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The Role of Glycans in Immune Evasion: The Human Fetoembryonic Defence System Hypothesis Revisted

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The Role of Glycans in Immune Evasion: The Human Fetoembryonic Defence System Hypothesis Revised
NEW RESEARCH HORIZON Review

The role of glycans in immune evasion: the human fetoembryonic defence system hypothesis revisited

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ABSTRACT: Emerging data suggest that mechanisms to evade the human immune system may be shared by the conceptus, tumour cells, persistent pathogens and viruses. It is therefore timely to revisit the human fetoembryonic defense system (Hu-FEDS) hypothesis that was proposed in two papers in the 1990s. The initial paper suggested that glycoconjugates expressed in the human reproductive system inhibited immune responses directed against gametes and the developing human by employing their carbohydrate sequences as functional groups. These glycoconjugates were proposed to block specific binding interactions and interact with lectins linked to signal transduction pathways that modulated immune cell functions. The second article suggested that aggressive tumour cells and persistent pathogens (HIV, H. pylori, schistosomes) either mimicked or acquired the same carbohydrate functional groups employed in this system to evade immune responses. This subterfuge enabled these pathogens and tumour cells to couple their survival to the human reproductive imperative. The Hu-FEDS model has been repeatedly tested since its inception. Data relevant to this model have also been obtained in other studies. Herein, the Hu-FEDS hypothesis is revisited in the context of these more recent findings. Far more supportive evidence for this model now exists than when it was first proposed, and many of the original predictions have been validated. This type of subterfuge by pathogens and tumour cells likely applies to all sexually reproducing metazoans that must protect their gametes from immune responses. Intervention in these pathological states will likely remain problematic until this system of immune evasion is fully understood and appreciated.

Key words: AIDS / galectins / Siglecs / Lewis antigens / immune evasion

Introduction

Recent data suggest that mechanisms to evade the human immune system may be shared by the conceptus, tumour cells, persistent pathogens and viruses. It is therefore timely to revisit the human fetoembryonic defense system (Hu-FEDS) hypothesis that was proposed in two papers in the 1990s (Clark et al., 1996; 1997). At that time there was little evidence to support this hypothesis. Herein, the Hu-FEDS hypothesis is revisited in the context of more recent studies that are relevant to this experimental model.

In 1953, Sir Peter Medawar defined one of the greatest enigmas in immunology. His specific question was ‘how does the pregnant mother nourish within itself for many weeks or months a fetus that is antigenically a foreign body?’ (Medawar, 1953). He cited three possible reasons why the ‘fetal transplant’ was not rejected by the mother: (i) antigenic immaturity of the fetus; (ii) immunological indolence or inertness of the mother and (iii) anatomical separation from the mother (Medawar, 1953).

Immunity is readily induced in skin transplantation tests by injections of fetal tissue, confirming that the fetus is antigenically mature (Billingham et al., 1956). Women respond to pathogens during pregnancy, arguing against maternal indolence (Head and Billingham, 1986). Human endovascular trophoblasts of placental origin invade and remodel the maternal spiral arteries to enable increased blood flow to the placenta, indicating intimate fetomaternal contact (Burton and Jauniaux, 2004). In short, the hypotheses proposed by Medawar were not supported by subsequent investigations.

In the first Hu-FEDS hypothesis article (Clark et al., 1996), a specific glycoprotein [glycodelin-A (GdA)] and mucins present in the placenta, amniotic fluid and decidua were implicated as factors that suppress the maternal immune response in the pregnant uterus. These glycoconjugates were suggested to manifest their effects by employing their glycans as functional groups to block immune cell binding or interact with lectin-like receptors coupled to signal transduction proteins that modulate immune responses. Another major emphasis of the Hu-
Immune recognition of human eggs

Human zona pellucida (ZP) glycans were predicted to mediate both sperm binding and immune recognition (Clark et al., 1996). This suggestion was based on the ability of fucoidan and the sialyl-Lewis \(^{x}\) tetrasaccharide (sLe\(^{x}\)) to inhibit sperm binding in the human hemizona assay (Table 1) (Huang et al., 1982; Clark et al., 1995). sLe\(^{x}\) is the universal ligand for selectins, cell adhesion molecules that mediate initial neutrophil binding to inflamed endothelium (Foxall et al., 1992). Fucoidan is a potent inhibitor of lymphocyte homing, a process that also relies on selectin-mediated adhesions (Imai et al., 1993). However, selectins were not detected on human sperm, leading to the proposal that the human sperm–egg binding involved a ‘selectin-like interaction’ (Patankar et al., 1993; Clark et al., 1995). Though not a selectin, the binding specificity of the human egg binding protein was anticipated to overlap with these cell adhesion molecules. The possibility that sLe\(^{x}\) was being employed for both immune and gamete adhesions suggested that immune cells could also recognize the human egg, perhaps evoking protective responses (Clark et al., 1996).

Recent studies have confirmed that the human ZP is profusely coated with sLe\(^{x}\) on both N- and O-glycans (Figs 1 and 2) (Pang et al., 2011). A minor amount of N-glycans terminated with another selectin ligand (sulpho-Lewis\(^{x}\)) was also detected (Table 1). sLe\(^{x}\) also inhibited human sperm–ZP binding in either the intact or multivalent neoglycoprotein form (Pang et al., 2011). These results confirmed the carbohydrate binding specificity for the human sperm–egg interaction that was originally proposed two decades ago (Patankar et al., 1993). sLe\(^{x}\) also binds to Siglec-9, an immunoglobulin-like lectin receptor that bears an immunoreceptor tyrosine-based inhibitory motif associated with many types of immune cells (Angata and Varki, 2000; Avril et al., 2004). The binding of sLe\(^{x}\) to Siglec-9 could induce an inhibitory signal in immune cells that encounter an egg. Activated neutrophils or other immune cells in the infected or inflamed uterus could bind to multivalent sLe\(^{x}\) on the human ZP, competing with sperm binding and inhibiting fertilization. Under normal quiescent conditions, multivalent sLe\(^{x}\) on the ZP could mediate human sperm binding and inhibit potential responses by Siglec-9 expressing immune cells. The presentation of sLe\(^{x}\) on the human ZP in this context could ensure that pregnancy proceeds only within a healthy uterine environment (Clark, 2013).

### Table 1 Terminal carbohydrate sequences referred to in the text.

<table>
<thead>
<tr>
<th>Name</th>
<th>Symbol</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sialyl-Le(^{x})</td>
<td>NeuAc(\alpha)2-3Gal(\beta)1-4GlcnAc(\beta)3</td>
<td>Fuc(\alpha)1</td>
</tr>
<tr>
<td>3-Sulpho-Le(^{x})</td>
<td>S-3Gal(\beta)1-4GlcnAc(\beta)3</td>
<td>Fuc(\alpha)1</td>
</tr>
<tr>
<td>Lewis(^{a})</td>
<td>Gal(\beta)1-4GlcNAc(\beta)3</td>
<td>Fuc(\alpha)1</td>
</tr>
<tr>
<td>Lewis(^{b})</td>
<td>Fuc(\alpha)1-2Gal(\beta)1-4GlcNAc(\beta)3</td>
<td>Fuc(\alpha)1</td>
</tr>
<tr>
<td>Fucosylated LacdiNAc</td>
<td>GalNAC(\beta)1-4GlcNAc(\beta)3</td>
<td>Fuc(\alpha)1</td>
</tr>
<tr>
<td>Pseudo-Lewis(^{x})</td>
<td>Fuc(\alpha)1-3Gal(\beta)1-4GlcNAc(\beta)3</td>
<td>Fuc(\alpha)1</td>
</tr>
</tbody>
</table>

N-acetylneuraminic acid; \(\alpha\)-glucose; \(\alpha\)-acetylgalactosamine; \(\alpha\)-acetylglucosamine; sulphate (S); \(\alpha\)-acetylgalactosaminine.
This immune privilege was initially explained by a blood–testis barrier (Setchell, 1967; Dym, 1973). However, autoantigens are present in the basal compartment of the testis, which lack this barrier (Yule et al., 1988; Saari et al., 1996). Despite repeated challenge with sperm proteins, only $\sim 2–3\%$ of women will ever develop antisperm antibodies associated with subfertility or infertility (Rumke and Hellinga, 1959; Lombardo et al., 2001). The incidence of allergic reactions to human sperm is also rare (Sublett and Bernstein, 2011). These results indicate that human sperm and seminal plasma must have very powerful means of attenuating immune responses directed against sperm autoantigens in the male and female reproductive systems.

Major histocompatibility (MHC) antigens in humans are referred to as human leukocyte antigens (HLAs). Sperm and eggs completely lack HLA class I and II antigens (Hutter and Dohr, 1998). Natural killer (NK) cells lyse cells lacking HLA class I antigens, a concept known as ‘missing self’ (Karre, 2002). NK cells are the predominant immune cell type in the human uterus, indicating that they could target sperm (King et al., 1991). Structural analysis of the oligosaccharides derived from classical HLA class I molecules confirms that 35–92% are biantennary bisecting type N-glycans (Fig. 3) (Barber et al., 1996). HLA class I negative K562 erythroleukemia cells evade lysis by NK cells if they express a sufficient level of these N-glycans on their plasma membranes, indicating that NK cells also survey the oligosaccharides on target cells (el Ouagari et al., 1991). Glycomic analysis of human sperm has now confirmed the substantial expression of biantennary bisecting type N-glycans on these cells (Pang et al., 2007). Most human sperm are immunostained with anti-Ley monoclonal antibody (mAb), and there is uniform binding of this mAb to the inner acrosomal membrane and acrosomal contents (Pang et al., 2007). Le$\text{a}^\text{N}$ and Le$\text{a}$ are carbohydrate ligands for DC-SIGN, a C-type lectin receptor (CLR) expressed on dendritic cells that is associated with potent immune tolerizing effects (Gringhuis et al., 2009).

**Human seminal plasma glycoproteins**

Seminal plasma contains several immune-deviating factors that do not rely on carbohydrate recognition (Kelly, 1999; Robertson et al., 2009). The possibility was considered that glycoproteins in this fluid could also evoke immune tolerance. Carbohydrate sequencing confirmed...
that seminal plasma glycoproteins are decorated with exactly the same types of unusual multivalent Le<sup>a</sup> and Le<sup>e</sup> type N-glycans observed in sperm (Pang et al., 2009). Mucin-associated O-glycans and free oligosaccharides in seminal plasma are also abundantly decorated with Le<sup>a</sup> and Le<sup>e</sup> (Hansich et al., 1986; Chalabi et al., 2002). Unlike other normal tissues, these Lewis carbohydrate antigens are prevalent in the male reproductive system.

Glycoprotein ligands for CLRs like DC-SIGN have been implicated as the preferred mediators of immune homeostasis (Garcia-Vallejo and van Kooyk, 2009). Clusterin, galectin-3-binding protein, prostatic acid phosphatase and protein C inhibitor were recently identified as the major endogenous glycoprotein ligands for DC-SIGN in human seminal plasma (Clark et al., 2012). These glycoproteins likely supplement transforming growth factor (TGF)-β, prostaglandins, spermine and prostasomes to evoke immune privilege in the male reproductive system (Kelly, 1999; Robertson et al., 2009). They also modulate immune responses in the female reproductive system after insemination (Clark and Schust, 2013).

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**Glycodelin-A**

GdA was originally designated as a major component of the Hu-FEDS hypothesis (Clark et al., 1996). GdA is a 27 kDa endometrial glycoprotein that is secreted from the mid-luteal phase until the end of the first trimester (Julkunen et al., 1985; Dalton et al., 1995). It is a major decidual product between 7 and 11 weeks of gestation, constituting 4–16% of the total protein in this tissue (Julkunen et al., 1991). GdA is secreted into the amniotic fluid, and taken up and concentrated in the placenta (Julkunen et al., 1986). The expression of GdA declines precipitously in the uterus after the 20th week of gestation, becoming a minor decidual component at term (Julkunen et al., 1991).

