Breastfeeding is known to have many health benefits for a newborn. Not only does human milk provide an excellent source of nutrition, it also contains components that protect against infection from a wide range of pathogens. Some of the protective properties of human milk can be attributed to the immunoglobulins. Yet, there is another level of defense provided by the “sweet” protective agents that human milk contains, including free oligosaccharides, glycoproteins and glycolipids. Sugar epitopes in human milk are similar to the glycans that serve as pathogen adhesion sites in the human gastrointestinal tract and other epithelial cell surfaces; hence, the milk glycans can competitively bind to and remove the disease-causing microorganisms before they cause infection. The protective value of free oligosaccharides in human milk has been well researched and documented. Human milk glycoconjugates have received less attention but appear to play an equally important role. Here, we bring together the breadth of research that has focused on the protective mechanisms of human milk glycoconjugates, with a particular focus on the glycan moieties that may play a role in disease prevention. In addition, human milk glycoconjugates are compared with bovine milk glycoconjugates in terms of their health benefits for the human infant.

Keywords: glycoconjugate / glycolipid / glycoprotein / human milk / pathogen

Introduction

The immune system of a newborn is immature, leaving the infant susceptible to a vast range of infections. A mother’s milk provides a line of defense, helping to combat pathogenic organisms whilst nourishing the child with essential nutrients. The protective properties of human milk were once attributed entirely to the immunoglobulins (Ogra and Ogra 1978; Goldman et al. 1982), which are present at very high concentrations in colostrum then decrease as milk, and the immune system of the infant matures. It has since become apparent that other milk components also have antipathogenic properties (Holmgren et al. 1983; Cravioto et al. 1991; Newburg 1996). Many antipathogenic components contain glycan structures, sugars that exist either as free oligosaccharides (human milk oligosaccharides, HMOs) or bound to macromolecules in the form of glycoconjugates (glycoproteins and glycolipids).

The glycan structures in milk resist digestion and generally remain intact during transit through the digestive tract (Chaturvedi et al. 2001; Newburg et al. 2005). They are synthesized by enzymes (glycosyltransferases) that also form the glycans adorning molecules on epithelial surfaces throughout the body, which provide recognition and attachment sites for harmful disease causing pathogens. As a consequence, human milk glycans are able to mimic the pathogen recognition sites, bind to and block the pathogen from adhering to cell surfaces, thereby preventing infection (Figure 1). This is a superb natural and sustainable form of disease protection, sometimes referred to as “immune exclusion” (Lamm 1997; Royle et al. 2003). As the mechanism does not rely on microbiocidal activity, development of resistant strains is less likely to occur.

The majority of the functional analysis of glycans in human milk has concentrated on the ability of HMOs to inhibit gut pathogens (comprehensively reviewed in Bode 2012). However, evidence is also accumulating for the value of glycoconjugates for disease prevention. Some glycoconjugates such as secretory immunoglobulin A (sIgA) and lactoferrin have bactericidal properties that can be mostly attributed to their protein backbone. However, as a second line of defense, the attached glycans of these and other human milk glycoconjugates can act as binding epitopes to remove pathogens in a similar manner as soluble oligosaccharides. In addition, glycoconjugates typically exist as numerous glycoforms with variations in the incidence, composition and structures of their glycans, resulting in a diversity of binding sites and a greater flexibility for pathogen adhesion. The multivalent presentation of the sometimes long and extensively branched oligosaccharide chains adorning the macromolecules could also provide a superior mechanism for pathogen capture as the comparatively simple and small structures typical of HMOs (Bernardi et al. 2013).

This review presents the emerging body of research that focuses on glycoconjugates in human milk as protective agents against infection, particularly of the gastrointestinal tract. Evidence for protection against some of the leading causes of
Glycoproteins from human milk with known antipathogenic effects that can be at least partly attributed to their glycan moieties are slgA, κ-casein, lactoferrin and proteins from the MFGM [lactadherin, mucins and bile salt-stimulated lipase (BSSL)]. This aspect of research on each of these glycoproteins is described below, and a summary is provided in Table I.

**Secretory IgA**

The glycoprotein slgA is the dominant antibody fraction in human milk. It can also be found in the mucosal lining of the gastrointestinal and respiratory tract, as well as in tears and saliva, and plays a major role in the protection of many vulnerable epithelial surfaces in the human body via Fab-mediated neutralization of bacteria, viruses and toxins (Norderhaug et al. 1999). Additional to its immunological properties, slgA from human milk also presents a secondary form of antipathogenic activity for the breastfed infant by means of the glycan moieties that adorn the slgA protein backbone. There are two known sites of N-linked glycosylation on slgA at Asn-263 and Asn-459, which carry a large proportion of complex glycans with terminal N-acetylenuraminic acid (sialic acid; Neu5Ac), N-acetylglucosamine (GlcNAc) and mannose (Man) residues that are known to form potential-binding epitopes for pathogens (Arnold et al. 2007). The slgA1 subtype is also O-glycosylated in the hinge region at Thr-225, Thr-228, Ser-230, Ser-232 and Thr-236, predominantly by GalNAc-Gal disaccharides (Takahashi et al. 2010; Zauner et al. 2013). In addition, slgA dimers are complexed to a highly glycosylated secretory component that has seven N-linked glycosylation sites (Wold et al. 1990).

