat cells death after 48 hours treatment. Viable, necrotic and apoptotic cells were identified and quantified by bivariate Yo-Pro®-1/PI flow cytometry. Cells without stain were counted as viable cell. Cells labeled with Yo-Pro®-1 only were counted as apoptotic cells (blue). Cells labeled with both Yo-Pro®-1 and PI were counted as necrotic cells (Red).

Figure 8. Effect of glycoprotein on IL-2 secretion and viability of PBMCs and Jurkat cells after 16 hours treatment. PBMCs primed by PHA (5 μg/ml) and Jurkat cells were incubated with 1 μg/ml of glycoprotein for 16 hours. IL-2 secretion and Cell viability were quantified by ELISA and flow cytometry respectively. Data are mean ± S.E.M., N=4, * and ** P<0.05 and 0.01 vs corresponding control, respectively.

Figure 9. Sialidase activity on the intact cumulus cells. Sialidase activity of intact cumulus cells was determined by incubation with 4MU-NANA at pH 7.4 for 2-8 hours at 37°C. The results represent the mean of three independent experiments ± S.E.M and are expressed as percentage of activity at time 0. *P<0.05 when compared to the corresponding control at the same time point.

Table 1. Binding of lectins with glycoprotein-C, -A, -S and -F. Data are presented as means ± S.E.M. (N=5). The control coated with 10000 ng/ml BSA instead of lectins had OD between 0.07-0.13. a,b,c,d,e,f P<0.05 within the same row (ANOVA on rank).

Table 2. GC-MS linkage analyses of partially methylated alditol acetates obtained from the PNGase F released N-glycans of glycoprotein-A, glycoprotein-F and glycoprotein-C. Permethylated N-glycans were hydrolyzed, reduced, acetylated and analyzed by GC-MS (“Experimental Procedures”). Note the presence of the 3,4-linked galactose which provides evidence for the Sda epitope, and 3,4,6-linked mannose for bisecting GlcNAc.

Table 3. Effect of Glycodelins on viability of Jurkat and PBMCs by XTT assay. Cells of 3x10^5 were incubated with 0.001, 0.01, 0.1 and 1 μg/ml of glycoprotein for 36 hours, XTT labeling mixture was added 12 hours before measurement. Percentage stimulation (%) = (Abs Gd - Abs blank) / (Abs control – Abs blank) x 100%. Data are mean ± S.E.M, N=8. P values are shown for significant differences as compared to control without treatment (One-way ANOVA).

Table 4. Effect of deglycosylation and desialylation of GdA on cell death of Jurkat and PBMCs. Jurkat cells and PBMCs were incubated with 1 μg/ml of glycoprotein A, F, S, C and deglycosylated glycoprotein (De-Gd). Viable, necrotic and apoptotic cells were identified and quantified by bivariate Yo-Pro®-1/PI flow cytometry. Cells without stain were counted as viable cell. Cells labeled with Yo-Pro®-1 only were counted as apoptotic cells. Cells labeled with both Yo-Pro®-1 and PI was counted as necrotic cells. Data are mean ± S.E.M, N=4. * and ** P<0.01 and 0.001, P values are shown for significant differences as compared to
control without treatment (student’s t-test).

**Supplementary Figure S1.** MALDI-TOF mass spectra of N-glycans from two GdA preparations. Regions from the MALDI MS spectra for both batches are expanded for clarity. Data are presented in sets of spectra with the upper panel of each subfigures being the first batch of GdA [GdA - batch (i); 50 µg starting material] and the lower panel, the second batch [GdA - batch (ii); 50 µg starting material]. Subfigure S1-A, m/z 1560-2000; Subfigure S1-B, m/z 2000-2300; Subfigure S1-C, m/z 2300-2600; Subfigure S1-D, m/z 2600-2900; Subfigure S1-E, m/z 2900-3200; Subfigure S1-F, m/z 3200-3500; Subfigure S1-G, m/z 3500-3800; Subfigure S1-H, m/z 3800-4090; Subfigure S1-I, m/z 4090-4550; Subfigure S1-J, m/z 4500-5200 (GdA - batch (i) only because batch (ii) appeared to contain a little less sample and signal to noise was poor above m/z 4500).