The immune-deviating effects of GdA have been the subject of a recent review (Clark and Schust, 2013). This glycoprotein: (i) blocks mitogen-induced proliferation of T cells; (ii) inhibits IL-2 production in activated T cells; (iii) promotes apoptosis in activated T cells; (iv) interacts with CD45 on T cells by employing a lectin-like activity; (v) blocks NK cell lysis of K562 erythroleukemia cells; (vi) decreases the release of IgM and the expression of MHC class II molecules by B lymphocytes; (vii) inhibits the chemoattractant-stimulated migration of monocytes; (viii) blocks E-selectin-mediated adhesion of neutrophils and (ix) induces the release of IL-6 from monocytes and macrophages by binding to L-selectin and an extracellular signal-regulated kinase. This glycoprotein is also a ligand for Siglec-6, an immune lectin expressed on the surface of human syncytiotrophoblasts and cytotrophoblasts (Lam et al., 2011).

GdA is also a potent inhibitor of sperm binding in the human hemizona assay (Oehninger et al., 1995).

Seminal plasma also contains an isoform of GdA designated GdS (Julkunen et al., 1984). This isoform has the same protein backbone as GdA, but does not induce any of the same immune-deviating effects. GdA and GdS are also decorated with very different oligosaccharides, implicating the N-glycans linked to GdA as functional groups (Fig. 5)(Dell et al., 1995; Morris et al., 1996; Lee et al., 2009). The major antennae on GdA-associated N-glycans are α2–6 sialylated or fucosylated lacdiNAc sequences (Dell et al., 1995; Lee et al., 2009). The fucosylated lacdiNAc sequence was previously implicated as a selectin ligand, consistent with the observation that GdA inhibits E-selectin-mediated adhesions (Table I)(Grinnell et al., 1994; Jeschke et al., 2003). Like Le<sup>a</sup> and Le<sup>e</sup>, the fucosylated lacdiNAc sequence is also a carbohydrate

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**Figure 3** Restricted heterogeneity of N-glycans associated with classical class I molecules (HLA-A, -B, -C). These structures are referred to as triantennary N-glycan (A), biantennary bisecting type N-glycans (B) or truncated N-glycans (A and B). In A, the exact positions of the N-acetylneuraminic residues were not defined.

**Figure 4** The expression of N-glycans with multivalent Le<sup>e</sup> on human sperm and seminal plasma glycoproteins. Biantennary, (A), triantennary (B and C) and tetraantennary N-glycans (D) terminated on each antenna with Le<sup>e</sup> (Table I) are expressed on human sperm and seminal plasma glycoproteins (Pang et al., 2007, 2009). Heterogeneous intermediates terminated with different combinations of Le<sup>e</sup>, Le<sup>a</sup> or lacNAc (Galβ1-4GlcNAc) on their antenna are also present.
ligand for DC-SIGN, as noted previously a CLR associated with several immunomodulatory effects (van Liempt et al., 2006; Gringhuis et al., 2009).

The highly elevated expression of GdA plus its known activities should confirm that this glycoprotein is a major immunomodulatory factor during pregnancy. Nonetheless, GdA is virtually never mentioned in

Figure 5 Glycans associated with GdA and GdS. A total of 44 different N-glycans were previously identified in GdA (Lee et al., 2009). This glycoprotein is decorated primarily with complex type N-glycans and only very marginal amounts of high mannosetype hybrid type N-glycans. Antenna are attached to biantennary (Bi), triantennary (Tri) and tetraantennary N-glycans (Tetra) via β1–2, β1–4 or β1–6 linkages to the trimannosyl core (L1–L3, respectively). The majority of the antennae contain the unusual lacdINAc sequence (GalNAcβ1–4 GlcNAc) in intact, fucosylated or sialylated forms (A1–A3). The remaining antennae (A4–A8) are based on the conventional lacNAc sequence (Galβ1–4GlcNAc). The terminal lacNAc sequence on A8 is also modified with fucose, N-acetylgalactosamine and sialic acid to generate the same sequences shown in A5–A7. In contrast, the N-glycans linked to GdS are high mannos type biantennary complex types with only nine structures identified (Morris et al., 1996). GdS does not bind to DC-SIGN, even though this glycoprotein bears high mannos type N-glycans, Lewis+ and Lewis−. This CLR displays preferential binding to seminal plasma glycoproteins bearing triantennary and tetraantennary N-glycans terminated with multivalent Lewis+ and Lewis− sequences (Clark et al., 2012).
reviews focused on the induction of the tolerant state during human pregnancy (Moffett and Loke, 2006; Trowsdale and Betz, 2006; Arck and Hecher, 2013; Erlebacher, 2013). GdA is not a classical immune molecule nor is there a murine analogue, precluding knockout strategies that test its physiological significance in mice. However, there are numerous anatomical, biochemical and physiological differences between murine and human reproduction (Duc-Goiran et al., 1999; Arck and Hecher, 2013).

Humans and other higher primates display a deeper level of haemochorial implantation than other eutherians (Duc-Goiran et al., 1999; Clancy, 2009; Pijnenborg et al., 2011). Pre-eclampsia is a disease of pregnant women and a small number of chimpanzees and gorillas (Pijnenborg et al., 2011). This pathological condition has been linked to an inadequate depth of haemochorial implantation and deficient remodeling of the spiral arteries (Pennington et al., 2012). GdA could play a crucial role in human implantation. Surplus chorionic villous sampling tissues were collected at 10–12 week of gestational age, banked and analysed for gene expression by microarray analysis after pregnancy outcomes were determined. The mRNA for GdA was decreased by 15.6-fold in the decidua of women who developed pre-eclampsia compared with term pregnancies (Founds et al., 2009). This finding indicates that GdA could be a key factor that promotes deep haemochorial implantation in humans, and explain why a functional analogue is not present in mice. Aberrant glycosylation of GdA could also result in the development of pre-eclampsia. Clearly, this evidence indicates that GdA is essential for the development of normal human pregnancies.

Cancer antigen 125 (CA125, MUC16)

CA125 was not discussed at all in the Hu-FEDS papers because its glycosylation was not defined at that time. CA125 was initially identified as a tumour-associated antigen in ovarian cancer patients (Bast et al., 1981). It is an enormous mucinous glycoprotein (24,000 amino acids) that is highly decorated with both N- and O-glycans (Kui Wong et al., 2003). Like GdA, CA125 is an endometrial product that is highly up-regulated in uterine flushings during the mid-luteal phase and first trimester, placing it in a temporospatial position where it could act as a major immune-deviating factor during the early stages of pregnancy (Dalton et al., 1995).

The initial question that arose during preliminary studies was which immune cell type could be targeted by CA125 during both early pregnancy and ovarian tumour development. Uterine NK (uNK) cells are present in small numbers in the proliferative and early secretory phase endometrium, but their numbers increase substantially during the late secretory phase, becoming the predominant immune cell type (King et al., 1991). NK cells in the peripheral blood can be divided into two different populations, CD16<sup>pos</sup>/CD56<sup>dim</sup> cytotoxic cells that constitute 90% of the total and CD16<sup>dim/neg</sup>/CD56<sup>bright</sup> cells lacking cytotoxic activity that make up the remaining 10% (Nagler et al., 1989). uNK cells are also CD16<sup>dim/neg</sup>/CD56<sup>bright</sup> cells with low cytotoxic activity (King et al., 1991; Kopcow et al., 2005).

Trophoblasts secrete a chemokine (MIP-1α), which sequesters uNK cells at the site of implantation, where they come into direct contact with the human embryo (Drake et al., 2001). Syncytiotrophoblasts on the surface of the implanting human embryo lack HLA class I molecules, which could make them sensitive to lysis by uNK cells based on the ‘missing self’ hypothesis (Carbone et al., 1996; Blaschitz et al., 2001). However, syncytiotrophoblasts are completely resistant to killing by uNK cells, and only partially susceptible to lymphokine-activated uNK cells (King and Loke, 1990). These changes in marker expression and lytic activity led to the hypothesis that CA125 could be a major factor that affects the cytotoxicity of uNK cells and NK cells in the peritoneal cavity of ovarian cancer patients.

CA125 was isolated from an ovarian carcinoma cell line (OVCAR-3) and analysed for its carbohydrate expression (Kui Wong et al., 2003). Incubation of peripheral blood NK cells with CA125 at physiological concentrations present in the pregnant uterus for 3 days led to a 50–70% decline in their ability to kill K562 target cells (Patankar et al., 2005). Lymphokine-activated NK cells were inhibited to the same extent as circulating NK cells. This exposure did not change the expression of any NK cell marker except for CD16, which was reduced by 40–70% (Patankar et al., 2005). As noted previously, uNK cells are CD16<sup>dim/neg</sup> cells (King et al., 1991). The cytolytic activity of uNK cells is decreased by 85% compared with peripheral blood NK cells (Kopcow et al., 2005). These findings indicate that CA125 promotes the uNK phenotype.

The immune-deviating activity of CA125 on NK cell cytotoxicity and CD16 marker expression relies completely on its ability to bind to Siglec-9 on the surface of NK cells (Belisle et al., 2010). CA125 does not bind to NK cells after the removal of its terminal sialic acid residues. These results indicate that CA125 employs its carbohydrate sequences as functional groups to induce immune deviation in NK cells.

Uromodulin

Uromodulin was also not discussed in the original Hu-FEDS papers. This immune-deviating glycoprotein was initially detected in the urine of pregnant women (Muchmore and Decker, 1985). Subsequent studies confirmed that uromodulin is a pregnancy-associated isoform of Tamm–Horsfall glycoprotein (THP) (Tamm and Horsfall, 1952; Hession et al., 1987). Uromodulin is a considerably more potent inhibitor of antigen-induced T cell proliferation than THP derived from either men or non-pregnant women (Hession et al., 1987).

In early studies, the glycans linked to uromodulin were implicated as potential functional groups that enabled its immune-deviating activities (Muchmore et al., 1987). However, this claim was not confirmed in 1996. Dell and coworkers later performed extensive structural analysis of uromodulin and THP to determine if any differences existed between the isoforms (Easton et al., 2000). They observed no major changes in N-glycosylation between them. However, unusual core 2 type O-glycans bearing one, two or three sLex terminals were detected on uromodulin (Fig. 6). THP obtained from non-pregnant females and males was decorated with simple core 1 type O-glycans (Easton et al., 2000).

The glycosylation of uromodulin shifted almost completely back to the THP glycoforms 2 months after parturition (Easton et al., 2000). The immune-deviating activity of CA125 on NK cell cytotoxicity and CD16 marker expression relies completely on its ability to bind to Siglec-9 on the surface of NK cells (Belisle et al., 2010). CA125 does not bind to NK cells after the removal of its terminal sialic acid residues. These results indicate that CA125 employs its carbohydrate sequences as functional groups to induce immune deviation in NK cells.
Galectins

Galectins are a family of small soluble lectins that have one or two carbohydrate recognition domains with affinity for lactose and/or N-acetyllactosamine (lactNAc) (Barondes et al., 1994). Galectins often possess domains that accommodate modifications of lactNAc with other monosaccharides (Cummins and Liu, 2009). Multivalent presentations of lactNAc or polyvalent forms of this disaccharide (polylactosamine) substantially increase the affinity of some galectins for specific glycans bearing such sequences (Hirabayashi et al., 2002; Stowell et al., 2008a; Vasta et al., 2012). Because they often dimerize and in some cases oligomerize, galectins form lattices between glycoproteins on the surface of cells and between cells (Demetriou et al., 2001; Vasta et al., 2012). This property enables them to promote a plethora of biological activities in many different biological pathways (Vasta et al., 2012).

Before 1999, galectins had not been detected in the eutherian uterus. Interest in the role of galectins during development was heightened by the observation that human placental protein (PP13) is a galectin (Gal-13) (Than et al., 1999). There is currently evidence for the expression of 16 different human galectin genes at the fetomaternal interface (Than et al., 2009). Galectins induce many immune-deviating effects in T cells, B cells, neutrophils, macrophages, eosinophils, mast cells and basophils (Cummins and Liu, 2009; Than et al., 2012).