Numerous in vitro studies have revealed the ability of the diverse array of slgA glycans to bind to pathogens that can threaten the health of the newborn. For example, slgA from human colostrum bound to mannose-specific lectins of the most common adherence organelle of *Escherichia coli*, namely the Type I fimbria, and thus prevented *E. coli* adhesion to IEC-29 (human colonic epithelial carcinoma) cells; furthermore, the abundance of the high mannose oligosaccharide Manα6- (Manα6-3) Manβ6- (Manβ6-3) Manβ6-1→4 GlcNAcβ1-4 GlcNAc in various slgA preparations positively correlated with binding inhibition (Wold et al. 1990). slgA from colostrum and mature milk also inhibited the localized adherence of the diarrhea-causing enteropathogenic *E. coli* (EPEC) to HEP-2 (human epithelial laryngeal carcinoma) cells. This mechanism was attributed to the binding of slgA fucosylated glycans to a plasmid encoded outer membrane bacterial protein called the EPEC adherence factor (Cravito et al. 1991). Conversely, S-fimbriated *E. coli*, a cause of infantile sepsis and meningitis, bound to sialic acid residues (Neu5Ac α2-3 Gal) on slgA from human colostrum, thereby reducing adhesion to buccal epithelial cells (Schroten et al. 1998). Human colostrum slgA also inhibited the binding of the stomach pathogen *Helicobacter pylori* to gastrointestinal mucosa cells isolated from human gastric epithelial tissue. In this case, the inhibitory action of slgA was based on the competitive binding of the bacteria to a fucose-containing glycan epitope on the immunoglobulin; enzymatic removal of terminal fucose residues reduced the inhibition of binding by slgA (Falk et al. 1993). Toxin A from *Clostridium difficile*, a serious cause of antibiotic-associated diarrhea, was...
<table>
<thead>
<tr>
<th>Glycoprotein</th>
<th>Target</th>
<th>Experimental evidence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>sIgA High mannose glycans</td>
<td>Type 1 fimbriated E. coli</td>
<td>Inhibited binding to HT-29 (human epithelial colorectal adenocarcinoma) cells</td>
<td>Wold et al. (1990)</td>
</tr>
<tr>
<td>sIgA Fucose-containing glycans</td>
<td>Enteropathogenic E. coli (membrane bound EPEC adherence factor)</td>
<td>Inhibited binding to HEp-2 (human epithelial laryngeal carcinoma) cells</td>
<td>Cravioto et al. (1991)</td>
</tr>
<tr>
<td>sIgA Fucose-containing glycans</td>
<td>Helicobacter pylori</td>
<td>Inhibited binding to mucosa cells from human gastric epithelial tissue</td>
<td>Falk et al. (1993)</td>
</tr>
<tr>
<td>sIgA Sialic acid-containing glycans</td>
<td>S-fimbriated E. coli</td>
<td>Inhibited binding to human buccal epithelial cells</td>
<td>Schroten et al. (1998)</td>
</tr>
<tr>
<td>sIgA Glycans (undefined)</td>
<td>Toxin A of C. difficile</td>
<td>Inhibited binding to hamster intestinal brush border membranes</td>
<td>Dallas and Rolfe (1998)</td>
</tr>
<tr>
<td>sIgA Heat labile-toxin of E. coli</td>
<td></td>
<td>Infected children fed human milk containing high levels of sIgA remained asymptomatic</td>
<td>Cruz et al. (1988)</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>Enterotoxigenic E. coli (ETEC)</td>
<td>Inhibited ETEC induced hemagglutination of human erythrocytes in vitro</td>
<td>Giugliano et al. (1995)</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>Enteropathogenic E. coli (EPEC)</td>
<td>Inhibited binding to HeLa (human epithelial cervical adenocarcinoma) cells</td>
<td>de Araújo and Giugliano (2001)</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>Shigella flexneri</td>
<td>Inhibited binding to HeLa (human epithelial cervical adenocarcinoma) cells</td>
<td>Gomez et al. (2003)</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>Salmonella typhimurium</td>
<td>Inhibited binding to HeLa (human epithelial cervical adenocarcinoma) cells</td>
<td>Bessler et al. (2006)</td>
</tr>
<tr>
<td>Lactoferrin N-Glycans</td>
<td>Listeria monocytogenes, Salmonella enterica</td>
<td>Inhibited adhesion to human Caco-2 (human epithelial colorectal adenocarcinoma) cells</td>
<td>Barboza et al. (2012)</td>
</tr>
<tr>
<td>Lactoferrin Terminal fucose</td>
<td>Salmonella enterica (Typhimurium)</td>
<td>Inhibited adhesion to human Caco-2 (human epithelial colorectal adenocarcinoma) cells</td>
<td>Barboza et al. (2012)</td>
</tr>
<tr>
<td>Lactoferrin Terminal fucose</td>
<td>Salmonella enterica (Heidelberg)</td>
<td>Inhibited adhesion to human Caco-2 (human epithelial colorectal adenocarcinoma) cells</td>
<td>Barboza et al. (2012)</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>Cytomegalovirus, HIV-1</td>
<td>Blocked human cytomegalovirus invasion of human fetal lung fibroblasts and inhibited HIV-1 induced cytopathic effect on human MT4 T cells</td>
<td>Harmsen et al. (1995)</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>Rotavirus</td>
<td>Inhibited rotavirus hemagglutination and binding in human erythrocytes and HT-29 (human epithelial colorectal adenocarcinoma) cells</td>
<td>Superti et al. (1997)</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>RSV and cytomegalovirus (CMV)</td>
<td>Inhibited RSV invasion of HEp-2 cells and CMV invasion of human embryonic lung cells</td>
<td>Portelli et al. (1998)</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>Poliovirus</td>
<td>Inhibited infection of Vero (African green monkey kidney) cells</td>
<td>Marchetti et al. (1999)</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>HIV-1</td>
<td>Inhibited virus-cell fusion and entry into human MT4 T cells</td>
<td>Swart et al. (1996)</td>
</tr>
<tr>
<td>Lactoferrin and lactoferricin</td>
<td>Herpes simplex virus 1 and 2 (HSV-1 and HSV-2)</td>
<td>Inhibited cell-to-cell spread of HSV-1 and HSV-2 in Vero (African green monkey kidney) cells</td>
<td>Jenssen et al. (2008)</td>
</tr>
</tbody>
</table>
similarly inhibited from binding to hamster intestinal brush border membranes by slgA, with deglycosylation of slgA decreasing its binding to the toxin (Dallas and Rolfe 1998).

The above in vitro studies demonstrate a diversity in the glycan epitopes that are used by different pathogens to bind to epithelial cell surfaces, a phenomenon that is impressively countered by an equally diverse range of glycan decoys on the slgA glycoprotein. Examining the protective value of human milk slgA in vivo is far more complex. Nevertheless, a case study by Cruz et al. (1988) provides one example in which the value of milk slgA antibodies in protecting children from the effects of the heat-labile toxin from *E. coli* was shown. Children infected with the toxin-producing *E. coli* remained asymptomatic when fed breast milk containing high levels of slgA, whereas infants receiving breast milk with significantly lower levels of slgA developed gastroenteritis.

