Molecular Human Reproduction, Vol.20, No.3 pp. 185–199, 2014
Advanced Access publication on September 15, 2013 doi:10.1093/molehr/gat064

molecular human reproduction
MHR | basic science of reproductive medicine

NEW RESEARCH HORIZON ***Review***

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

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Submitted on July 10, 2013; resubmitted on August 15, 2013; accepted on September 3, 2013

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.

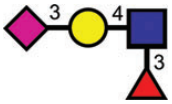
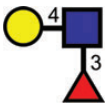
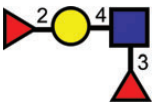
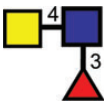
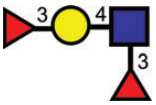
In the first Hu-FEDS hypothesis article (Clark *et al.*, 1996), a specific glycoprotein [glycodelin-A (GdA)] and mucins present in the placenta, amniotic fluid and decidua were implicated as factors that suppress the maternal immune response in the pregnant uterus. These glycoconjugates were suggested 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-

FEDS model was the concept that the carbohydrate sequences on the surface of human gametes are also employed as functional groups for immune deviation (Clark *et al.*, 1996). Factors in human seminal plasma and the pregnant uterus had previously been shown to inhibit immune responses *in vitro* (Bolton *et al.*, 1987; Kelly and Critchley, 1997). However, the concept that human gametes could present specific signals to modulate immune responses was novel.

In the second Hu-FEDS hypothesis article, this model was further expanded (Clark *et al.*, 1997). The suggestion was made that persistent pathogens and aggressive tumour cells either acquired or mimicked the same carbohydrate functional groups that were employed to modulate immune responses directed against gametes and the developing human *in utero*. HIV, schistosomes and *H. pylori* were presented as major pathogens that exploited this system of protection (Clark *et al.*, 1997).

Since 1996, many different pathways of immune modulation have been implicated in the induction of tolerance to the developing eutherian *in utero* that are independent of carbohydrate recognition. They have been extensively reviewed (Moffett and Loke, 2006; Trowsdale and Betz, 2006; Prins *et al.*, 2012; Arck and Hecher, 2013; Erlebacher, 2013). Similarly, pathogens and tumour cells employ many pathways of immune evasion that do not rely on lectin-like interactions (Cohen, 1985; Whiteside, 2009; Wilson, 2012; Matsuura, 2013). The Hu-FEDS hypothesis is concerned primarily with the roles of glycoconjugates and lectins in the induction of tolerance required to fulfill the reproductive imperative and their linkages to pathogenesis. This model has been repeatedly tested since it was initially proposed. The results of many other studies have also greatly impacted this paradigm, and this evidence will be presented in this review.

Table I Terminal carbohydrate sequences referred to in the text.

Name	Symbol	Sequence
Sialyl-Le^x		NeuAcα2-3Galβ1-4GlcNAc 3 Fucα1
3-Sulpho-Le^x		**S**-3Galβ1-4GlcNAc 3 Fucα1
Lewis^x		Galβ1-4GlcNAc 3 Fucα1
Lewis^y		Fucα1-2Galβ1-4GlcNAc 3 Fucα1
Fucosylated LacdiNAc		GalNAcβ1-4GlcNAc 3 Fucα1
Pseudo-Lewis^y		Fucα1-3Galβ1-4GlcNAc 3 Fucα1

◆, *N*-acetylneuraminic acid; ●, galactose; ■, *N*-acetylglucosamine; ▶, fucose; ●, sulphate (S); ■, *N*-acetylgalactosamine.

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 I) (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 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 I). 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).

Immune recognition of human sperm

Testicular germ cells differentiate into sperm following the initiation of puberty, long after the period of thymic education (Fijak and Meinhardt, 2006). Sperm-specific proteins that are not tolerized during early development are autoantigens (neoantigens). The induction of autoimmune orchitis following the injection of autologous testicular homogenates distal to the testes confirms that such antigens are foreign (Tung *et al.*, 1981). However, the testis is an immune privileged site due to its ability to tolerate allografts and xenografts (Head and Billingham,