The glycodelin N-glycans were released by PNGase F, permethylated, and subsequently subjected to Sep-Pak cleanup ("Experimental Procedures"). Data were acquired in the positive ion mode [M+Na]. Peak assignments are based on $^{12}$C isotopic composition together with knowledge of the biosynthetic pathways, and structures were confirmed by MS/MS and linkage analyses. The assignments for the peaks with boxed m/z values were confirmed by MS/MS. The sugar symbols are those employed by the Consortium for Functional Glycomics for the representation of glycan structures. Structures shown outside a bracket have not been unequivocally defined. For simplicity, specific linkages are not assigned in the mass spectra and only one branching pattern for tri-antennary structures is shown. Therefore, the position of an antenna in a cartoon does not imply designation of a specific arm. Peaks which are labelled with an "x" are due to known contaminants.

**Supplementary Figure S2.** MALDI-TOF mass spectra of N-glycans from two GdC preparations. Regions from the MALDI MS spectra for both batches are expanded for clarity. Data are presented in sets of spectra with the upper panel of each subfigures being the first batch of GdC [GdC - batch (i); 50 µg starting material] and the lower panel, the second batch [GdC - batch (ii); 50 µg starting material]. Subfigure S2-A, m/z 1560-2000; Subfigure S2-B, m/z 2000-2300; Subfigure S2-C, m/z 2300-2600; Subfigure S2-D, m/z 2600-2900; Subfigure S2-E, m/z 2900-3200; Subfigure S2-F, m/z 3200-3500; Subfigure S2-G, m/z 3500-3800; Subfigures S2-H (m/z 3800-4090) and S2-I (m/z 4090-4500) are data only from GdC - batch (i) because batch (ii) appeared to contain a little less sample and signal to noise was poor above m/z 3600. For experimental details see Figure S1.

**Supplementary Figure S3.** MALDI-TOF mass spectra of N-glycans from two GdF preparations. Regions from the MALDI MS spectra for both batches are expanded for clarity. Data are presented in sets of spectra with the upper panel of each subfigures being the first batch of GdF [GdF - batch (i); 50 µg starting material] and the lower panel, the second batch [GdF - batch (ii); 25 µg starting material]. Subfigure S3-A, m/z 1560-2000; Subfigure S3-B, m/z 2000-2300; Subfigure S3-C, m/z 2300-2600; Subfigure S3-D, m/z 2600-2900; Subfigure S3-E, m/z 2900-3200; Subfigure S3-F, m/z 3200-3500; Subfigures S3-G (m/z 3500-3800), S3-H (m/z 3800-4090) and S3-I (m/z 4090-4500) are data only from GdF - batch (i) because the batch (ii) had half the amount of sample compared to batch (i) and signal to noise was poor above m/z 3500. For experimental details see Figure S1.

**Supplementary Figure S4.** Electron impact mass spectra (EI-MS) of the partially methylated alditol acetate (PMAA) derived from 3,4-linked galactose, which provides the evidence for the Sda epitope: Upper Panel, GdA; Lower Panel, GdC. GC-MS linkage analyses of partially methylated alditol acetates obtained from the PNGase-F released N-glycans of GdA and GdC. Permethylated N-glycans were hydrolyzed, reduced, acetylated and analyzed by GC-MS ("Experimental Procedures"). The retention time on the capillary GC column and the fragment ions observed in the EI-MS spectra are used to identify the 3,4-linked galactose. Characteristic fragment ions of 3,4-linked galactose PMAA shown in the inset.
Supplementary Table 1. The structures of the Sda-containing glycans. The table is tabulated from the signals observed in the mass spectra (Supplementary Figures S1 to S3). The m/z values are rounded up to whole numbers for the ease of comparison. Values which are more than m/z 3200 are adjusted by the addition of m/z 1 to make the rounding more accurate.