**Lactoferrin**

Human milk lactoferrin is an 80 kDa glycoprotein containing highly sialylated and fucosylated glycans (Wakabayashi et al. 2006; Picariello et al. 2008) predominantly on two major glycosylation sites (Asn-138 and Asn-479), with limited glycosylation also appearing on Asn-624 (van Berkel et al. 1996). Lactoferrin

<table>
<thead>
<tr>
<th>Glycoprotein (binding epitope if known)</th>
<th>Target</th>
<th>Experimental evidence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>κ-casein GlcNAcβ1-3Gal-</td>
<td><em>Streptococcus pneumoniae, Haemophilus influenzae</em></td>
<td>Inhibited binding to human respiratory tract epithelial cells</td>
<td>Aniansson et al. (1990)</td>
</tr>
<tr>
<td>κ-casein Sialic acid-containing glycans</td>
<td><em>Streptococcus mutans</em> GS-5</td>
<td>Inhibited binding to saliva-coated hydroxyapatite</td>
<td>Vacca-Smith et al. (1994)</td>
</tr>
<tr>
<td>Mucin Sialic acid motif (NeuAcα2-3)</td>
<td><em>S</em>-fimbriated <em>E. coli</em></td>
<td>Inhibited binding to buccal epithelial cells</td>
<td>Schroten et al. (1992, 1993)</td>
</tr>
<tr>
<td>MUC 1 and MUC 4</td>
<td><em>Salmonella enterica</em> (Typhimurium)</td>
<td>Inhibited invasion of Caco-2 (human epithelial colorectal adenocarcinoma) cells and human fetal small intestine cells (FHS 74 Int)</td>
<td>Liu et al. (2012)</td>
</tr>
<tr>
<td>Mucin Sialic acid-containing glycans</td>
<td>Rotavirus</td>
<td>Inhibited infection of MA-104 (African green monkey kidney) cells, virus replication in tissue and gastroenteritis in mice</td>
<td>Yolken et al. (1992)</td>
</tr>
<tr>
<td>Mucin Secretor and Lewis epitopes (fucosyl side chains)</td>
<td><em>Norovirus</em></td>
<td>Blocked the adhesion of recombinant norovirus-like particles to saliva</td>
<td>Jiang et al. (2004)</td>
</tr>
<tr>
<td>MUC1 Lewis X Galβ1-4(Fucα1-3)GlcNAc</td>
<td>HIV (gp120 envelope protein)</td>
<td>Blocked interaction with dendritic cells, and transmission from dendritic cells to CD4+ T cells</td>
<td>Saeland et al. (2009)</td>
</tr>
<tr>
<td>BSSL Glycan involvement unknown</td>
<td><em>Giardia lamblia</em></td>
<td>Induced swelling and lysis of trophozoites</td>
<td>Gillin et al. (1983)</td>
</tr>
<tr>
<td>BSSL α1-2-linked fucose</td>
<td><em>Norovirus</em></td>
<td>Inhibited the attachment of recombinant norovirus-like particles to saliva</td>
<td>Ruoën-Clouet et al. (2006)</td>
</tr>
<tr>
<td>BSSL Lewis X Galβ1-4(Fucα1-3)GlcNAc</td>
<td>HIV-1</td>
<td>Bound to DC-SIGN on dendritic cells. Prevents transfer of HIV-1 transfer to CD4+ T cells</td>
<td>Naarding et al. (2005, 2006)</td>
</tr>
<tr>
<td>BSSL</td>
<td><em>Streptococcus mutans</em></td>
<td>Inhibited binding to saliva- and gp340 (salivary agglutinin)-coated hydroxyapatite</td>
<td>Danielsson Niemi et al. (2009)</td>
</tr>
<tr>
<td>Lactadherin Sialic acid-containing glycans</td>
<td>Rotavirus</td>
<td>Inhibited infection of MA-104 (African green monkey kidney) cells, virus replication in tissue and gastroenteritis in mice</td>
<td>Yolken et al. (1992)</td>
</tr>
<tr>
<td>Lactadherin</td>
<td>Rotavirus</td>
<td>Decreased incidence of severe diarrhea in infected infants fed with human milk containing high levels of lactadherin</td>
<td>Newburg et al. (1998)</td>
</tr>
<tr>
<td>Lactadherin</td>
<td>Rotavirus</td>
<td>Preincubation prevented rotavirus infection of Caco-2 (human epithelial colorectal adenocarcinoma) cells</td>
<td>Kvistgaard et al. (2004)</td>
</tr>
</tbody>
</table>
is also found in human tears and saliva, as well as most other exocrine secretions (Berluti et al. 2011) that continually “wash” cells to keep them lubricated and free of infection.

Lactoferrin has bactericidal activity that can be partly attributed to the ability of the glycoprotein to bind to iron, which limits the availability of this essential metal for microbial growth (Brock 1980; Baker and Baker 2005). A peptide derived from the N-terminal domain of the protein, known as lactoferricin, can also bind and release lipopolysaccharides (LPSs) from the outer membranes of Gram-negative bacteria, causing osmotic damage and cell death. The basis of these bactericidal (killing) mechanisms has been primarily attributed to the protein backbone; however, the attached glycans may play an important, though indirect role by increasing the stability of the molecule against proteolysis and by other, as yet unclear, molecular interactions. For example, sialic residues on bovine lactoferrin have been shown to effectively bind to $\text{Ca}^{2+}$ ions that otherwise appear to stabilize LPS on the bacterial outer membrane (Rossi et al. 2002); it seems quite feasible that the sialic acid moieties of human lactoferrin could function in a similar manner.