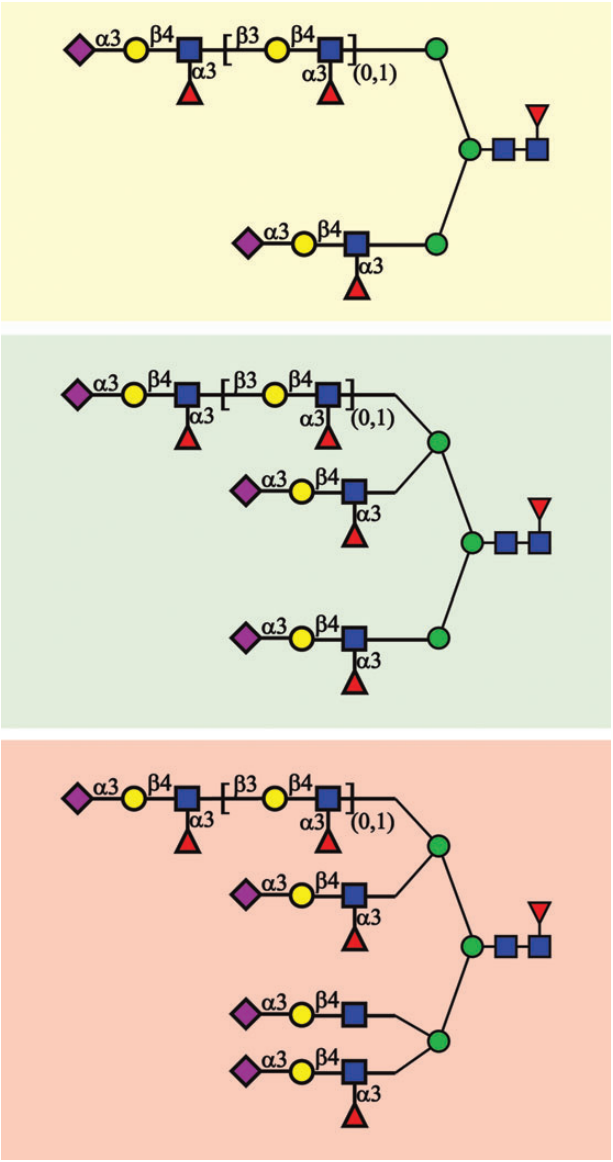


Figure 1 Major human ZP *N*-glycans. sLe^x sequences are in dashed boxes. This figure is adapted from a previous study (Pang *et al.*, 2011).

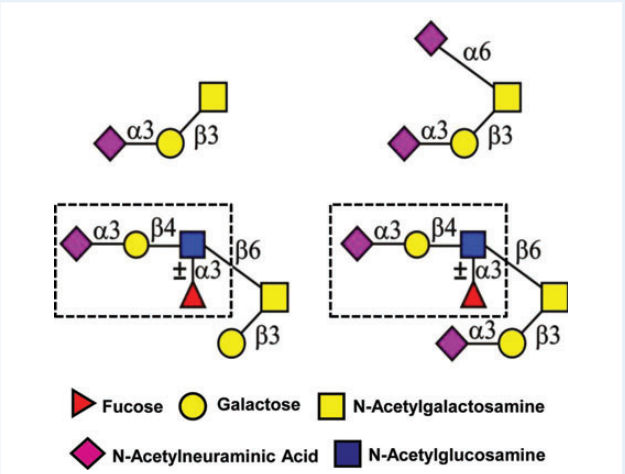


Figure 2 Human ZP-associated *O*-glycans. sLe^x sequences are in the dashed boxes. This figure is adapted from a previous study (Pang *et al.*, 2011).

1985). 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 ~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.*, 1995; Yoshimura *et al.*, 1996).

Glycomic analysis of human sperm has now confirmed the substantial expression of biantennary bisecting type *N*-glycans on these cells (Pang *et al.*, 2007). These *N*-glycans bind to erythroagglutinating phytohemagglutinin (E-PHA) (Cummings and Kornfeld, 1982; Yamashita *et al.*, 1983). The binding of E-PHA to the plasma membrane of human sperm indicates that these *N*-glycans are localized to the surface of these gametes (Lee and Damjanov, 1985; Cross and Overstreet, 1987). Sequencing of human sperm oligosaccharides also revealed the profligate expression of Lewis antigens (Le^x, Le^y), many in tri- and tetravalent presentations on a single *N*-glycan (Table I, Fig. 4) (Pang *et al.*, 2007). Most human sperm are immunostained with anti-Le^y monoclonal antibody (mAb), and there is uniform binding of this mAb to the inner acrosomal membrane and acrosomal contents (Pang *et al.*, 2007). Le^x and Le^y 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^x and Le^y 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^x and Le^y (Hanisch *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).