Gal-I is the best studied galectin among those up-regulated in the human placenta during pregnancy (Bildner and Rabinovich, 2013). Serum levels of Gal-I increase during the first trimester, peak during the second trimester and remain elevated until parturition in pregnant women (Tirado-Gonzalez et al., 2013). Gal-I promotes the apoptosis of alloreactive T cells as well as the development of tolerogenic DCs and T regulatory cells (Bilos et al., 2007; Kopcow et al., 2008). This galectin also induces the expression of HL-A-G on trophoblasts during the initial stages of human pregnancy (Tirado-Gonzalez et al., 2013). Another galectin (Gal-3) is present in human semen (Jones et al., 2010). This galectin promotes the apoptosis of primary-activated human T cells, indicating that it could induce immune deviations in the male and female reproductive systems (Stowell et al., 2008b). Ligands for galectins are also expressed on the surface of the murine ZP, suggesting that these gametes could be protected by these immune modulators (Clark and Dell, 2006). Galectins are up-regulated in tumour cells and bind to pathogens, indicating that they could be employed for immune deviations that promote their survival (Vasta, 2009; Cedeno-Laurent and Dimitroff, 2012; Rabinovich and Croci, 2012).

HIV and AIDS

A major focus of the second Hu-FEDS paper was to define how HIV could employ its carbohydrate functional groups to modify immune responses leading to the development of AIDS (Clark et al., 1997). The key linchpin was considered to be the ability of HIV to specifically infect CD4+ T cells, providing access to the glycosylation machinery in this cell type (Barre-Sinoussi et al., 1983). This integration enabled HIV glycoproteins to acquire glycans that normally acted as functional groups during T cell interactions. In 1996, there were many reports indicating that structural similarities existed between HIV glycoproteins and native T cell glycoproteins (Golding et al., 1988, 1989; Imberti et al., 1991; Dalgleish et al., 1992; Levy, 1993). The three-dimensional structure of a protein combined with the location of its specific glycosylation sites are essential for determining the sequence of the glycans linked to a glycoprotein. This complementarity meant that HIV could direct the synthesis of proteins that could aberrantly activate or suppress immune responses. Because of the high mutation rate of HIV, many glycoproteins with slightly different protein structures could be generated, thus enabling mutant viruses to acquire different carbohydrate functional groups (Clark et al., 1997).

Several effects of this manoeuvering were predicted including: (i) HIV isotypes expressing the ‘right’ carbohydrate sequences could suppress immune responses directed against them; (ii) the glycans on these variant HIV could bind to the carbohydrate receptors of different cell types, enabling them to infect other immune and non-immune cell types and (iii) soluble or cell surface-associated HIV glycoproteins could inhibit normal immune functions or induce aberrant activation of the immune responses. HIV mutants could be selected for appropriate composite glycoproteins that could overcome the human immune system by combining their high mutational rate with the T cell lineage glycosylation system.

Another interesting linkage was also suggested in the original Hu-FEDS model. Leβ is normally expressed on a low percentage (5–8%) of CD4+ and CD8+ T lymphocytes in the circulation (Table I). However, after HIV infection, the proportion of Leβ positive CD4+ and CD8+ T lymphocytes gradually increases over time to 20–25% (Adachi et al., 1988; Kashiwagi et al., 1994). This elevation is positively correlated with the severity of the immune suppression observed in patients. This increased expression is likely directly induced by HIV, since the percentage of human H9 lymphoblastoid cells bearing Leβ increases from 12 to 97% after infection (Adachi et al., 1988; Kashiwagi et al., 1994). This aberrant expression of a DC-SIGN ligand could promote inappropriate T cell binding and signalling interactions with dendritic cells. Such interactions could also selectively protect HIV-infected cells, providing a reservoir for the virus. As outlined in this review, Leβ or a close structural analogue is expressed on many persistent pathogens and human sperm.

The glycans associated with the major capsid glycoprotein of HIV (gp120) were subsequently implicated in the promotion of viral infection. gp120 contains a very high percentage of high mannose type N-glycans (Geyer et al., 1988; Mizuochi et al., 1990; Bonomelli et al., 2011). DC-SIGN binds not only to fucosylated sequences like Leβ but also to high mannosse type N-glycans (Feinberg et al., 2001). HIV
binds to dendritic cells via the interaction of its high mannose type N-glycans on gp120 with DC-SIGN (Geijtenbeek et al., 2000). This presentation promotes efficient HIV infection of CD4+ T cells. The interaction of DC-SIGN with different carbohydrate sequences on gp120 could promote signalling that leads to either immune suppression or activation (Geijtenbeek and Gringhuis, 2009). This flexibility provides HIV with the ability to modulate adaptive immune responses depending on the glycosylation of its glycoproteins. HIV promotes immune activation following initial infection, but induces immune suppression during the development of AIDS (Fauci, 1993). Viral capsid-associated gp120 almost exclusively acquires high mannose type N-glycans in human and monkey cell types infected in vitro (Geyer et al., 1988; Bonomelli et al., 2011). However, the N-glycans acquired by gp120 in vivo when AIDS is manifested have not been defined.

Simian immunodeficiency virus

Connections to simian immunodeficiency virus (SIV) infections were not considered in the second Hu-FEDS article. However, they were discussed in another paper published later that same year (Clark and Patankar, 1997). Modern AIDS vaccination strategies seek to block HIV infection, but this pathway is not required to prevent the development of AIDS in the natural hosts of SIV. The predominant mechanism for escaping the pathological effects of SIV is the induction of tolerance. Many species of African monkeys are infected with their own species-specific variant of SIV, but very few ever develop symptoms associated with AIDS (Daniel et al., 1987; Aghokeng and Peeters, 2005). The two major experimental animal models for investigating the effects of homologous SIV infection are African green monkeys (AGM) and sooty mangabeys (SM) infected with SIVagm and SIVsm, respectively (Kraus et al., 1989; Silvestri et al., 2003). AGMs and SMs display a profound lifelong viremia that is greater than or equal to the highest levels of circulating HIV observed during infection in humans without developing AIDS (Broussard et al., 2001).

There are currently four hypotheses that have been proposed to explain how SIV<sub>sm</sub> fails to induce simian AIDS in its natural hosts (Chahroudi et al., 2012). However, differential glycosylation of SIV glycoproteins could contribute to this tolerizing effect. The possibility was suggested that SIV<sub>agm</sub> and SIV<sub>sm</sub> acquire the same carbohydrate functional groups that are employed to induce tolerance to gametes or the developing monkey in utero (Clark and Patankar, 1997). The immune system of African monkeys would be activated only during the initial stages of infection with their own SIV subtype, and subsequently develop only mild responses to these virions.

There is inferential evidence that supports this hypothesis. Human H9 lymphoblastoid cells were infected with either HIV-1 or SIV<sub>env</sub> in previous studies (Geyer et al., 1988; Holschbach et al., 1990). The relative proportion of high mannose type N-glycans was substantially elevated in gp120 isolated from the HIV capsid compared with the corresponding SIV<sub>sm</sub> glycoprotein (gp130) (92 versus 24%). More importantly, 37% of the N-glycans linked to gp130 were identified as tetraantennary types, in some cases bearing one or two additional LacNAc sequences (Fig. 7) (Holschbach et al., 1990). N-glycans with this structure are high affinity ligands for galectins designated Gal-1 and Gal-3 (Hirabayashi et al., 2002; Stowell et al., 2008a; Song et al., 2009). As discussed previously, galectins possess diverse immune modulating activities (Cummings and Liu, 2009; Than et al., 2012). No tetraantennary N-glycans were detected in gp120 isolated from HIV propagated in H9 cells (Geyer et al., 1988; Mizuochi et al., 1990). However, definitive glycomic analysis of gp130 isolated from SIV<sub>sm</sub> in the circulation of SMs must be performed to determine if these virions actually acquire galectin ligands. If this acquisition is confirmed, it could help to explain how tolerance to SIV<sub>env</sub> is induced in its natural host.

In contrast, heterologous infection of macaques with SIV<sub>env</sub> or SIV<sub>agm</sub> results in the development of simian AIDS (Hirsch et al., 1995; Villinger

![Figure 7](https://example.com/galectin_ligands.png) Galectin ligands are abundantly expressed on the major viral capsid glycoprotein isolated from SIV<sub>env</sub> produced by infected human H9 lymphoblastoid cells. N-Glycans were isolated from the major viral coat glycoprotein (gp130) of SIV<sub>env</sub> propagated in human H9 lymphoblastoid cells (Holschbach et al., 1990). Complex type N-Glycans were digested with neuraminidase to remove sialic acid in α2–3 and α2–6 linkage and sequenced. The percentage of the total N-Glycan fraction is indicated for each oligosaccharide.
et al., 1996). This differential response was proposed to occur because SIV\textsubscript{agm} and SIV\textsubscript{sm} do not acquire the appropriate carbohydrate functional groups necessary to evoke tolerance in macaques (Clark and Patankar, 1997). Comparative glycomic analysis of gpl30 isolated from SIV\textsubscript{sm} propagated in rhesus monkeys and SMs must be performed to determine if they are differentially glycosylated. Heterologous infection with chimpanzee SIV (SIV\textsubscript{c Taiwanese}) is considered to be how HIV-1 was initially introduced into the human population (Sharp and Hahn, 2011).

In conclusion, the results that have been obtained during the investigation of SIV infection of natural and heterologous hosts are consistent with the Hu-FEDS model for AIDS pathogenesis. However, more careful experimentation is necessary to validate the potential linkages discussed here.

**Helicobacter pylori**

This bacterial species was also proposed to be a Hu-FEDS pathogen (Clark et al., 1997). Infection with *H. pylori* is the major cause of gastric ulcers and cancers in humans (Marshall, 1983; 1993). This bacterium infected modern humans before they migrated out of Africa, indicating an ancient association with this pathogen (Linz et al., 2007). Thought to be specifically noteworthy about *H. pylori* in 1996 was the expression of Le\(^\text{a}\) and Le\(^\text{b}\) on the terminal ends of the lipopolysaccharides associated with 81% of all strains (Aspinall et al., 1995; Simoons-Smit et al., 1996). As described earlier, increased Le\(^\text{a}\) expression had also been detected on CD4+ and CD8+ T cells following HIV infection (Adachi et al., 1988; Kashigi et al., 1994).

DC-SIGN binds to both Le\(^\text{a}\) and Le\(^\text{b}\) (Appelmelk et al., 2003; Van Die et al., 2003). *H. pylori* lipopolysaccharides bearing these Lewis antigens have been shown to modulate Th1/Th2 responses in favour of tolerance via their interactions with DC-SIGN (Bergman et al., 2004). *H. pylori* modulates the expression of these Lewis antigens on its lipopolysaccharides (i.e. phase-variable expression) depending on the level of inflammation that these bacteria encounter (Bergman et al., 2006). More recent studies indicate that lipopolysaccharides bearing Le\(^{\text{a}}\) and Le\(^{\text{b}}\) actively dissociate the KSR1-CNKK-Raf-1 complex from the signalosome after binding to DC-SIGN (Gringhuis et al., 2009). This dissociation results in the increased secretion of IL-10 from dendritic cells, and decreased expression of IL-12 and IL-6 in a Raf-1-independent but LSP1-dependent manner. This signalling pathway is the basis for a Th1 to Th2 shift in T cell responses that favours tolerance of this bacterial pathogen (Gringhuis et al., 2009). This same skewing of the immune response could also be beneficial for blocking adaptive immune responses directed against human sperm, seminal plasma neoantigens and HIV-infected T cells that express Le\(^{\text{b}}\).

**Schistosomes and schistosomiasis**

Schistosomes are intravascular helminthic parasites that were also previously designated as a Hu-FEDS pathogen (Clark et al., 1997). Chronic infection with schistosomes (schistosomiasis) shifts the immune system from a Th1 to a Th2 response, resulting in immune suppression (Pearce et al., 1991). Mature schistosomes are highly resistant to the human immune response.

Schistosomes were initially suggested to be a Hu-FEDS pathogen because of the considerable expression of terminal fucosylated lactosamine and Le\(^{\text{b}}\) on their tegumental surfaces (Ko et al., 1990; Srivatsan et al., 1992). The fucosylated lactosamine sequence is a major antenna associated with GdA-derived N-glycans (Fig. 5) (Dell et al., 1995). As noted previously, this sequence has been implicated as a ligand for both selectins and DC-SIGN (Grinnell et al., 1994; van Lier et al., 2006). The glycolipids associated with the cercarial forms of Schistosoma mansoni are primarily terminated with Le\(^{\text{a}}\) and a close structural analogue of Le\(^{\text{a}}\) known as pseudo-Le\(^{\text{a}}\), another DC-SIGN ligand (Table 1) (Wuhrer et al., 2000; Meyer et al., 2005).