In addition to the above antibacterial activities of lactoferrin, the glycoprotein also appears to play an important role in preventing bacterial adhesion to receptor sites in the host. Lactoferrin binds to various microbial pathogens including $E.\ coli$, $Salmonella\ typhimurium$ (Naidu et al. 1993), $Staphylococcus\ aureus$ (Naidu et al. 1992), $Neisseria\ meningitidis$ (Schryvers and Morris 1988) and $Haemophilus\ influenzae$ (Schryvers 1989). Consequently, lactoferrin could act as a decoy, deflecting these pathogens away from the receptor sites on host cell surfaces. In support of this view, human milk lactoferrin inhibited the binding of $S.\ typhimurium$ (Bessler et al. 2006), Shigella flexneri (Gomez et al. 2003) and enteropathogenic $E.\ coli$ to HeLa (human epithelial cervical adenocarcinoma) cells (de Araújo and Giugliano 2001) and inhibited enterotoxigenic $E.\ coli$-induced hemagglutination of human erythrocytes in vitro (Giugliano et al. 1995).

Recently, a more thorough investigation was conducted to determine the role that the glycan moieties of human milk lactoferrin may play in inhibiting pathogen adhesion (Barboza et al. 2012). Human milk lactoferrin and glycoforms, in which terminal monosaccharides were removed, were assessed for their ability to inhibit adherence and invasion of the infant enteropathogens $Listeria\ monocytogenes$, $E.\ coli$ O157:H7 and $Salmonella\ enterica$ (serotypes Typhimurium, Enteritidis and Heidelberg) to Caco-2 (human epithelial colorectal adenocarcinoma) cells. In addition, $N$-glycans were released from lactoferrin and the impact of these oligosaccharides on pathogen adhesion assessed. The lactoferrin $N$-glycans significantly reduced adherence of $Listeria$ and all $Salmonella$ strains, but the inhibition mechanism of $E.\ coli$ was attributed entirely to the protein backbone. Enzymatic removal of terminal fucose residues of lactoferrin resulted in a significant increase in $S.\ enterica$ Typhimurium adhesion, implicating the involvement of the fucose residue in the binding inhibition by milk lactoferrin for this particular bacterial strain; however, the same phenomenon was not observed for Enteritidis and Heidelberg strains. Conversely, the removal of terminal galactose residues increased adhesion of Heidelberg only.

The work by Barboza et al. (2012) also revealed that the glycosylation of lactoferrin changes dramatically throughout the lactation period. Lactoferrin from colostrum exhibited a high level of glycosylation, thereafter overall glycosylation decreased over the first 2 weeks of lactation, corresponding to the transition from colostrum to mature milk. However, an increase in fucosylation occurred throughout the remaining lactation period corresponding to increased expression of fucosyltransferase genes, determined by RNA sequencing. Changes in glycosylation of human milk glycoproteins throughout the lactation period has been reported previously (Landberg et al. 2000; Wilson et al. 2008; Froehlich et al. 2010), yet the corresponding effect on anti-pathogenic activity remains an area inviting further investigation.

Lactoferrin from human milk also has antiviral properties, although the role that glycosylation plays in these reactions has not been investigated. Human milk lactoferrin inhibited the growth of respiratory syncytial virus (RSV) and cytomegalovirus in HEp-2 (human epithelial laryngeal carcinoma) cells and human embryo lung cells, respectively (Portelli et al. 1998). Cell infection by poliovirus (Marchetti et al. 1999), HIV-1 (Swart et al. 1996), hepatitis C virus (Yi et al. 1997) and rotavirus (Superti et al. 1997) was also inhibited in vitro by human milk lactoferrin. Cell-to-cell spread of the herpes simplex virus 1 and 2 was inhibited by lactoferrin and its peptide derivative lactoferricin in Vero (African green monkey kidney) cells (Jenssen et al. 2008). Lactoferrin from human milk and colostrum could also completely block human cytomegalovirus invading human fetal lung fibroblasts and inhibit HIV-1-induced cytopathic effect on human MT4 T cells (Harmsen et al. 1995).

$\kappa$-casein

Although $\beta$-casein is the most abundant form of casein in human milk (representing $\sim$75% of total casein), it contains no known glycosylation sites. In comparison, $\kappa$-casein (representing $\sim$25% of total human milk casein; Räähä 1985) has seven O-glycosylation sites in the C-terminal portion of the molecule (Fiat et al. 1980). Together $\kappa$-casein and $\beta$-casein form micelles containing essential amino acids and minerals for the growing child. However, the glycosylated $\kappa$-casein appears to be the main contributor to antimicrobial activity. Once inside the gut, $\kappa$-casein is cleaved by proteases to form the insoluble peptide para-$\kappa$-casein, and the soluble hydrophilic glycopeptide caseinomacropetide (CMP; also known as glycomacropetide or GMP; Ward et al. 1997).

The protective value of $\kappa$-casein for preventing gut infections in infants is multifaceted. On one level, $\kappa$-casein, and specifically CMP, promotes the growth of beneficial microbiota including $Bifidobacterium\ infantis$ (Azuma et al. 1984) and $Lactobacillus\ bifidus$ (Bezkorovainy et al. 1979) in the infant’s gut, thus reducing colonization by disease causing pathogens. In addition, $\kappa$-casein has an inhibitory effect on the adhesion of pathogens to the gut and respiratory cell surfaces of the infant. For example, $\kappa$-casein inhibited the adhesion of fluoroisothiocyanate-labeled $H.\ pylori$ to human gastric mucosa and of $Streptococcus\ pneumoniae$ and $Helicobacter\ influenzae$ to human respiratory tract epithelial cells, most likely via GlicNAc $\beta 1\rightarrow3$Gal moieties on the $\kappa$-casein that mimicked pathogen-binding sites (Aniansson et al. 1996). Binding of the oral pathogen $S.\ mutans$ to saliva-coated hydroxyapatite was also prevented in the presence of $\kappa$-casein, an interaction that relied on the presence of sialic acid residues on the human milk glycoproteins (Vacca-Smith et al. 1994).
Proteins from the MFGM
The fat globules in milk are surrounded by a membrane comprising cholesterol, phospholipids and proteins that protect the globules from coalescence in the otherwise aqueous environment of milk. The MFGM originates from the apical plasma membrane of the lactating epithelial cells; therefore, the proteins it contains resemble those on other epithelial surfaces of the body (Newburg 2001). Many of the MFGM proteins are glycosylated and thus present a diverse array of glycan-binding epitopes across the external surface of the membrane that can act as decoys to pathogens and prevent attachment to the epithelial surfaces. Human MFGM glycoproteins that appear to provide decoy receptors for pathogens include mucin, bile-salt stimulated lipase and lactadherin.