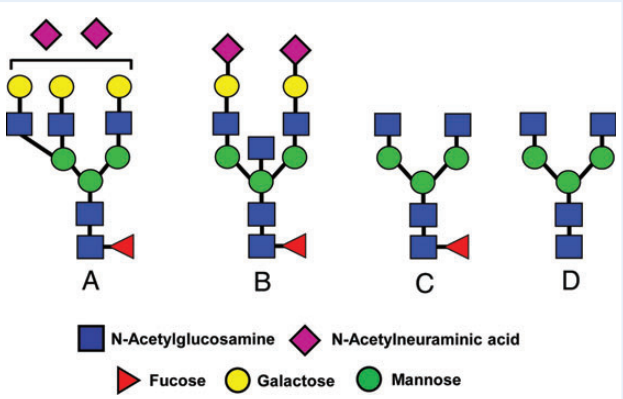


Figure 3 Restricted heterogeneity of N-glycans associated with classical class I molecules (HLA-A, -B, -C). These structures are referred to as triantennary N-glycan (**A**), biantennary bisecting type N-glycans (**B**) or truncated N-glycans (**A** and **B**). In **A**, the exact positions of the N-acetylneuraminic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

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 and Sd^a 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^x and Le^y, the fucosylated lacdiNAc sequence is also a carbohydrate

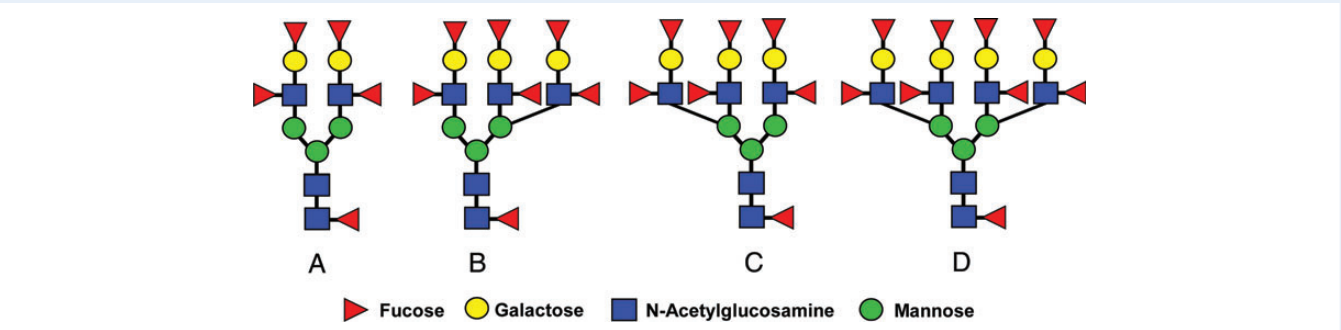


Figure 4 The expression of *N*-glycans with multivalent Le^y on human sperm and seminal plasma glycoproteins. Biantennary, (**A**), triantennary (**B** and **C**) and tetraantennary *N*-glycans (**D**) terminated on each antenna with Le^y (Table I) are expressed on human sperm and seminal plasma glycoproteins (Pang *et al.*, 2007, 2009). Heterogeneous intermediates terminated with different combinations of Le^x, Le^y or lacNAc (Galβ1-4 GlcNAc) on their antenna are also present.