**Cancer and Hu-FEDS**

Cancer was another pathological state that was originally linked to the Hu-FEDS hypothesis (Clark et al., 1997). As noted earlier, K562 human erythroleukemia cells are protected from NK cell cytotoxicity by up-regulating their surface expression of biantennary bisecting type N-glycans (Fig. 3) (el Ouagani et al., 1995; Yoshimura et al., 1996). Lectin-binding studies available in 1996 suggested that bisecting type N-glycans were expressed on human sperm and ZP (Cross and Overstreet, 1987; Patankar et al., 1997). However, definitive carbohydrate sequencing studies confirmed the presence of these glycans on human sperm but not ZP (Pang et al., 2007; 2011).

Investigators began isolating mAb that would selectively bind to tumour cells but not to progenitor cells over three decades ago (Ritz et al., 1980). A mouse mAb designated CSLEX was specifically bound to tumour cells associated with stomach, colorectal, lung, esophageal, ovarian, breast, bladder and pancreatic cancers, but not to normal cells or tissues. The carbohydrate epitope recognized by CSLEX turned out to be sLe\(^{\text{a}}\) (Fukushima et al., 1984). This study led to the designation of sLe\(^{\text{a}}\) as a tumour-associated carbohydrate antigen. Glycomic studies have confirmed that sLe\(^{\text{a}}\)-bearing N-glycans are substantially increased on serum glycoproteins in patients with breast, lung, stomach and ovarian cancer compared with normal controls (Saldova et al., 2007; Abd Hamid et al., 2008; Arnold et al., 2011; Bones et al., 2011; Julien et al., 2011).

Glycoconjugates bearing sLe\(^{\text{a}}\) could interfere with many key immune functions in cancer patients. Expression of this sequence on tumour cells has been implicated in their binding to selectins on endothelial surfaces and metastasis (Laubli and Borsig, 2010). Tumour cells expressing sLe\(^{\text{a}}\) on their plasma membrane-associated glycoconjugates could also block immune cell-mediated responses directed against them via interaction with Siglec-9 (Angata and Varki, 2000; Avril et al., 2004). Metastatic tumour cells present in lymph nodes could inhibit lymphocyte homing and antigen presentation in the lymph system by secreting glycoproteins terminated with multivalent sLe\(^{\text{a}}\) (Johnson, 1999). Studies with uromodulin indicate that glycoproteins bearing multivalent sLe\(^{\text{a}}\) could also inhibit the antigen-induced proliferation of T cells, which is required for adaptive immune responses (Muchmore and Decker, 1985; Easton et al., 2000). In summary, the multiple effects of aberrant sLe\(^{\text{a}}\) expression could completely paralyse the immune response in cancer patients.

Another mAb designated AH6 was developed against human tumour cells (Abe et al., 1983). The ligand for AH6 is Le\(^{\text{b}}\), but this mAb also cross-reacted with another blood group determinant (H2 antigen). A mAb with a strict specificity for Le\(^{\text{b}}\) was bound to many different types of organ-specific tumour cells, but not to normal tissues. These findings confirmed that Le\(^{\text{b}}\) is also a tumour-associated cancer antigen that is neoexpressed on about 70% of all tumours of epithelial origin (Hellstrom et al., 1997). Comparative glycomic analysis of gp130 isolated from SIV\textsubscript{sm} propagated in rhesus monkeys and SMs must be performed to determine if they are differentially glycosylated. Heterologous infection with chimpanzee SIV (SIV\textsubscript{c Taiwanese}) is considered to be how HIV-1 was initially introduced into the human population (Sharp and Hahn, 2011).
The expression of ligands for DC-SIGN like Le$^\text{a}$ could evoke immune deviations that protect tumour cells from adaptive immune responses. Le$^\text{a}$ is abundantly expressed on human sperm and seminal plasma glycoconjugates (Table I, Fig. 4) (Hanisch et al., 1986; Chalabi et al., 2002; Pang et al., 2007, 2009). It is noteworthy that three endogenous glycoprotein ligands for DC-SIGN in seminal plasma (clusterin, galectin-3 binding protein, prostatic acid phosphatase) were previously identified as tumour-associated glycoprotein markers (Huggins and Hodges, 1941; Fukaya et al., 2008; Pucci et al., 2009; Clark et al., 2012).

It is clear from this discussion that human tumour cells express Le$^\text{a}$ and Lewis$^\text{a}$/Lewis$^\text{b}$ sequences that are associated with human gametes. However, tumour cells also become more like human gametes in other important ways. As mentioned previously, human gametes do not express HLA class I antigens (Hutter and Dohr, 1998). Tumour cells also become HLA class I negative during the progression of cancer (Algarra et al., 2004). Like the gametes, the absence of these antigens makes these tumour cells insensitive to MHC class I restricted CTL responses (Zinkernagel and Doherty, 1977). If tumour cells also up-regulate the expression of bisecting biantennary type N-glycans that are expressed on human sperm, then they can also become resistant to NK cells. The evidence indicates that aggressive tumour cells escape the human immune response by employing the same immune-deviating pathways associated with human gametes.

**Summary**

The evidence supporting the Hu-FEDS hypothesis was limited in 1996. However, evidence obtained since then clearly indicates that glycans act as functional groups to elicit tolerizing effects that protect human gametes and offspring in utero. Many persistent pathogens and aggressive tumour cells also either mimic or acquire these same carbohydrate sequences, enabling them to couple their survival to the human reproductive imperative. Though not reviewed here, results from several studies indicate that this system of immune subterfuge is also operating in other eutherian mammals. For this reason, this model is now referred to as the eutherian fetoembryonic defense system (Eu-FEDS) hypothesis (Clark et al., 2001).

This system of protection may not be limited to eutherians. Immune destruction of gametes in any obligate sexually reproducing metazoan will prevent that individual from contributing its genes to future generations. This powerful selection pressure ensures that these germ cells must be insulated from any type of immune response that they might normally encounter. If a pathogen or tumour cell acquires or mimics the glycans employed for the protection of gametes in lower species, they could likely also evade the host’s immune system. The metazoan immune system may not be as imperious as many investigators believe. Rather, data suggest that it is tightly constrained by the reproductive imperative under normal physiological conditions.

There is evidence for this type of restriction, and recent data confirm the unpleasant finding that inoculation with AIDS vaccines actually increases the odds of becoming HIV infected (Cohen, 2013). Similar discouraging results have also been obtained with cancer vaccines (Goldman and DeFrancesco, 2009). Vaccines directed against H. pylori and schistosomes have been similarly unsuccessful (McWilliam et al., 2012; Sutton and Chio, 2013). These results suggest that pathogens and tumour cells that can integrate themselves into the same immune-deviating pathways that are necessary for human reproduction are unlikely to be viable candidates for vaccination. These findings are quite demoralizing, to say the least. However, ignoring such effects will make it much more difficult if not impossible to treat these recalcitrant pathological states. In contrast, adoption of this logic and acting upon it could mean the resolution of many pathological states in diverse sexually reproducing organisms, including humans.

**Dedication**

Those of us who knew Robert Edwards were saddened to hear about his recent passing on 10 April after a long illness. However, we will certainly remember his razor sharp mind and keen wit, in addition to his many scientific contributions in the area of reproductive biology. The Hu-FEDS hypothesis papers were published in the ESHRE journals in the 1990s with encouragement from Bob Edwards who was then Editor-in-Chief. His fascination for the subject was clear in several telephone conversations and he predicted at that time that there would never be an AIDS vaccine. His insights continue to be relevant and this article is dedicated to him.

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**Conflict of interest**

None declared.

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Glycosylation Related Actions of Glycodelin: Gamete, Cumulus Cell, Immune Cell and Clinical Associations
Glycosylation related actions of glycodelin: gamete, cumulus cell, immune cell and clinical associations

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Glycodelin is an example of a glycoprotein whose complex-type glycans mediate biological actions in human reproduction and immune reactions. Being attached to an identical protein backbone, glycodelin oligosaccharides vary significantly from one reproductive tissue to another and have an effect on its own secretion and role in cell communication. For instance, uterine glycodelin-A inhibits sperm–oocyte interaction by binding on the sperm head. This is a glycosylation-dependent phenomenon, in which fucosyltransferase-5 plays a key role. Glycodelin-S from seminal plasma binds evenly around the sperm head and maintains an uncapacitated state in the spermatozoa, until the isoform is detached during sperm passage through the cervix. Glycodelin-F from follicular fluid and Fallopian tube binds to the acrosomal region of the sperm head, thereby inhibiting both the sperm–oocyte binding and premature progesterone-induced acrosome reaction. The cumulus cells surrounding the oocyte can capture glycodelin-A and -F from the surrounding environment and convert these isoforms to a cumulus cell isoform, glycodelin-C. It differs by glycosylation from the other isoforms, and it too attaches on the sperm head, with the highest density in the equatorial region. Glycodelin-C is capable of detaching the sperm-bound inhibitory isoforms so that the sperm–oocyte binding is enhanced. Glycodelin-A also has immunosuppressive actions directed to cellular, humoral and innate immunity. Although these actions depend mainly on the protein backbone, glycosylation also plays a part. Glycosylated glycodelin may be involved in the protection of spermatozoa against maternal immune reactions, and glycodelin also has apoptogenic activity. Some glycosylation patterns of glycodelin may mask its apoptogenic domain. This review updates the recent research and clinical associations of glycodelin, highlighting the role of glycosylation.

Key words: cumulus cells/fertilization/immunosuppression

Immunological and structural background

Nomenclature, antibodies and immunodetection

The name ‘glycodelin’ was proposed by the Helsinki team to their collaborators who had resolved the glycan structures and biological actions of purified glycoprotein (Riittinen et al., 1991), previously known as placental protein 14 or progesterone associated endometrial protein (Dell et al., 1995; Kämäräinen et al., 1996; Koistinen et al., 1996; Morris et al., 1996). The name ‘glycodelin’ was subsequently agreed by the pioneers who had launched these and a variety of other names for this protein into the literature (reviewed in Seppälä et al., 1998). The name ‘glycodelin’ highlights the importance of glycosylation for the biological activity of the glycoprotein that is formed in various sites of the body.

Glycodelin is sugar-rich, comprising 17.5 wt.% carbohydrate (Bohn et al., 1982). On the basis of the differences in glycosylation, the isoforms so far characterized are designated as glycodelin-A (amniotic fluid, endometrium/decidua and maternal serum) (Dell et al., 1995; Koistinen et al., 2003), glycodelin-S (seminal plasma and seminal vesicles) (Morris et al., 1996), glycodelin-F (follicular fluid and the oviduct) (Tse et al., 2002) and glycodelin-C (cumulus oophorus) (Chiu et al., 2006). Polyclonal and monoclonal anti-glycodelin antibodies generated using purified glycodelin protein from amniotic fluid and seminal plasma show similar immunoreactivity against these four glycodelin isoforms. Although carbohydrates play an active role in protein folding and participate in the formation of discontinuous epitopes, the differentially glycosylated glycodelin isoforms, such as glycodelin-A and glycodelin-S, share similar thermodynamic parameters of reversible denaturation, suggesting that their native folding is not influenced by different glycosylation patterns (Koistinen et al., 1999). This may explain why the generation of antibodies specific for either glycodelin-A or glycodelin-S has turned out to be difficult, and all the four
glycosylated isoforms react in the same way with anti-glycodelin antibodies.