Mucins. Mucins protect many epithelial surfaces of the human body, including the gastrointestinal and respiratory tracts. They exist in two forms: secreted mucins and membrane-bound mucins. The MFGM mucin proteins are the latter form and consist of a membrane-bound region, a short cytoplasmic segment and an extensive highly O-glycosylated exterior portion. It is the glycan chains of the MFGM mucins that are thought to act as decoys to reduce pathogen attachment to the epithelial cells of the infant’s body anywhere along the digestive tract, from the mouth to final excretion (Peterson et al. 1998). Evidence of mucin in the feces of breastfed infants (Patton 1994) demonstrates a resistance to digestion, in line with a primary function of pathogen removal rather than nutrition.

Two key mucins have been identified in the human MFGM, MUC1 and MUC4 (Patton et al. 1995). Of these, MUC1 predominate and has been the focus of more extensive research, particularly for the role its glycan moieties play in pathogen adhesion (Peterson et al. 1998; Wilson et al. 2008). Buccal epithelial cells line the oral cavity and provide an early attachment point for pathogenic bacteria in the newborn. MUC1 has been shown to inhibit the adhesion of S-fimbriated (meningitis-causing) E. coli to human buccal epithelial cells in vitro via a sialic acid (Neu5Acα2-3) determinant (Schroten et al. 1992).

Recently, both MUC1 and MUC4 from human MFGM were shown to inhibit S. enterica serovar Typhimurium (SL1344) invasion of human intestinal epithelial cells in vitro (Liu et al. 2012). Two epithelial cell lines were used in this study, one derived from human colorectal adenocarcinoma (Caco-2) and another derived from normal human fetal small intestine (FHs 74 Int). The FHs 74 Int cells were considered to provide a better representation of the immature infant gut than the adult cancer model in terms of morphology, mucosa and tyrosine kinase-dependent response to human milk. Invasion of the intestinal cells by fluorescein isothiocyanate labeled Salmonella was determined by confocal fluorescent microscopy and quantified by flow cytometry. Interestingly, there was little difference between the cell lines. MUC1 and MUC4 significantly inhibited invasion of both Caco-2 and FHs 74 Int cells at concentrations of 150 µg/mL (typical of human milk), with MUC1 inhibiting invasion more strongly than MUC4; however, the specific involvement of the mucin glycans in the inhibition of bacterial invasion was not examined.

Experimental support for the potential of human milk mucins to prevent viral gastrointestinal infection has also accumulated. For instance, sialylated human milk mucin inhibited rotavirus replication in tissue culture and prevented rotavirus gastroenteritis in a mouse model; deglycosylation of the mucin resulted in loss of antiviral activity (Yolken et al. 1992). Mucins containing Secretor and Lewis epitopes (histo-blood group antigens containing fucosyl side chains; Figure 2) also blocked the adhesion of recombinant norovirus-like particles to saliva (Jiang et al. 2004).

The transmission of HIV-1 from mother to child could be inhibited by MUC1 from human milk (Saeland et al. 2009). A protective mechanism was proposed in which Lewis X glycans (Figure 2) on MUC1 bind to the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) receptors on dendritic cells lining the intestinal tract, thus blocking binding of the gp120 envelope protein that initiates HIV infection. In support of this view, Lewis X epitopes on MUC1 from human milk blocked the interaction of fluorescent beads coated with HIV-gp120-Fc with dendritic cells. Furthermore, MUC1 blocked DC-SIGN-mediated transmission of HIV from dendritic cells to CD4+ T cells (Saeland et al. 2009).

Bile salt-stimulated lipase. BSSL is the chief enzyme found in milk that aids in the digestion of fats (Bläckberg and Hernell 1981). The enzyme is also secreted by the pancreas and is not

![Fig. 2. The blood group H-antigen and Lewis structures found in glycan-containing molecules (oligosaccharides and glycoconjugates) that can act as bacterial adhesion receptors. Type 1 chains are characterized by Galβ1-3GalNAc, whereas Type 2 chains have a Galβ1-4GalNAc linkage (shown in brackets). Those in the population classed as secretors have a functional FUT2 gene and fucose residues can be added to glycans via an α1-2 linkage, whereas non-secretors do not have glycans containing fucosyl residues linked by an α1-2 linkage.](image-url)
active until stimulated by bile salts in the intestine. Young infants only secrete small amounts of the pancreatic lipase and so human milk BSSL provides a much needed aid to digestion until the mature digestive system develops (Naarding et al. 2006). Human milk BSSL is a highly glycosylated protein, with a mucin-like C-terminal region containing 10 potential O-linked glycosylation sites, heavily adorned with carbohydrates containing fucose, galactose, glucosamine, galactosamine and sialic acid in molar ratios of 1:3:2:1:0.3, respectively (Wang et al. 1995). The glycosylation of BSSL has been shown to vary depending on the blood group phenotype of the mother and across the course of lactation (Landberg et al. 2000). Total glycosylation was greater in the first month, with a large proportion of sialic acid residues compared with later in lactation. The number of fucosylated structures including the Lewis X epitope (Figure 2) increased throughout the lactation period.

A number of potential antipathogenic properties have been attributed to BSSL from human milk. In one early study, BSSL from human milk was specifically found to kill Giardia lamblia (Gillin et al. 1983). The human milk BSSL induced swelling and lysis in the Giardia trophozoites. Later, BSSL from the milk of women with the Secretor blood phenotype (those with a functional FUT2 gene, producing α1,2-fucose-containing glycans; Figure 2) inhibited the attachment of recombinant norovirus-like particles to saliva or synthetic H-type 1 oligosaccharides, suggesting that α1-2-linked fucose residues could act as decoy receptors and prevent norovirus binding to gastrointestinal cells (Ruvöen-Clouet et al. 2006). Furthermore, the Lewis X epitope on BSSL bound to DC-SIGN and prevented the transfer of HIV-1 gp120 envelope protein to CD4+ T cells (Naarding et al. 2005, 2006). BSSL was also found to inhibit the binding of the oral pathogen Streptococcus mutans to saliva and salivary agglutinin (gp340 glycoprotein)-coated hydroxyapatite (Danielsson Niemi et al. 2009).