Supplementary Table S2. Binding of GdA with or without sialidase treatment to wheat germ agglutinin (WGA).
### Figure 1

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<th>GdA</th>
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</table>
Figure 2

A

B

C

Key: △ Rucose (Ruc) □ Mannose (Man) ○ Galactose (Gal) □ N-acetylglucosamine (GlcNAc) 
□ N-acetylgalactosamine (GalNAc) ▼ N-acetyleneuraminic acid (NeuAc)
Figure 3
Figure 4

A

B

Key: ▲ Fucose (Fuc)  ● Mannose (Man)  ○ Galactose (Gal)  □ N-acetylglucosamine (GlcNAc)
□ N-acetylgalactosamine (GalNAc)  ◼ N-acetylneuraminic acid (NeuAc)
Figure 5

A

B

Key: ▲ Fucose (Fuc)  ● Mannose (Man)  ○ Galactose (Gal)  □ N-acetylgalcosamine (GlcNAc)  ■ N-acetylagalactosamine (GalNAc)  ◊ N-acetylhеuraminic acid (NeuAc)
Figure 6

A (GdA/F)

B (GdC)

C GdA

m/z 2646  m/z 3007  m/z 3211

Key:

▲ Fucose (Fuc)
○ Mannose (Man)
○ Galactose (Gal)
■ N-acetylglucosamine (GlcNAc)
□ N-acetylgalactosamine (GalNAc)
☆ N-acetylneuraminic acid (NeuAc)

GdC

m/z 2285  m/z 2489  m/z 2592

GdF

m/z 2040  m/z 2244  m/z 2489
Figure 7

Control De-Gd GdA GdF GdS GdC De-sialylated GdA De-sialylated GdF

PBMCs

Jurkat
Figure 8

IL-2 secretion (pg/ml)

- **PBMC**
- **Jurkat**

Cell viability (%)

- **PBMC**
- **Jurkat**

Control GdA GdF GdS GdC De-Gd
Figure 9

[Graph showing the effect of time on sialidase activity with and without cumulus cells. The graph compares the activity levels between the two conditions over a 8-hour period, with error bars indicating variability.]
Table 1

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<th>Lectin [specificity]</th>
<th>Lectin-immunoassay at OD$_{490}$ (Mean ± SEM)</th>
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<th>GdF</th>
<th>GdA</th>
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Data are presented as means ± S.E.M. (N=5).
The control coated with 10000 ng/ml BSA instead of lectins had OD between 0.07-0.13.

$^{a,b,c,d,e,f}$ P<0.05 within the same row (ANOVA on rank).
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Data are mean ± S.E.M (N=8).

Suppression Index (S.I. ± SEM) = (Abs Gd - Abs blank) / (Abs control–Abs blank) x 100%
P values were shown for significant differences as compared to control without treatment (One-way ANOVA).
Table 4

<table>
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<th></th>
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<tr>
<td>Viable</td>
<td>87.3 ± 0.4%</td>
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<td>86.3 ± 1.0%</td>
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<td>14.2 ± 1.2% **</td>
<td>87.6 ± 0.3%</td>
<td>83.2 ± 2.5%</td>
<td>82.5 ± 2.9%</td>
<td>83.4 ± 1.6</td>
<td>82.9 ± 1.6</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>3.8 ± 0.5%</td>
<td>8.9 ± 0.9% **</td>
<td>8.7 ± 0.9% **</td>
<td>3.0 ± 0.2%</td>
<td>4.1 ± 0.7%</td>
<td>4.9 ± 1.1%</td>
<td>9.8 ± 1.7</td>
<td>9.3 ± 1.4</td>
</tr>
<tr>
<td>Necrosis</td>
<td>7.6 ± 0.4%</td>
<td>73.9 ± 2.7% **</td>
<td>75.8 ± 1.9% **</td>
<td>8.9 ± 0.3%</td>
<td>12.0 ± 1.8%</td>
<td>11.8 ± 1.6%</td>
<td>6.8 ± 1.0</td>
<td>7.7 ± 1.0</td>
</tr>
</tbody>
</table>

Data are mean ± S.E.M (N=4).
* and ** p< 0.01 and 0.001, P values were shown for significant differences as compared to control without treatment (Student’s t-test).