In addition to the conventional monoclonal and polyclonal antibodies generated with the use of native glycosylated glycodelin that has been purified from biological materials, polyclonal antibodies have been produced against a synthetic linear femtopeptide consisting of amino acids 69–83 of the glycodelin sequence – NH₂-Lys-Lys-Val-Leu-Gly-Glu-Lys-Thr-Glu-Asn-Pro-Lys-Lys-Phe-Lys-COOH (Poddar et al., 1998). Not unexpectedly, the results obtained with the use of this peptide antibody (Horowitz et al., 2001; Song et al., 2001) are different from those obtained with the anti-glycodelin antibodies (Julkunen et al., 1986a; Waites et al., 1990; Mandelin et al., 2003). The difference is seen both in normal tissues and in tumours, and cross-testing of the two types of antibodies (R. Koistinen and M. Seppälä, unpublished observation) revealed that, while both react with glycodelin, the femtopeptide antibody shows a wider spread of immunostaining in tumours, normal tissues and, notably, in blood vessels in which the glycodelin antibodies show no reactivity. Most importantly, not all immunoreactivity of the femtopeptide antibody can be abolished by absorption with purified glycodelin-A, demonstrating that its additional reactivity is glycodelin unrelated. Given that about one half of the sequence of the linear femtopeptide used for immunization (Poddar et al., 1998) is similar to the sequences present in many human proteins, such as SCP-1 peptide (100% identity with the first seven amino acids of the femtopeptide), cutaneous T cell lymphoma (CTCL) tumour antigen se2-1, bullous pemphigoid antigen 1, dynein heavy chain domain 3, KIAA1503 protein, dystonin isoform 1eA precursor and a number of other proteins, any of these proteins/peptides may potentially cross-react with the femtopeptide antibody. Therefore, until the nature of the glycodelin unrelated additional immunoreactivity of the peptide antibody has been specified, the name ‘glycodelin’ is misleading in this context. Perhaps, a name ‘glycodelin-derived peptide’ should more accurately describe this immunoreactivity to distinguish it from glycodelin.

**Glycodelin gene**

The Human Genome Organization (HUGO) has registered progestagen-associated endometrial protein (PAEP) as the official symbol of the glycodelin gene (Kämäräinen et al., 1991). The gene is 5.05 kb long and, like many other lipocalin genes, it is divided into seven exons (Vaisse et al., 1990). The nucleotide sequence encoding the retinol-binding motif of β-lactoglobulins is conserved in the glycodelin gene that comprises four putative glucocorticoid/progesterone response elements (PRE) in the promoter region (Vaisse et al., 1990). The presence of PREs is compatible with the observations that progesterone is involved in the regulation of glycodelin synthesis.

**Primary structure**

Glycodelin is a member of the lipocalin family of proteins. Its primary sequence of 180 amino acid residues (Julkunen et al., 1988) has significant similarity with β-lactoglobulins from several species. The crystal structure of glycodelin is not known, and information on its tertiary structure rests on the Swiss model deduced from the crystal structure of bovine β-lactoglobulin (Koistinen et al., 1999).

**Carbohydrate moieties**

The amino acid sequence of glycodelin comprises three potential N-glycosylation sites, at positions 28, 63 and 85 (Julkunen et al., 1988). Two of them (Asn 28 and Asn 63) are glycosylated in uterine glycodelin-A and seminal plasma glycodelin-S. Nevertheless, there are striking differences in the glycosylation patterns between these two isoforms (Figure 1) (Dell et al., 1995; Morris et al., 1996). Among the complex-type glycans of uterine glycodelin-A, the major non-reducing epitopes are lAcNAC, lAcNAC, sialylated lAcNAC, sialylated lAcNAC, blood group Lewis⁸ and the lAcNAC analogue of Lewis⁸ (Dell et al., 1995) (Figure 1). A substantial subset of the glycans are fucosylated on the penultimate GlcNAc, yielding the fucosylated lAcNAC type sequence (GalNAcβ1-4[Fucα1-3]GlcNAc). In addition to their physico-chemical and immunological similarities, purified glycodelin from amniotic fluid, endometrium, decidua and pregnancy serum is similar in respect of their glycans and may be subgrouped as glycodelin-A (Koistinen et al., 2003). The high degree of sialylation and fucosylation are important characteristics of this isoform. Glycodelin-S differs from glycodelin-A in that it contains no sialylated glycans. Glycosylation of glycodelin-S is highly site-specific, as Asn 63 carries only complex-type structures, whereas only high mannose structures are found at Asn 28. Moreover, glycodelin-S glycans are unusually fucose-rich, so that more than 80% of the complex-type glycans at Asn 63 have 3–5 fucose residues per glycan. Each biantennary glycan in glycodelin-S is core fucosylated and the remaining fucoses are attached to lAcNAC antennae, resulting in Lewis⁸ and Lewis⁹ blood group epitopes (Morris et al., 1996). The third well-defined isoform, glycodelin-F, from follicular fluid also has the same protein core.

**Figure 1.** Representative examples of the major complex-type glycans present at the N-glycosylation sites Asn 28 and Asn 63 of glycodelin-A and glycodelin-S. Characteristic epitopes are marked by broken line. Adapted with permission from authors Dell et al. (1995) and Morris et al. (1996) and the American Society for Biochemistry and Molecular Biology.
Glycodelin, gametes, cumulus cells and immune reactions

as glycodelin-A and glycodelin-S, but it too has its own specific glycosylation, as indicated by studies employing fluorophore-assisted carbohydrate electrophoresis and lectin binding characteristics (Chiu et al., 2003b). The fourth isoform, glycodelin-C, is modified from glycodelin-A and -F by the cumulus cells. Compared with its parent isoforms, its molecular size is smaller and the isoelectric point is higher, and their lectin binding properties are different. Glycodelin-C reacts strongly with concanavalin-A, Wisteria floribunda agglutinin, Ricinus communis agglutinin, Ulex europaeus agglutinin and Dolichos biflorus agglutinin but, unlike its parent isoforms, not with Sambucus nigra bark agglutinin that reacts with the α-NeuNAc(2-6)gal/galNAc residues (Table I).

Glycodelin produced by recombinant technologies may be either non-glycosylated or glycosylated, depending on the cells used for synthesis. For instance, recombinant glycodelin from Chinese hamster ovary cells is glycosylated and it reacts immuno-logically in the same way as glycodelin-A, yet their glycosylation patterns are different. By contrast, glycodelin produced in the human embryonic kidney 293 cells fulfills both immunological and glycosylation based criteria of glycodelin-A (Van den Nieuwenhof et al., 2000). Therefore, in the absence of specific information on the type of glycosylation, term glycodelin without any isoform designation is recommended.

**Cellular origin and secretion in reproductive tissues**

Glycodelin-A is synthesized in glandular and luminal surface epithelium of progesterone- or progestagen-exposed endometrium (Julkunen et al., 1986a; Mandelin et al., 1997, 2001) (Table I). During the estrogen dominated fertile window of a normal ovulatory cycle, absence of glycodelin-A synthesis in the endometrium is significant, because the glycoprotein has anti-fertilization activity (discussed later). In a normal ovulatory cycle, glycodelin expression peaks in the secretory endometrium 8–10 days after ovulation, whereas in maternal serum and amniotic fluid, the levels are highest at 10 and 16 weeks of pregnancy, respectively (Julkunen et al., 1985). Glycodelin-A is secreted mainly into endometrial/decidual gland lumen, from there to uterine fluid or amniotic fluid, and less to serum. Results of a study employing mutagenesis of the asparagines at the N-glycosylation sites (Asn 28 and Asn 63) suggest that glycosylation at Asn 28 plays a key role in the extracellular secretion of glycodelin, as mutation of Asn 28 brings about a significant decrease in the amount of secreted protein. Loss of both glycosylation sites dramatically reduces the secretion (Jayachandran et al., 2004). This study shows that glycosylation is essential for glycodelin secretion.

Glycodelin-S is one of the major secretory proteins in the seminal plasma, produced in seminal vesicle glands (Petrunin et al., 1980; Bohn et al., 1982; Julkunen et al., 1984; Koistinen et al., 1997). No clinically meaningful association has been found between the glycodelin concentration in seminal plasma and sperm pathology (Julkunen et al., 1984), or the success of in vitro fertilization (IVF) (Koistinen et al., 2000).

Glycodelin-F is synthesized in luteinized granulosa cells of the ovary and it is present in follicular fluid (Table I). Glycodelin has also been found in the oviduct, synthesized by its epithelial cells (Julkunen et al., 1986b, 1990; Laird et al., 1995). Part of oviductal glycodelin is similar to glycodelin-F (Yeung et al., 2006). Cumulus oophorus cells contain glycodelin immunoreactivity but not glycodelin mRNA, indicating uptake rather than synthesis (Tse et al., 2002). The cumulus cell isoform is immunologically similar but it differs from the other three isoforms by glycosylation and charge (Chiu et al., 2006).

Outside the pelvic organs, glycodelin has been found in haematopoietic cells of the bone marrow (Kämäräinen et al., 1994; Morrow et al., 1994), normal breast (Kämäräinen et al., 1999), glandular tissues in the lung and eccrine sweat glands (Kämäräinen et al., 1997). The type of glycosylation of glycodelin from these tissues remains to be characterized.

**Biological behaviour and actions in the reproductive system**

The difference in glycosylation between glycodelin and β-lactoglobulin is reflected in their different biological actions. Unlike β-lactoglobulin and many other lipocalins, glycodelin-A has not been found to bind retinol, retinoic acid or other lipocalin ligands (Koistinen et al., 1999). Glycodelin inhibits E-selectin mediated cell adhesion (Jeschke et al., 2003). This propensity is compatible with an observation that oligosaccharides with fucosylated lactNAc antennae present in glycodelin-A have been shown to block selectin-mediated adhesions, and a biantennary N-linked oligosaccharide bearing GalNAcβ1-4(Fucα1-3)GlcNAc antennae is an inhibitor of E-selectin-mediated adhesion (Grinnell et al., 1994). Glycodelin has also been shown to take part in cell differentiation, and it may be involved in tissue modelling (Kämäräinen et al., 1997; Koistinen et al., 2005; Uchida et al., 2005).

### Table I. Sperm-binding glycodelin isoforms

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Origin</th>
<th>Typical carbohydrates</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GdA</td>
<td>Endometrium, amniotic fluid, pregnancy serum</td>
<td>Sialylated and/or fucosylated lacNAc or lacdNAc E-selectin ligands</td>
<td>Dell et al., (1995)</td>
</tr>
<tr>
<td>GdF</td>
<td>Luteinized granulosa cells, follicular fluid, oviduct</td>
<td>Like GdA but more N-acetylgalcosamine</td>
<td>Koistinen et al., (2003)</td>
</tr>
<tr>
<td>GdS</td>
<td>Seminal vesicles, seminal plasma</td>
<td>Fucosylated Lewis-x and Lewis-y, high mannose</td>
<td>Tse et al., (2002)</td>
</tr>
<tr>
<td>GdC</td>
<td>Cumulus cells</td>
<td>α-man, galNAc, β-gal, α-L-fuc</td>
<td>Yeung et al., (2006)</td>
</tr>
</tbody>
</table>

Gd, glycodelin.
Glycodelin-C

Glycoconjugate biosynthesis of the sialyl Lewisx or sialyl Lewisa

ferases that incorporate fucosyl residues into glycolipid or glyco-

Action

Inhibits spermatozoa–zona pellucida

Protection spermatozoa against lymphocyte attack

Kinetic data have been extracted from Chiu et al. (1996). It is believed that the function of glycosyltransferases is likely to allow detachment of glycodelin-S from spermatozoa. Like glycodelin-A, glycodelin-S binds on the human sperm head via two binding sites that are saturable and reversible (Chiu et al., 2005). The binding sites of glycodelin-S are different because glycodelin-A and -F cannot displace glycodelin-S from its binding sites. Although the binding of glycodelin-S is specific, its affinity is low. The low affinity binding sites are more abundant than the high affinity binding sites. On the basis of indirect immunofluorescence staining the sperm-bound glycodelin-S covers the sperm head completely and this immunoreactivity is removed when spermatozoa migrate through cervical mucus surrogates. The low binding affinity of the carbohydrate-based interactions is likely to allow detachment of glycodelin-S from spermatozoa.

In follicular fluid, glycodelin-F is the main sperm–oocyte binding inhibitory isoform (Chiu et al., 2003a), but follicular fluid also contains small amounts of glycodelin-A (P.C.N. Chiu and W.S.B. Yeung, unpublished observation). Glycodelin-C has been converted from glycodelin-A and -F by the cumulus cells (Chiu et al., 2006). Its protein core is identical with that of the other glycodelin isoforms, but glycodelin-C has a smaller molecular size, a higher isoelectric point and different lectin binding properties compared with the other isoforms (Table I).