**Lactadherin.** Lactadherin is a mucin-associated sialylated glycoprotein in the MFGM and contains five N-linked glycosylation sites (Picariello et al. 2008). The antipathogenic properties of lactadherin for the human infant have been mainly associated with the prevention of rotavirus infection. Work carried out by Yolken et al. (1992) revealed that a mucin-associated 46 kDa glycoprotein (later designated as lactadherin) inhibited rotavirus infection of MA-104 (African green monkey kidney) cells, a phenomenon that was replicated in tissue culture and in mice. Binding of lactadherin to rotavirus was substantially reduced after chemical hydrolysis of sialic acid, implicating the role of the sialic acid determinant in the interaction. Several years later, a human case study supported the role of lactadherin for rotavirus prevention in vivo (Newburg et al. 1998). The incidence of rotavirus infection and associated symptoms was monitored among 200 infants from Mexico City and compared with the lactadherin content in their mother’s breast milk. Among infected infants, those who were fed milk containing high level of lactadherin remained asymptomatic, whereas severe diarrhea resulted in infants fed milk containing low levels of lactadherin (Newburg et al. 1998).

In 2004, the mechanisms by which lactadherin inhibits rotavirus infection was studied further in vitro, using the Caco-2 (human epithelial colorectal adenocarcinoma) cell line (Kvistgaard et al. 2004). Human milk lactadherin inhibited infection when incubated with the virus prior to application to the cells, but not when pre-incubated with the intestinal cells prior to the infection by the virus or when cells and virus were pre-incubated subsequently to lactadherin application. This suggests that lactadherin thus protects infants against infection primarily via an inhibitory mechanism; however, the role of the glycan moieties involved in the inhibition was not examined in this instance.

Table I provides a summary of the glycoproteins from human milk that have been experimentally associated with pathogen inhibition. Where possible, the binding glycan epitope implicated in pathogen or toxin adhesion is specified.

**Milk glycolipids with antipathogenic properties**

The glycolipids in human milk are mainly sialic acid-containing glycosphingolipids, known as gangliosides, and are exclusively associated with the MFGM. They consist of an 18 carbon sphingosine base and an amide-linked acyl group to form a ceramide, to which glycans are attached (Newburg 1996). The ceramide portion of the molecule is hydrophobic and embeds in the lipid bilayer of the MFGM, leaving the glycan chains exposed to the exterior. This arrangement mimics that of glycoproteins that form part of epithelial cell membranes, to which pathogens can attach. Therefore, analogous to human milk glycoproteins, the gangliosides of the MFGM are natural decoys that can prevent pathogen attachment to the epithelial cells of a young infant and thus prevent infection.

Gangliosides are present in human colostrum and mature human milk at concentrations ranging from 9.51 ± 1.16 mg lipid-bound sialic acid (LBSA)/L to 9.07 ± 1.15 mg LBSA/L, respectively (Bode et al. 2004). In human colostrum, the predominant ganglioside is GD3 (Neu5Ac α2-8 Neu5Ac α2-3 Gal β1-4 Glic β1-1 ceramide, comprising 65% of total LBSA; Takamizawa et al. 1986), whereas in mature human milk, GM3 (Neu5Ac α2-3 Gal β1-4 Glic β1-1 ceramide) is the major ganglioside accounting for ~70% of the total glycolipid content, and GD3 accounts for ~25% (Laegreid et al. 1986). Minor glycolipid components in mature human milk include the gangliosides GM2 (GalNAcβ1-4[Neu5Ac α2-3]Galβ1-4Glic β1-1 ceramide; ~2%) and GM1 (Galβ1-3GalNAcβ1-4[Neu5Ac α2-3] Galβ1-4Glcβ1-1 ceramide; ~0.1%) and the neutral glycolipid Gb3 (Galβ1-3Galβ1-4Glcβ1-1 ceramide; ~2%; Newburg and Chaturvedi 1992). The structures of these molecules are shown in Table II, along with research that has demonstrated the ability of human milk glycolipids to inhibit pathogen adhesion and infection. Further details of this work are provided below.

The effects of human milk gangliosides on the adhesion of gut pathogens have been investigated in a number of studies. The adhesion of enterotoxigenic E. coli to Caco-2 (human epithelial colorectal adenocarcinoma) cells was most effectively inhibited by GM1 (80% inhibition), followed by GM3 (69% inhibition) and GD3 (16% inhibition; Idota and Kawakami 1995). Binding of enteropathogenic E. coli to Caco-2 cells was inhibited by GD3 (Idota and Kawakami 1995), and notably, the terminal Neu5Ac α2-8Neu5Ac disaccharide of GD3 is also a preferred binding epitope of S-fimbriated E. coli (Hanisch et al. 1986).
### Table II. Human milk glycolipids that may inhibit the adhesion of pathogens and/or their toxins to human epithelial cells (nomenclature is based on that of Svennerholm 1963)