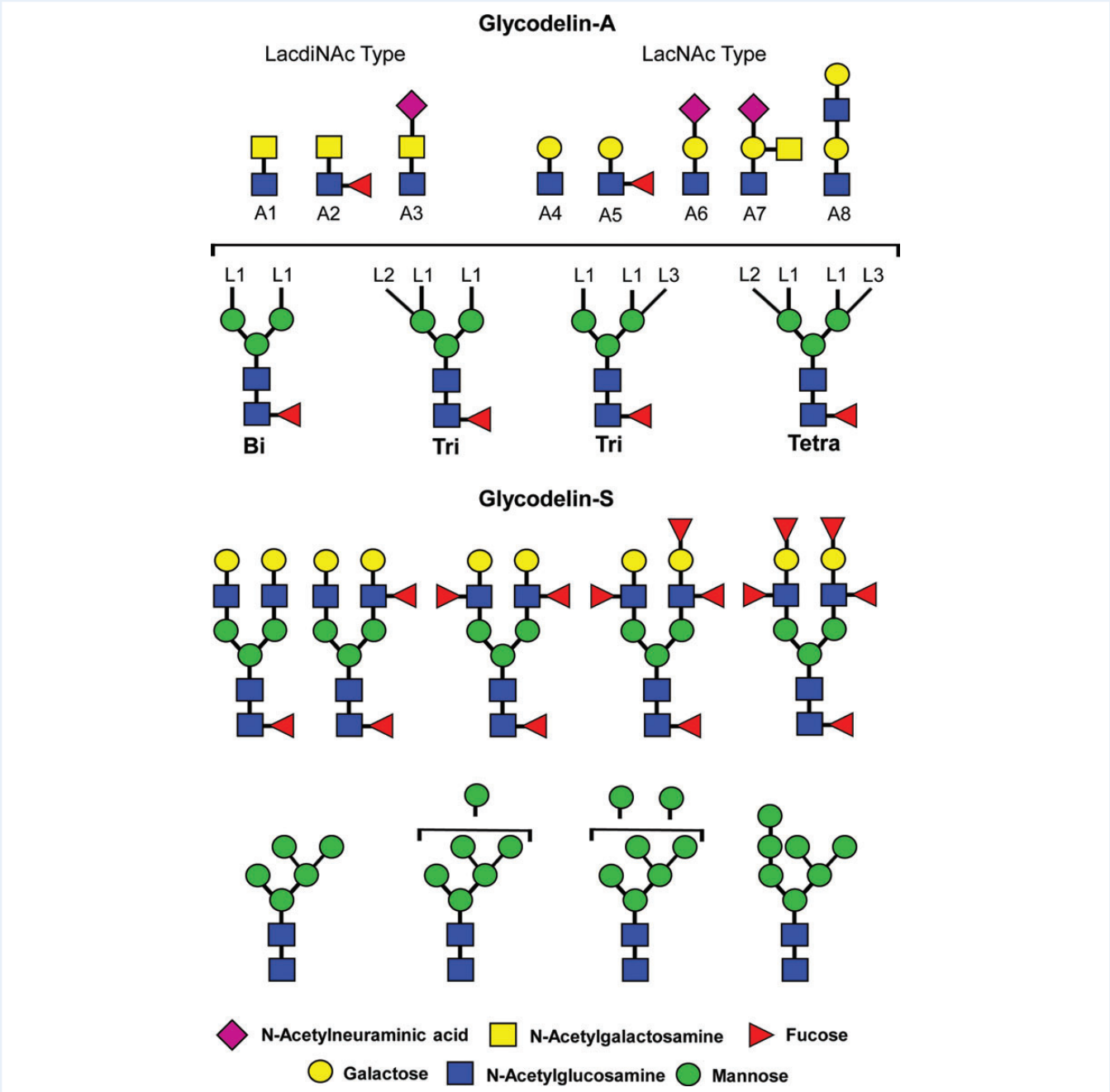


Figure 5 Glycans associated with GdA and GdS. A total of 44 different N-glycans were previously identified in GdA (Lee *et al.*, 2009). This glycoprotein is decorated primarily with complex type *N*-glycans and only very marginal amounts of high mannose/hybrid type *N*-glycans. Antenna are attached to 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 mannose and 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 mannose type *N*-glycans, Lewis^x and Lewis^y. This CLR displays preferential binding to seminal plasma glycoproteins bearing triantennary and tetraantennary *N*-glycans terminated with multivalent Lewis^x and Lewis^y sequences (Clark *et al.*, 2012).

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

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, $CD16^{pos}CD56^{dim}$ cytotoxic cells that constitute 90% of the total and $CD16^{dim/neg}CD56^{bright}$ cells lacking cytotoxic activity that make up the remaining 10% (Nagler *et al.*, 1989). uNK cells are also $CD16^{dim/neg}CD56^{bright}$ 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 $CD16^{dim/neg}$ 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 sLe^x 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). No other changes were detected in uromodulin compared with THP, confirming that differential *O*-glycosylation was responsible for the greatly enhanced immune-deviating activity of uromodulin.