Table II. Binding of glycodelin-A isoforms on different cell types

<table>
<thead>
<tr>
<th>Glycodelin-A</th>
<th>Spermatozoa</th>
<th>Monocyte</th>
<th>T cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High affinity</td>
<td>Low affinity</td>
<td>CD14+ cell from PBMC</td>
</tr>
<tr>
<td>K_D (nM)</td>
<td>N/A</td>
<td>21 ± 2</td>
<td>6.7 ± 1.1</td>
</tr>
<tr>
<td>Binding sites /cell</td>
<td>N/A</td>
<td>1 884 260 ± 66 220</td>
<td>9 510–35 820</td>
</tr>
<tr>
<td>Possible receptor</td>
<td>N/A</td>
<td>FUT5</td>
<td>N/A</td>
</tr>
<tr>
<td>Action</td>
<td>Inhibits spermatozoa–zona pellucida binding</td>
<td>Protects spermatozoa against lymphocyte attack</td>
<td></td>
</tr>
</tbody>
</table>

Table III. Binding of glycodelin-F, -S and -C on spermatozoa

<table>
<thead>
<tr>
<th>Glycodelin-F</th>
<th>Spermatozoa</th>
<th>Spermatozoa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High affinity</td>
<td>Low affinity</td>
</tr>
<tr>
<td>K_D (nM)</td>
<td>3.9 ± 0.1</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>Binding sites /cell</td>
<td>8 187 200 ± 18 060</td>
<td>2 028 740 ± 36 120</td>
</tr>
<tr>
<td>Possible receptor</td>
<td>N/A</td>
<td>FUT5</td>
</tr>
<tr>
<td>Action</td>
<td>Inhibits spermatozoa–zona pellucida binding</td>
<td>Prevents premature acrosome reaction</td>
</tr>
<tr>
<td>Glycodelin-S</td>
<td>Spermatozoa</td>
<td>Spermatozoa</td>
</tr>
<tr>
<td>K_D (nM)</td>
<td>104 ± 20</td>
<td>1 413 ± 316</td>
</tr>
<tr>
<td>Binding sites /cell</td>
<td>2 408 000 ± 301 000</td>
<td>5 117 000 ± 903 000</td>
</tr>
<tr>
<td>Possible receptor</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Action</td>
<td>Maintains spermatozoa in an uncapacitated state</td>
<td></td>
</tr>
<tr>
<td>Glycodelin-C</td>
<td>Spermatozoa</td>
<td>Spermatozoa</td>
</tr>
<tr>
<td>K_D (nM)</td>
<td>Positive co-operativity among binding sites</td>
<td></td>
</tr>
<tr>
<td>Binding sites /cell</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Possible receptor</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Action</td>
<td>Stimulates spermatozoa–zona pellucida binding</td>
<td>Displaces sperm-bound glycodelin-A/F</td>
</tr>
</tbody>
</table>

Kinetic data have been extracted from Chiu et al. (2003b), Miller et al. (1998), Vigne et al. (2001) and Rachmilewitz et al. (2003). Data are expressed as mean and standard error of the mean (SEM). N/A, not available; K_D, equilibrium dissociation constant, FUT5, sperm fucosyltransferase-5; PBMC, peripheral blood mononuclear cells.

Order of tables:
1. Table II. Binding of glycodelin-A isoforms on different cell types
2. Table III. Binding of glycodelin-F, -S and -C on spermatozoa

**Binding of glycodelin isoforms on spermatozoa**

All the four isoforms, glycodelin-A, -S, -F and -C, bind on the human sperm head (Tables II and III). Glycodelin-F has two binding sites. One of them is shared with glycodelin-A, and glycodelin-A can displace maximally 70% of labelled glycodelin-F bound on the spermatozoa (Chiu et al., 2003b). Immunochemical staining localizes glycodelin-F binding sites to the acrosomal region of the human spermatozoa. Studies on neoglycoproteins have shown that the binding of glycodelin-A to spermatozoa involves mannos, fucose and possibly E-selectin ligands, whereas that of glycodelin-F involves mannos, fucose and N-acetylglucosamine, but not the selectin ligands (Chiu et al., 2004).

Fucosyltransferases (FUT) constitute a family of glycosyltransferases that incorporate fucosyl residues into glycoplipid or glycoprotein glycans, providing one of the possible termination steps of glycoconjugate biosynthesis of the sialyl Lewis^a^ or sialyl Lewis^b^ determinant that plays a role in cell–cell interaction (Borsig et al., 1996). It is believed that the function of glycosyltransferases on the cell surface is confined to their carbohydrate binding ability rather than glycosyltransferase function because of a lack of sugar nucleotide donors (Colley, 1997). Using chemical cross-linking and anti-glycodelin antibody immunoprecipitation, the glycosylreceptor complex was isolated and analysed by mass spectrometry and Western blot analysis using specific anti-FUT5 antibodies (Chiu et al., 2007). The results suggested that FUT5 serves as the receptor of glycodelin-A on human spermatozoa (Table IV). Differential extraction of the surface labelled sperm proteins and immunofluorescence staining suggested that sperm FUT5 is an externally oriented integral membrane protein in the acrosomal region of the human spermatozoa. Subsequently, Chiu et al. (2007) succeeded in purification of biologically active FUT5 from human spermatozoa and, in co-immunoprecipitation experiments, they confirmed that the interaction between glycodelin-A and sperm FUT5 or recombinant FUT5 is highly specific. According to binding kinetic analyses, the K_D of sperm FUT5 binding to solubilized zona pellucida is 43 pmol ml^-1_. The ability of sperm FUT5 to bind to both glycodelin-A and -F, and to the zona pellucida, suggests that human sperm FUT5 is a receptor of glycodelin-A (and -F) and zona pellucida glycoproteins (Chiu et al., 2007). The likely mechanism by which these glycodelin isoforms inhibit spermatozoa–zona pellucida binding is by blocking the binding of sperm FUT5 to the zona pellucida.

Like glycodelin-A, glycodelin-S binds on the human sperm head via two binding sites that are saturable and reversible (Chiu et al., 2005). The binding sites of glycodelin-S are different because glycodelin-A and -F cannot displace glycodelin-S from its binding sites. Although the binding of glycodelin-S is specific, its affinity is low. The low affinity binding sites are more abundant than the high affinity binding sites. On the basis of indirect immunofluorescence staining the sperm-bound glycodelin-S covers the sperm head completely and this immunoreactivity is removed when spermatozoa migrate through cervical mucus surrogates. The low binding affinity of the carbohydrate-based interactions is likely to allow detachment of glycodelin-S from spermatozoa.

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In co-immunoprecipitation, solubilized zona pellucida reduces the binding of glycodelin-A to sperm FUT5

Substrate of FUT5 specifically blocks the binding of glycodelin-A to spermatozoa in competition binding assay

Anti-FUT5 antibody and FUT5 acceptors inhibit spermatozoa-zona pellucida binding in hemizona binding assay

Indirect immunofluorescence staining localizes FUT5 to the acrosome region of human spermatozoa, a region that binds glycodelin-A and zona pellucida (Chiu et al., 2003a)

Fluorophore-labelled sperm FUT5 binds strongly to intact and solubilized human zona pellucida

**Capacitation**

This is defined as a series of transformations that spermatozoa normally undergo during their migration through the female genital tract to reach and bind to the zona pellucida. During capacitation, extensive changes take place in all sperm compartments. Ion fluxes induce biochemical modifications, membrane lipids, extensive changes take place in all sperm compartments. Ion fluxes and capacitation are initiated (de Lamirande et al., 1997). At high physiological concentrations (>900 pmol ml⁻¹) glycodelin-S significantly suppresses bovine serum albumin-induced capacitation of human spermatozoa, suggesting that glycodelin-S may contribute to an uncapacitated state of human spermatozoa in seminal plasma and prevent premature capacitation (Chiu et al., 2005). Deglycosylated glycodelin-S has no similar inhibitory effect, demonstrating the importance of glycosylation in this process. Interestingly, another study employing recombinant glycodelin has shown that whereas glycosylated glycodelin stimulates human sperm capacitation, non-glycosylated glycodelin stimulates it (Dutta et al., 2001). Compared with the binding kinetics of the other glycodelin isoforms, glycodelin-S binds to and detaches from spermatozoa at a faster rate than the other two isoforms do. The fast kinetics is obviously important for its biological action to take place because spermatozoa will be in contact with seminal plasma during a short time after ejaculation only. The weak binding affinity of glycodelin-S may also explain why labelled glycodelin immunoreactivity can be demonstrated on spermatozoa only when the cells are treated with high physiological concentration of glycodelin-S (Chiu et al., 2003b, 2005). It also explains the readiness of removal of bound glycodelin-S from spermatozoa during migration through the cervical mucus. Interestingly, glycodelin immunoreactivity has been found in the cervical mucus (Pockley et al., 1989), but its origin remains to be determined.

There is cholesterol efflux from human spermatozoa during bovine serum albumin and cyclodextrin induced capacitation of human spermatozoa. Glycodelin-S significantly reduces this efflux induced by either of the stimulators, and it exerts this effect upstream of protein kinase activation in the adenyl cyclase/protein kinase A/tyrosine kinase signalling pathway. Again, these findings demonstrate the importance of carbohydrate moieties of glycodelin-S for its biological action, particularly because the said processes are activated upon removal of glycodelin-S from the spermatozoa. In vivo, the dissociation probably takes place during the passage of spermatozoa through the cervix, as suggested by in vitro experiments employing a cervical fluid surrogate (Chiu et al., 2005). In view of these observations glycodelin-S appears to play a role in maintaining an uncapacitated state in the human spermatozoa before their passage through the cervix.

**Inhibition of sperm binding to the zona pellucida**

Glycodelin-A was the first endogenous human glycoprotein that was found to potently and dose-dependently inhibit the binding of spermatozoa to the zona pellucida (Oehninger et al., 1995). This observation came from studies employing a hemizona assay (HZA) that was originally developed to predict the fertilizing potential of spermatozoa in the human, without inadvertent fertilization in vitro (Burkman et al., 1988). The HZA uses the bisected matching halves of a human zona pellucida, providing an internal control on zona to zona variability. One of the two halves can be exposed to a test substance, whereas the other serves as a control. In the case of glycodelin, the inhibition of sperm–oocyte binding was observed in HZA after a short pre-incubation time of glycodelin and the spermatozoa. This propensity of uterine glycodelin-A was glycosylation dependent, because differently glycosylated glycodelin-S had no similar activity (Morris et al., 1996). The result indicates that uterine glycodelin-A has anti-fertilizing activity (Table II). This overtly surprising finding does not contradict with the current knowledge of the sequence of events in human reproduction, because glycodelin secretion is cyclically absent from endometrium during the fertile midcycle when the spermatozoa migrate through the uterine cavity to fertilize an oocyte in the fallopian tube.

**Acrosome reaction**

Initial sperm–oocyte binding in the mammals involves recognition of glycosylated proteins of the zona pellucida by glycosylated proteins on sperm surfaces (Benoff, 1997). Once the binding of spermatozoa to the zona pellucida is engaged, acrosome reaction is induced via a G protein-mediated event (Ward and Kopf, 1993). Glycodelin-F participates in the regulation of the acrosome reaction in an interesting fashion. The isoform is secreted from luteinized ovarian granulosa cells into pre-ovulatory follicular fluid (Tse et al., 2002) and transferred with the cumulus oophorus complex into the oviduct at ovulation. Glycodelin-F binds on the acrosome region of the sperm head, thereby inhibiting progesterone induced acrosome reaction. Glycodelin-A does not have this property. Glycodelin-F has even stronger an inhibitory activity on sperm-zona binding than glycodelin-A does (Chiu et al., 2003a), and this capacity is lost upon deglycosylation (Chiu et al., 2003b).

**Role of the cumulus oophorus**

At ovulation the oocyte and its associated cumulus cell mass containing follicular fluid are released and transported to the oviduct. The cumulus cells that surround the oocyte secrete...
hormones (Shutt and Lopata, 1981), and they have been suggested to be involved in nutrition, selecting spermatozoa with normal morphology, induction of the acrosome reaction and support of oocyte nuclear maturation, fertilization and the subsequent embryo development (Carrell et al., 1993; Yanagimachi, 1994; Wongsrikeao et al., 2005). Failure of cumulus oophorus formation results in problems in fertilization. For fertilization of the oocyte, the spermatozoa have to migrate through the cumulus cell mass.