<table>
<thead>
<tr>
<th>Glycolipid (binding epitope)*</th>
<th>Target</th>
<th>Experimental evidence</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM1</td>
<td>Enterotoxigenic <em>E. coli</em> (ETEC)</td>
<td>Inhibited binding to Caco-2 (human epithelial colorectal adenocarcinoma) cells, 80% inhibition</td>
<td>Idota and Kawakami (1995)</td>
</tr>
<tr>
<td>GM1, GM2</td>
<td><em>E. coli</em> heat-labile enterotoxin (LT); <em>Vibrio cholerae</em> cholera toxin</td>
<td>Inhibited LT binding to ELISA plates and reduced the in vivo effects of cholera toxin in rabbit intestinal loop experiments</td>
<td>Otnaess et al. (1983); Laegreid and Kolstø Otnaess (1987)</td>
</tr>
<tr>
<td>GM3</td>
<td>Enterotoxigenic <em>E. coli</em> (ETEC)</td>
<td>Inhibited binding to Caco-2 (human epithelial colorectal adenocarcinoma) cells, 69% inhibition</td>
<td>Idota and Kawakami (1995)</td>
</tr>
<tr>
<td>GM1, GM3, GD3, Neu5Ac</td>
<td><em>Campylobacter jejuni</em>, <em>L. monocytogenes</em>, <em>Salmonella enterica</em> (Typhi), <em>S. sonnei</em>, <em>H. pylori</em></td>
<td>Inhibited binding to Caco-2 (human epithelial colorectal adenocarcinoma) cells</td>
<td>Salcedo et al. (2013)</td>
</tr>
<tr>
<td>GD3</td>
<td>Enterotoxigenic <em>E. coli</em> (ETEC), enteropathogenic <em>E. coli</em> (EPEC)</td>
<td>Inhibited binding to Caco-2 (human epithelial colorectal adenocarcinoma) cells, 16% inhibition</td>
<td>Idota and Kawakami (1995)</td>
</tr>
<tr>
<td>GB3</td>
<td><em>Shigella dysenteriae</em> Shiga toxin</td>
<td>Bound to Shiga toxin in a solid-phase binding assay</td>
<td>Newburg and Chaturvedi (1992)</td>
</tr>
<tr>
<td>NeuAcα2-3Gal, NeuAcα2-6Gal</td>
<td>EV71</td>
<td>Inhibited infection of DLD-1 (human epithelial colorectal adenocarcinoma) cells</td>
<td>Yang et al. (2009)</td>
</tr>
<tr>
<td>Sulfated glycolipids-Sulfatide ceramide, Sulfated lactosyl</td>
<td>HIV gp120</td>
<td>Inhibited the binding of recombinant HIV surface glycoprotein gp120 in cultured human colonic and vaginal epithelial cells</td>
<td>Newburg and Chaturvedi (1997)</td>
</tr>
</tbody>
</table>

*Structures: GM1, GM2, GM3, GD3, Neu5Ac, NeuAcα2-3Gal, NeuAcα2-6Gal*
infections of DLD-1 (human epithelial colorectal adenocarcinoma) cells (Yang et al. 2009). Enterovirus causes infant hand-foot-mouth disease, which often leads to fatal encephalitis in many Asian countries (Chang 2008). The sulfated glycolipids sulfatide and sulfated lactosyl ceramide inhibited the binding of recombinant HIV surface glycoprotein gp120 in cultured human colonic and vaginal epithelial cell lines in a solid phase assay (Newburg and Chaturvedi 1997), suggesting that protection against HIV infection could be provided by sulfated glycolipids from human milk.

Table II provides a summary of research conducted on the antipathogenic properties of glycolipids from human milk, the glycan moieties implicated in the interactions, and the bacteria, toxins and viruses against which they protect. In comparison with free HMOs (Bode 2012) and glycoproteins (Table I), the paucity of data on the antipathogenic properties of human milk glycolipids is notable. Table III provides some examples of research implicating pathogen interactions with glycan moieties of specific glycolipids from non-milk sources, including those from bovine brain and erythrocytes. The significance of including these studies in this review lies in the fact that the glycan moieties are the same on each class of ganglioside regardless of their source. It could be anticipated that the glycans from the equivalent glycolipid classes in human milk could be a beneficial supply of effective decoys to prevent adhesion of these pathogens to receptor sites in the body. However, it should be noted that the sphingosine and fatty acid moieties of non-milk glycolipids may vary in length and degree of hydroxylation and saturation compared with human milk glycolipids, which could in turn impact on the presentation of the attached glycans (Varki et al. 2009).

Prebiotic potential of human milk glycoconjugates

Infants fed human milk are known to develop a population of beneficial microorganism in their gut, including Bifidobacterium and Lactobacillus species. These “friendly” bacteria can assist digestion of nutrients and block adhesion and colonization of pathogenic bacteria, thereby providing protection against disease and allergy development (Conroy et al. 2009; Le Huërou-Luron et al. 2010). The development of beneficial microbial communities has been partly attributed to HMOs, which serve as a food source for the bacteria (Bode 2012). More recently, the ability of microorganisms to use the glycans from human milk glycoproteins as a carbon source has been revealed. The bacteria produce extracellular glycosidases that cleave the glycan chains on proteins and conceivably may generate free glycans that contribute to the HMOs that have been found to inhibit pathogen adherence. Bifidobacteria, for example, can utilize mucin O-linked glycans by the secretion of endo-α-N-acetylgalactosaminidase (limited to removal of only Gal-GaINAc) and 1,2-α-L-fucosidase (Ruas-Madiedo et al. 2008) and can cleave N-glycans from lactoferrin and immunoglobulins by an endo-β-N-acetylgalactosaminidase (Garrido et al. 2012).

Glycoconjugates in bovine milk

While human milk may provide the best food source for the newborn infant, formulae derived from bovine milk are often used as an acceptable alternative. The dietary value of formula-based products has received much attention and high-quality goods providing all the nutritional requirements of an infant are now widely available. Also desirable is that the formula based products provide antipathogenic attributes akin to those provided by human milk. Since the glycan structures in human milk, as free oligosaccharides or moieties of glycoconjugates, are now considered a particular feature of human milk that assists in pathogen deflection, research is beginning to address whether bovine milk contains similar glycan structures and similar anti-pathogenic effects.