The precise functional role of uromodulin during pregnancy remains to be determined. Nonetheless, these results support the concept that uromodulin *O*-glycans act as functional groups to enable its immune-deviating activities during pregnancy.

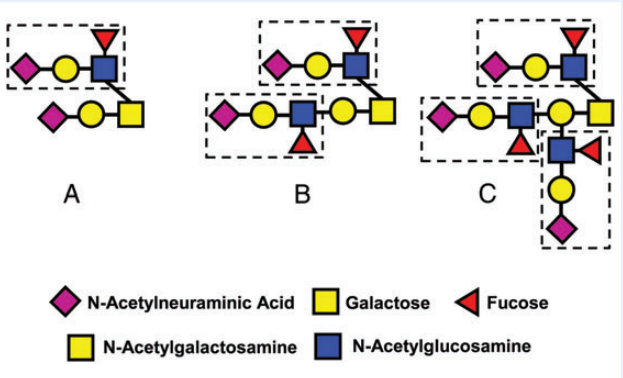


Figure 6 *O*-Glycans associated with uromodulin. sLe^x is in each dashed box. *O*-Glycans bearing one (**A**), two (**B**) or three of these sequences are present in uromodulin, but not in THP.

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 (lacNAc) (Barondes *et al.*, 1994). Galectins often possess domains that accommodate modifications of lacNAc with other monosaccharides (Cummings and Liu, 2009). Multivalent presentations of lacNAc 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 (Cummings and Liu, 2009; Than *et al.*, 2012).

Gal-1 is the best studied galectin among those up-regulated in the human placenta during pregnancy (Blidner and Rabinovich, 2013). Serum levels of Gal-1 increase during the first trimester, peak during the second trimester and remain elevated until parturition in pregnant women (Tirado-Gonzalez *et al.*, 2013). Gal-1 promotes the apoptosis of alloreactive T cells as well as the development of tolerogenic DCs and T regulatory cells (Blois *et al.*, 2007; Kopcow *et al.*, 2008). This galectin also induces the expression of HLA-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 would 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. Le^y is normally expressed on a low percentage (5–8%) of CD4+ and CD8+ T lymphocytes in the circulation (Table 1). However, after HIV infection, the proportion of Le^y 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 Le^y 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, Le^y 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 Le^x and Le^y, but also to high mannose 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 SIV_{agm} and SIV_{sm}, 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_{sm} 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_{agm} and SIV_{sm} 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_{sm} 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_{sm} 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_{sm} 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_{sm} is induced in its natural host.

In contrast, heterologous infection of macaques with SIV_{sm} or SIV_{agm} results in the development of simian AIDS (Hirsch *et al.*, 1995; Villinger