An interesting set of observations led to the discovery of the role of glycodelin-F in the fertilization process. Human follicular fluid was found to inhibit human sperm–zona pellucida binding (Yao et al., 1996), and the cumulus cells reduced this effect (Hong et al., 2003). To clarify the factors behind these observations, collaborative research between Hong Kong and Helsinki identified glycodelin as an important effector molecule in this phenomenon. As the sperm cells migrated through the cumulus oophorus matrix, both the glycodelin-A and glycodelin-F dependent inhibitory activities on gamete interaction were reduced (Figure 2). The uptake of these glycodelin isoforms by the cumulus cells was found to be unique among proteins of the lipocalin family. In addition to the uptake of glycodelin, the cumulus cells also partially deglycosylated glycodelin-F, yielding an interesting glycodelin isomer, glycodelin-C, that had opposite effects, i.e. it stimulated sperm–zona pellucida binding (Chiu et al., 2006). These observations may explain why removal of the cumulus cells before IVF does not improve the results, rather to the contrary (Magier et al., 1990). The results suggest that the biological role of glycodelin-F may be related to the prevention of premature progesterone-induced acrosome reaction before the spermatozoa have penetrated through the cumulus oophorus matrix on their way to bind to the zona pellucida. But there still are open questions. The stoichiometry between the cumulus cells and the spermatozoa that pass through this layer has not been determined, and it is not known if all the spermatozoa arriving at this site are bound to glycodelin-F in vivo. Furthermore, it is not known if all the spermatozoa that have migrated through the cumulus cell layer are free of glycodelin-F as they reach the zona pellucida, or how many of them carry the stimulatory glycodelin-C. It seems that the cumulus penetration of spermatozoa modifies them in two ways. The first is removal of the inhibitory effects of glycodelin-A and -F on sperm–oocyte binding and the acrosome reaction and the second is stimulation by glycodelin-C of the zona pellucida binding capacity of the spermatozoa (Chiu et al., 2006). Even if the cumulus cells did not remove or modify the inhibitory isoforms from all the spermatozoa, the results strongly suggest that the fertilizing spermatozoa is likely to be free of the unmodified, inhibitory isoforms. Whether the fertilizing spermatozoon requires stimulatory glycodelin-C bound on its head remains to be studied.

The immune system

Glycodelin is a rare human glycoprotein because it manifests both immunosuppressive and anti-fertilization activities, suggesting convergence of human immune and gamete recognition systems (Clark et al., 1996b).

The first identification of immunosuppressive activity came from the observations that purified glycodelin and decidual tissue extract inhibit thymidine uptake in mixed lymphocyte culture and glycodelin antibodies neutralized the antiproliferative effects of decidual tissue extract (Bolton et al., 1987; Pockley and Bolton, 1989, 1990). A receptor for glycodelin has been found on the human monocytes (Miller et al., 1998). Glycodelin also binds to pregnancy zone protein and α2-macroglobulin that may enhance its immunosuppressive activity (Skornicka et al., 2004).

T cells

Glycodelin inhibits T cell proliferation (Rachmilewitz et al., 1999), and it renders T cells less sensitive to stimulation (Rachmilewitz et al., 2001) (Table V). The inhibitory activity of glycodelin is mainly directed to the Th1 type cytokine response by selectively inhibiting the expression of a chemokine receptor (CXCR3) associated with the Th1 subtype and preventing repression of the transcriptional factor GATA-3, an event that is essential for differentiation along the Th1 lineage (Mishan-Eisenberg et al., 2004). Glycodelin appears to negatively regulate T cell activation by diminishing their responses in the contact site at the time of T cell receptor triggering (Rachmilewitz et al., 2002). This activity appears to be mediated by CD45, the tyrosine phosphatase receptor (Rachmilewitz et al., 2003). As glycodelin binds to the CD45 receptor on T cells, it may act as a
Table V. Immunosuppressive activity of glycodelin

<table>
<thead>
<tr>
<th>Type of immune cell</th>
<th>Effect by glycodelin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cells</td>
<td>Inhibits T cell proliferation</td>
<td>Rachmilewitz et al., (1999)</td>
</tr>
<tr>
<td></td>
<td>Renders T cells less sensitive to stimulation</td>
<td>Rachmilewitz et al., (2001)</td>
</tr>
<tr>
<td></td>
<td>Diminishes T cell responses in the contact site at the time of T cell receptor triggering</td>
<td>Rachmilewitz et al., (2002)</td>
</tr>
<tr>
<td></td>
<td>Inhibition mediated by CD45, the tyrosine phosphatase receptor</td>
<td>Rachmilewitz et al., (2003)</td>
</tr>
<tr>
<td>B cells</td>
<td>Inhibits B cell proliferation, IgM secretion and the surface expression of MHC class II</td>
<td>Yaniv et al., (2003)</td>
</tr>
<tr>
<td>NK cells</td>
<td>Inhibits cytotoxic activity of NK cells from peripheral blood</td>
<td>Yaniv et al., (2003)</td>
</tr>
<tr>
<td></td>
<td>Increased expression in uterine NK cells suggesting local activity</td>
<td>Okamoto et al., (1991)</td>
</tr>
</tbody>
</table>

calcium-dependent lectin to bind other T cell surface glycoproteins to mediate its immunoregulatory activities (Ish-Shalom et al., 2006). Importantly, glycodelin binding to T cells can be competitively inhibited with oligosaccharides, showing that glycodelin binds to T cell surfaces in a carbohydrate-dependent manner. Glycodelin has been suggested to act through its distinct receptors that are decorated by carbohydrates and expressed in different cells, wherein one of its surface molecular targets, CD45, mediates its T cell inhibitory activity (Yaniv et al., 2003).

**B cells**

Glycodelin also has an effect on the B cells (Table V). A human B cell inhibitory receptor, CD22, binds to the sialylated lacNac sequences (Powell and Varki, 1994). It has been suggested that the oligosaccharides bearing sialylated lacNac or lacdiNac antennae present in glycodelin-A may manifest immunosuppressive effects by specifically blocking the adhesive and activation related events mediated by CD22 (Dell et al., 1995). Preliminary evidence indicates that glycodelin inhibits B cell receptor mediated activation of human B cells (Yaniv et al., 2003). Here, glycodelin inhibits B cell proliferation and the up-regulation of IgM and major histocompatibility complex (MHC), but not CD69 and CD86, regardless of the extent of B cell receptor triggering. These findings suggest that glycodelin affects some but not all B cell responses. The B cell inhibition by glycodelin is different from the T cell inhibition in that the extent of glycodelin-mediated inhibition does not correlate to the level of B cell receptor triggering. Suggesting that glycodelin interferes with late events of B cell receptor signalling (Yaniv et al., 2003). Interestingly, CD22 binds to CD45, the leukocyte specific receptor linked to phosphotyrosine phosphatase involved in T cell activation (Stamenkovic et al., 1991). Taken together, these studies suggest that glycodelin is a soluble regulatory factor capable of interacting with both T and B cells in a carbohydrate-dependent manner to affect both cellular and humoral immune responses (Yaniv et al., 2003).

**Innate immunity**

Besides its actions on humoral and cellular immunity, glycodelin inhibits cytotoxicity of peripheral blood natural killer (NK) cells (Okamoto et al., 1991) (Table V). These cells are of specific interest because they need no prior exposure to react with foreign antigens, such as bacteria, viruses or an embryo, a semiallograft. It is believed that uterine NK cells are derived from a subset of the NK cells in peripheral blood (reviewed in Dosiou and Giudice, 2005). There is selective, increased expression of glycodelin in the NK cells isolated from pregnancy decidua (Koopman et al., 2003). However, direct evidence that glycodelin inhibits cytotoxicity of uterine NK cells is not available yet, and the role of glycosylation in this process remains to be clarified.

**Immunoprotection of spermatozoa**

The expression of glycoconjugates by normal cells may protect them from immune responses, especially in those cases in which the MHC recognition is minimal or absent (Clark et al., 1997). In spite of the frequent exposure to antigens in spermatozoa and in seminal plasma, women are rarely immunized against spermatozoa. Should it happen, the question remains whether the various glycodelin glycoforms that bind on the human spermatozoa would protect them against immune responses in the female body. The immunosuppressive properties of the Lewis^x^ epitopes present in glycodelin-S may contribute to the low immunogenicity of sperm in women (Morris et al., 1996). Recent findings (R. Tsang and W.S.B. Yeung, unpublished results) indicate that glycodelin-A treatment maintains viability of human spermatozoa in co-culture with a human lymphocyte cell line (Figure 3), and glycodelin bound spermatozoa are less likely to activate the lymphocytes. Obviously, glycosylation plays an important part here because, in the absence of glycosylation, glycodelin does not bind on the spermatozoa (Chiu et al., 2003b), and deglycosylated glycodelin does not have the same effect. But, there are other mechanisms, e.g. attenuation of immune responses against the gametes or the blastocyst may be manifested by binding of selectin- or ‘selectin-like’ receptors on the spermatozoa to the oligosaccharide ligands on immune effector cells (Clark et al., 1996b).

**Other aspects**

Oligosaccharides with at least one fucosylated lacdiNAc antenna, such as present in glycodelin-A (Figure 1), have been shown to be 15-20-fold more potent inhibitors of selectin-mediated adhesions than either sialyl- or sulpho-Lewis^x^/a type oligosaccharides (Grinnell et al., 1994). The presence of such oligosaccharides in glycodelin suggests indirectly that glycodelin may manifest some of its immunosuppressive effects by blocking selectin-dependent adhesions (Clark et al., 1996a). More recently, direct evidence has been provided that glycodelin inhibits E-selectin mediated cell adhesion (Jeschke et al., 2003).

**Apoptosis**

Glycodelin-A but not glycodelin-S induces apoptosis in T cells, but not in monocytes (Mukhopadhyay et al., 2001). This function
appears to be dependent on the presence of sialic acid residues (Mukhopadhyay et al., 2004). The immunosuppressive activity of glycodelin is related to its apoptogenic activity (Jayachandran et al., 2004). Studies employing mutagenesis of the asparagines at the N-glycosylation sites (Asn 28 and Asn 63) to glutamine show that the apoptogenic activity of glycodelin resides mainly in the protein backbone. The apoptogenic activity is present in glycodelin-A, whereas no similar activity is present in glycodelin-S and recombinant glycodelin products expressed in Pichia pastoris, or in Chinese hamster ovary cells (Karande et al., 2005). Recombinant glycodelin expressed in the SF21 insect cell line also yields an apoptotically active glycodelin, whereas the same gene expressed in the Tni insect cell line produces apoptotically inactive glycodelin (Jayachandran et al., 2006). These results suggest that, although the apoptogenic activity of glycodelin resides mainly in the protein backbone, certain glycans may modulate this activity either by masking or unmasking the functional region in the glycodelin molecule.

**Micro-organisms**

Another emerging relationship is that the same unusual carbohydrate sequences present in immunosuppressive glycodelin are also expressed on intravascular helminthic parasites (Srivatsan et al., 1992), *Helicobacter pylori* (Aspinall and Monteiro, 1996) and HIV infected T lymphocytes (Kawashinagi et al., 1994). This similarity suggests that mimicry or acquisition of the glycans used in this system by the pathogens may enable them to subvert or misdirect the host immune response to their own advantage (Clark et al., 1997).

**Clinical aspects**

**Fertilization, implantation and placentation**

In clinical practice, many ways have been tried to improve the fertilizing capacity of spermatozoa, from mere washing to specific substances and media. The classical Percoll™ treatment removes glycodelin-S from the sperm head. There is no systematic study addressing dislodging of glycodelin by the various media used for this purpose. Nevertheless, the present knowledge indicates that, outside their appropriate timing, binding of any of the three inhibitory glycodelin isoforms (-A, -F and -S) on the sperm head should have negative effects on the fertilizing capacity of spermatozoa. In respect of fertilization, it is significant that the binding of glycodelin-S is loose and reversible.

Due to ethical constraints, experimental studies on human implantation have not been feasible, and the importance of glycodelin in human implantation rests on its established immunosuppressive activities and abundance at the fetomaternal interface. In a normal fertile cycle, implantation takes place 8 days after the luteinizing hormone surge. At that time, the endometrium contains a full array of immune cells. According to global gene profiling studies, glycodelin expression is significantly increased during the window of implantation (Kao et al., 2002), and this change is translated into increased glycodelin synthesis and secretion at this site (Julkunen et al., 1986a; reviewed in Seppälä et al., 2002). Given its inhibitory effect on the peripheral blood NK cells (Okamoto et al., 1991) and the likelihood that uterine NK cells derive from peripheral blood NK cells (Dosioi and Giudice, 2005), an immunoprotective role has been suggested for glycodelin-A during implantation and placentation (Okamoto et al., 1991; Clark et al., 1996a).