Bovine milk contains less diversity in its free oligosaccharide content compared with human milk (~40 bovine milk oligosaccharides/BMOS compared with over 100 HMOs), more sialylated glycans (~70% of BMOS compared with 10–20% of HMOs) and vastly fewer fucosylated glycans (~1% of BMOS compared with 50–80% of HMOs, depending on the blood group of the mother; data compiled by Bode 2012). In addition, BMOS contain N-glycolylneuraminic acid (~7% of total acidic BMOS; Tao et al. 2008), which is not found in human milk. Little is known about the protective value of BMOS against pathogens, despite the fact that HMOs appear to have substantial antipathogenic properties. Moreover, the concentration of BMOS (0.09–1.2 g/L) is appreciably lower than HMOs.
MUC1 inhibited the binding of Caco-2 cells (Parker et al. 2010). Bovine milk may better assist in the rectal adenocarcinoma) cells (Nakajima et al. 2005), and bovine and enterohemorrhagic E. coli inhibited the receptor. For example, high molecular mass mucin-like residue. Furthermore, the sialic acid-containing GMP from bovine milk were able to inhibit hemagglutination of components from bovine milk. Lights the possible implications of the fucose deficiency in bovine milk. However, the increased sialic acid structures in bovine milk may better assist in the deflection of other pathogens that adhere to these epitopes depending on the structure of the receptor. For example, high molecular mass mucin-like components from bovine milk were able to inhibit hemagglutination of H. pylori (Hirno et al. 1998) via a sialic acid residue. Furthermore, the sialic acid-containing GMP from bovine κ-casein inhibited the adhesion of Salmonella enteritidis and enterohemorrhagic E. coli to Caco-2 (human epithelial colorectal adenocarcinoma) cells (Nakajima et al. 2005), and bovine MUC1 inhibited the binding of E. coli and S. typhimurium to Caco-2 cells (Parker et al. 2010).

Lactoferrin is present in bovine milk at concentrations ranging from 0.1 to 0.4 mg/mL, a significantly lower level than in human milk (1–3 mg/mL; Wakabayashi et al. 2006). Similar to the human milk equivalents, bovine lactoferrin and the pepsin-derived fragment lactoferricin have antibacterial activity by altering the LPS molecules on the membranes of Gram-negative bacteria, such as E. coli CL99 1-2, S. typhimurium SL696 and S. montevideo SL5222 (Yamauchi et al. 1993). Bovine lactoferrin also has been shown to have antiviral properties by blocking human cytomegalovirus infection and inhibiting the cytopathic effect of HIV (Harmsen et al. 1995; Groot et al. 2005). In the case of HIV, bovine lactoferrin was more effective than human lactoferrin in blocking the HIV gp120 binding to its DC-SIGN receptor (Groot et al. 2005). Another clinical trial of preschool-aged children investigated the daily dose of 100 mg of bovine lactoferrin and monitored the incidence of rotaviral gastroenteritis over a 3-month period. While the incidence of rotaviral infection was equivalent between the control and test subjects, a significant improvement in the frequency and duration of both vomiting and diarrhea was observed in the group of children taking bovine lactoferrin (Egashira et al. 2007).

Human and bovine milk contain similar gangliosides; however, the overall ganglioside concentration is much lower in bovine milk (3.98 ± 0.25 mg LBSA/L) than human milk (9.07 ± 1.15 mg LBSA/L; Bode et al. 2004). GD3 dominates in bovine milk, whereas GM3 is the main ganglioside in human milk (Pan and Izumi 2000; Bode et al. 2004). The antibacterial properties of bovine milk glycolipids have rarely been investigated to date. In one study, bovine milk gangliosides were compared with human milk gangliosides for their inhibitory activity against the binding of enteropathogenic E. coli to Caco-2 cells (Idota and Kawakami 1995). Whereas the human milk gangliosides inhibited binding, the bovine gangliosides did not, indicating that the dominant bovine ganglioside GD3 is not biologically active against this pathogen. In comparison, enterotoxigenic E. coli bound to GM3 and GD3 from bovine milk and inhibited bacterial hemagglutination (Sanchez-Juanes et al. 2009).

The future in milk glycoconjugate research

With ever-advancing methodologies in glycan analysis and the study of pathogen interactions, the protective value of human milk glycoconjugates against infection invites further investigation. The latest studies have involved the use of experimental models that aim to more authentically represent the infant/pathogen interaction, e.g. by using cell lines derived from fetal intestinal cells rather than adult carcinomas (Liu et al. 2012) and by investigating the effect of other components also present in vivo, such as cholesterol and other glycoconjugates (Gallegos et al. 2012). The combined effect of the cocktail of glycoconjugates, oligosaccharides and other factors in human milk are an even greater challenge for analysis, as are the conditional requirements for interactions to occur. Nevertheless, these complexities may need to be addressed before we gain a more comprehensive understanding of glycoconjugate protective mechanisms.

An increasing sophistication in glycan structural analysis through advances in mass spectrometry and glycan microarrays paves the way for a more detailed assessment of glycan moieties that are critical to interactions (Everest-Dass et al. 2013; Smith and Cummings 2013). Furthermore, advances in microscopy facilitate the study of the adhesion apparatus of pathogens...
Glycosylation changes are known to occur in milk glycoconjugates throughout the lactation period (Landberg et al. 2000; Wilson et al. 2008; Froehlich et al. 2010; Barboza et al. 2012). How this affects protection against pathogen adhesion in the breastfed infant remains another question for the future.

The oligosaccharide structures adorning macromolecules in human milk differ from those in cow’s milk and appear to be closely adapted to the inhibition of binding of human pathogens. Further research into this difference may have benefits in the future. The development of supplements to mimic the protective agents naturally occurring in human milk is becoming a real possibility, and in the context of the increasing worldwide incidence of antibiotic resistance, a more natural mechanism of protection against disease in both infants and the wider population is very appealing.

Funding

This work was supported by Dairy Australia (VC113495; R.P., J.G., N.P.) and an International Macquarie University Research Excellence scholarship (W.Y.C.).

Conflict of interest

None declared.

Abbreviations

BSSL, bile salt-stimulated lipase; CMP, caseinomacropeptide; DC-SIGN, DC-specific ICAM-3-grabbing non-integrin; ELISA, enzyme-linked immunosorbent assay; EPEC, enteropathogenic E. coli; EV71, enterovirus 71; GlcNAc, N-acetylgalactosamine; GM, glycomacropoctide; HIV, human immunodeficiency virus; HMO, human milk oligosaccharide; LBLS, lipoid-bonded sialic acid; LPS, lipopolysaccharide; Man, mannose; MFGM, milk fat globule membrane; Neu5Ac, N-acetylenuraminic acid; RSV, respiratory syncytial virus; sIgA, secretory immunoglobulin A.

References


Garrido D, Nwosu C, Ruiz-Moyano S, Aldredge D, German JB, Lebrilla CB, Mills DA. 2012. Endo-