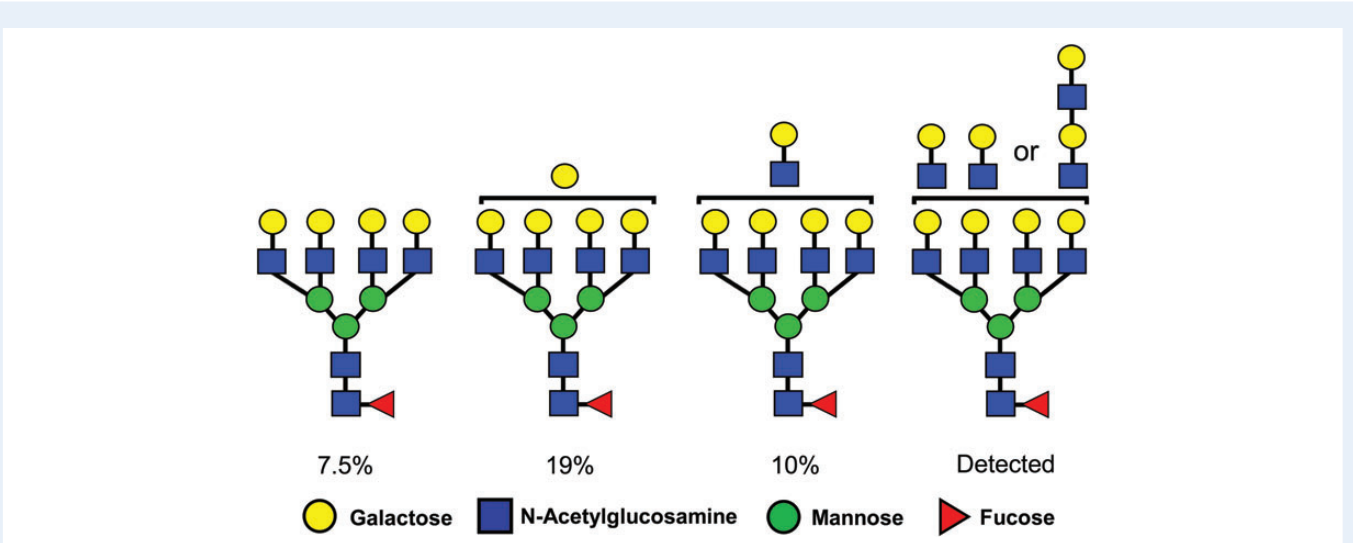


Figure 7 Galectin ligands are abundantly expressed on the major viral capsid glycoprotein isolated from SIV_{sm} produced by infected human H9 lymphoblastoid cells. *N*-Glycans were isolated from the major viral coat glycoprotein (gp130) of SIV_{sm} propagated in human H9 lymphoblastoid cells (Holschbach *et al.*, 1990). Complex type *N*-Glycans were digested with neuraminidase to remove sialic acid in $\alpha 2-3$ and $\alpha 2-6$ 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_{agm} and SIV_{sm} do not acquire the appropriate carbohydrate functional groups necessary to evoke tolerance in macaques (Clark and Patankar, 1997). Comparative glycomic analysis of gp130 isolated from SIV_{sm} propagated in rhesus monkeys and SMs must be performed to determine if they are differentially glycosylated. Heterologous infection with chimpanzee SIV (SIV_{cpz}) 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^x and Le^y 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^y expression had also been detected on CD4+ and CD8+ T cells following HIV infection (Adachi et al., 1988; Kashiwagi et al., 1994).

DC-SIGN binds to both Le^x and Le^y (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^x and Le^y actively dissociate the KSR1-CNK-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^y.

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 lacdiNAc and Le^x on their tegumental surfaces (Ko et al., 1990; Srivatsan et al., 1992). The fucosylated lacdiNAc 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 Liempt et al., 2006). The glycolipids associated with the cercarial forms of *Schistosoma mansoni* are primarily terminated with Le^x and a close structural analogue of Le^y known as pseudo-Le^y, another DC-SIGN ligand (Table I) (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 Ouagari 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^x (Fukushima et al., 1984). This study led to the designation of sLe^x as a tumour-associated carbohydrate antigen. Glycomic studies have confirmed that sLe^x-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^x 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^x 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^x (Johnson, 1999). Studies with uromodulin indicate that glycoproteins bearing multivalent sLe^x 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^x 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^y, but this mAb also cross-reacted with another blood group determinant (H2 antigen). A mAb with a strict specificity for Le^y was bound to many different types of organ-specific tumour cells, but not to normal tissues. These findings confirmed that Le^y is also a tumour-associated cancer antigen that is neoexpressed on about 70% of all tumours of epithelial origin (Hellstrom

et al., 1990). The expression of ligands for DC-SIGN like Le^y could evoke immune deviations that protect tumour cells from adaptive immune responses.

Le^y 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 sLe^x and $Lewis^x$/$Lewis^y$ 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, 1997). 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 impervious 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 Chionh, 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.

Acknowledgements

The author thanks Drs Anne Dell and Danny Schust for reviewing this manuscript and making useful suggestions. The author thanks Lynn Stevenson for her editorial assistance in preparing the manuscript.

Funding

Studies outlined by the author have been supported by the Life Sciences Mission Enhancement Reproductive Biology Program funded by the State of Missouri and a Research Board Grant (CB000500) supported by the University of Missouri System. Funding has also been obtained from the Breeden-Adams Foundation to investigate potential linkage to tumour evasion. The author has been supported in the past by grants from the Jeffress Memorial Trust of Virginia, the American Cancer Society, and the NIH.

Conflict of interest

None declared.

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