Failure in reproductive performance may manifest itself at the level of implantation due to undeveloped endometrial microenvir- 
onment. This may result in failure to implant or insufficient support of early pregnancy after implantation. Relevant clinical studies on glycodelin in such conditions are listed in Table VI.

The value of histologic dating of the timed endometrial biopsy for clinical assessment of the fertility status has been questioned, because an equal incidence of morphologic delay may occur in fertile and infertile women (Coutifaris et al., 2004; Krazer, 2004). Where a delay is observed, it is usually caught up within 2 or 3 days (Damario et al., 2001). Histologically retarded endo- 

metrium shows reduced glycodelin immunostaining, indicating functional failure that may adversely affect uterine receptivity during implantation and placentation (Klentzeris et al., 1994).

Pinopodes are protrusions on the endometrial surface that have been suggested to be ultrastructural markers of endometrial recep- 
tivity (Nikas et al., 1995). Glycodelin emerges in the endometrial glands at the time when the pinopodes appear, with an inverse correlation with the progesterone receptor B. Glycodelin has been detected on the pinopodes (Stavreus-Evers et al., 2006), but its expression is not limited to these structures. Taken together, these observations clearly show the presence of immuno- 
suppressive glycodelin-A in the receptive phase endometrium where it is likely to contribute to the fetomaternal defence mech- 

anisms by interacting with the immune cells that are replete at the same site.

**Ovarian failure, unexplained infertility and recurrent spontaneous abortion**

In women with premature ovarian failure who conceived after ovum donation and embryo transfer with exogenous steroid support, the serum glycodelin levels in early pregnancy were lower than in the women with normal ovarian function. This suggests that factors under the control of the maternal ovary are involved in glycodelin production by the endometrium (Critchley
et al., 1992). Besides progesterone that was replaced, relaxin appears to be another corpus luteum hormone that stimulates glycodelin secretion (Stewart et al., 1997; Tseng et al., 1999). Subnormal secretion or absence of ovarian relaxin secretion may have contributed to the observed difference in women with premature ovarian failure.

Subnormal peri-implantation phase glycodelin levels have been reported in the uterine fluid of non-pregnant patients with unexplained infertility (Mackenna et al., 1993) and also in women with a history of recurrent spontaneous abortion (Dalton et al., 1998; Salim et al., 2006). In the latter condition, also the circulating glycodelin concentration may be reduced at the mid-luteal phase (Tulppala et al., 1995). Although the glycodelin concentration of uterine secretions correlates well with endometrial morphology during the menstrual cycle (Li et al., 1993b), particularly during the peri-implantation period (Li et al., 1993a), the question remains whether this measurement would be clinically any more useful than immunohistochemical detection of glycodelin in endometrial biopsies, or the routine histologic dating. An advantage of determining glycodelin secretion from uterine flushings instead of endometrial biopsy is that glycodelin expression varies from one endometrial site to another (Li et al., 1991). Therefore, in spite of its invasive nature, the measurement of glycodelin from uterine flushings has clinical potential, should a standardized methodology become widely applicable.

**Polycystic ovary syndrome**

This condition is frequently associated with increased secretion of luteinizing hormone, androgen and insulin, resulting in problems with ovulation, implantation and early pregnancy loss. In ovulatory cycles of the women with polycystic ovary syndrome (PCOS), glycodelin serum level increases during treatment with metformin, an insulin-reducing agent (Jakubowicz et al., 2001). During pregnancy, reduced glycodelin serum concentrations in the first trimester are associated with early pregnancy loss, suggesting failure in placentaion (Jakubowicz et al., 2004). Interestingly, insulin has no acute glycodelin reducing effect (Seppälä et al., 2005), whereas its long-term effects on glycodelin secretion remain to be studied. Furthermore, it remains to be proven if the low glycodelin serum level plays any part in the pathogenesis of PCOS-related early pregnancy loss due to reduced fetomaternal defence mechanisms at the placentaion site.

### In vitro fertilization

Given the steep rise of serum glycodelin level from implantation onwards (Julkunen et al., 1985), prediction of the outcome of IVF by serum glycodelin levels has been thoroughly explored. Rather disappointedly, in many studies, the subnormal glycodelin serum levels at the implantation phase have not predicted fertile or infertile cycles in any consistent way (reviewed in Seppälä et al., 2002). More recent reports point to potential clinical utility under specific circumstances. For instance, there is a significant correlation between low serum glycodelin levels on day 21 of the pretreatment cycle and a higher pregnancy rate following IVF/ intracytoplasmic sperm injection in normogonadotrophic women subjected to the long protocol of pituitary down-regulation and gonadotrophin stimulation (Westergaard et al., 2004). In the subsequent treatment cycle, the glycodelin serum levels were significantly higher in conception than in non-conception cycles. The authors suggest that measuring the mid-luteal serum glycodelin level in the pretreatment cycle may offer a clinical test to decide whether infertility treatment should be initiated in that cycle or not. Perhaps, the low serum glycodelin level identified a specific group of women whose infertility was related to endometrial dysfunction, and this condition was corrected during ovarian stimulation for IVF, contributing to the high success rate.

Another approach is also of interest (Liu et al., 2006). In individual IVF cycles, comparison between serum glycodelin levels taken on the day of oocyte retrieval and on the day of embryo transfer gave information on success in terms of achieving a pregnancy. Although no overall difference in serum glycodelin was found on the day of oocyte retrieval or embryo transfer between the non-pregnant and pregnant groups, both the ratio and the difference of serum glycodelin levels on the days of embryo transfer and oocyte retrieval were higher in the pregnant group than in the non-pregnant group. This approach is of interest because it addresses intra-individual rather than inter-individual differences and, therefore, is free of the problem of wide individual variation in the glycodelin levels. The results are compatible with the fetoeembryonic defence system hypothesis (Clark et al., 1996a).

Both studies suggest clinical potential of the glycodelin test in predicting a short-term IVF success under specific situations, but
in different ways. The results are not mutually controversial because the first study addressed low pretreatment levels and the second study measured the difference from oocyte retrieval to embryo transfer in the hormonally stimulated treatment cycles.

Another viewpoint in IVF comes from the observed apoptotic activity of glycodelin, i.e., whether the glycodelin isoforms taken up (and possibly internalized) by the cumulus cells can exert apoptogenic activity before fertilization. The question is of interest because apoptosis has been used to estimate ovarian reserve in women undergoing IVF (Seifer et al., 1996). Here, the gametes and embryos derived from the cumulus complexes with no or minor apoptosis were found to have an increased chance of giving rise to optimum blastocysts (Corn et al., 2005).

Contraception

Contraceptive methods employing progestogens have an effect on endometrial glycodelin secretion. This has been shown in levonorgestrel-releasing intrauterine contraceptive system (Mandelin et al., 1997), subdermal implants (Mandelin et al., 2001) and levonorgestrel only containing pills taken for emergency contraception before the LH surge (Durand et al., 2005). In view of the anti-fertilizing effects of glycodelin-A, it would seem that induction of glycodelin synthesis before fertilization could contribute to the contraceptive effect of the above methods. However, the significance of this effect remains to be determined, because evidence of the magnitude of the effect in vivo is not available and the oocyte/cumulus cell complex can remove glycodelin from the spermatozoa and modify its activity before fertilization (see Role of the cumulus oophorus).

Cancer

Endometrial cancer

When endometrial adenocarcinoma cells (Ishikawa cells) were co-cultured with normal endometrial stromal cells in the presence of progesterone, proliferation was reduced concomitantly with induced glycodelin expression (Arnold et al., 2002). At the same time, the adenocarcinoma cells underwent conversion to a normal phenotype. These findings raised a question of whether glycodelin was a cause or a consequence of the reduction of malignant characteristics. Recent evidence indicates that glycodelin may be primary to the observed change. Experiments employing transfection of glycodelin cDNA into glycodelin-negative endometrial adenocarcinoma cells brought about induction of glycodelin expression at the same time as they showed increased differentiation and reduced tumour cell growth. A significant concomitant observation was reduced expression of the Bcl-XL gene, an anti-apoptotic survival gene involved in tumour cell growth and chemoresistance (Koistinen et al., 2005). The glycosylation profile of glycodelin induced by transfection in endometrial adenocarcinoma cells has not been determined, but the results are compatible with glycodelin’s apoptogenic isoforms (see Apoptosis). Given that progesterone and progesterone antagonists stimulate glycodelin gene expression, the results may encourage a reappraisal of glycodelin synthesis-stimulating pathways in supporting chemotherapy of malignant endometrial tumours, notably those expressing the progesterone receptor. However, the results obtained with the glycodelin femtopeptide antibody are contradictory to the above results, as they show increased ‘-glycodelin’ immunoreactivity in advanced malignant tumours (Horowitz et al., 2001). Obviously the glycodelin-unrelated specificity of the polyclonal anti-peptide antibody may contribute to the different results (see Nomenclature, antibodies and immunodetection).

Ovarian serous carcinoma

Ovarian cancer consists of many subtypes, serous carcinoma being the most common of them. Clinical stage and histological grade are the gold standards in the selection of clinical management, and many clinical and prognostic markers have been explored. Studies employing anti-glycodelin antibodies show that glycodelin expression in tumour cells is more frequent in well differentiated than in poorly differentiated carcinomas, and glycodelin expression is more frequent in early stage compared with advanced stage tumours (Mandelin et al., 2003). Importantly, in grade 1/stage III patients, the 5-year overall survival of the patients with glycodelin expressing tumours is significantly higher than in those patients whose tumours did not contain glyco delin. The study shows that glycodelin expression in ovarian serous carcinoma is a favourable prognostic sign. These results are at variance with those employing the glycodelin femtopeptide antibody (Horowitz et al., 2001; Song et al., 2001), as the femtopeptide antibody shows more intense staining in advanced tumours compared with local tumours or normal tissues, and it shows strong immunostaining in tumour blood vessels that are negative with the use of the anti-glycodelin antibody (Mandelin et al., 2003). Again, these differences are likely due to different specificities of the antibodies used in these studies.

Concluding remarks

It is now firmly established that glycodelin interacts by its unique carbohydrates with the cell surface of many cell types, particularly the gametes and the immune cells. In the gametes, most biological actions of glycodelin are inhibitory, such as the inhibition of sperm capacitation (glycodelin-S), sperm–oocyte binding (glycodelin-A and -F) and the acrosome reaction (glycodelin-F). All these activities involve binding of the specific glycodelin glycoform on the sperm head. Recent studies have uncovered an important role for the cumulus oophorus cells. These cells can take up glycodelin-A and -F and modify their glycans in such a way that the resulting glycodelin-C has stimulatory effects on the sperm– zona pellucida binding. The structure and biological role of the cumulus cell-modified glycodelin is now unfolding. Evidence for the involvement of glycodelin oligosaccharides in the cell signalling processes of the cellular, humoral and innate immune responses and apoptosis is also accumulating.

Finally, clinical research points to a number of areas in which significant changes take place in glycodelin secretion, providing functional information. As the removal of specific N-glycosylation sites by mutagenesis clearly shows that glycosylation is requisite for glycodelin secretion (Jayachandran et al., 2004), glycodelin secretion may be affected by either inappropriate glycosylation machinery or by another type of clinical dysfunction. So far, inappropriate glycosylation patterns of glycodelin have been addressed in one clinical study only, i.e. in respect of the fertilization potential of spermatozoa in vitro (Koistinen et al., 2000). The paucity of
such studies is not surprising because aberrant glycosylation of
glycodelin cannot be identified by any available routine test.
Nevertheless, research on glycodelin glycosylation has led the
way to a better understanding of the biology and physiology of
reproduction, immunology and even cancer. Today, detection of
inappropriate glycoprotein glycosylation remains a challenge for
clinical research and practice alike, an glycodelin provides a well-
characterized example of this complex issue for future studies.

Abbreviations
Asn, asparagine; Gly, glycine; Glu, glutamic acid; Leu, leucine; Lys,
lysine; Phe, phenylalanine; Pro, proline; Thr, threonine; Val, valine.

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