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Glycolipid receptor depletion as an approach to specific anti-microbial therapy

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Abstract

Mucosal pathogens recognise glycoconjugate receptors at the site of infection, and attachment is an essential first step in disease pathogenesis. Inhibition of attachment may thus prevent disease, and several approaches have been explored (Fig. 1). This review discusses the prevention of bacterial attachment and disease by agents that modify the glycosylation of cell surface glycoconjugates. The glycosylation inhibitors were tested in the urinary tract infection model, where P fimbriated *Escherichia coli* rely on glycosphingolipid receptors for attachment and tissue attack. *N*B-DNJ blocked the expression of glucosylceramidederived glycosphingolipids and attachment was reduced. Bacterial persistence in the kidneys was impaired and the inflammatory response was abrogated. *N*B-DNJ was inactive against strains, which failed to engage these receptors, including type 1 fimbriated or non-adhesive strains. In previous studies, *in vivo* attachment was successfully prevented by soluble receptor analogues, but there is little clinical experience of such inhibitors. Large-scale synthesis of complex carbohydrates, which could be used as inhibitors of attachment, remains a technical challenge. Antibodies to the bacterial lectins involved in attachment may be efficient inhibitors, and fimbrial vaccines have been developed. Glycosylation inhibitors have been shown to be safe and efficient in patients with lipid storage disease and might therefore be tested in urinary tract infection. This approach would differ from current therapies, including antibiotics, in that it would target the pathogens which recognise these receptors.

Introduction

Many pathogenic microbes rely on glycoconjugate receptors for their interaction with the host (Källenius *et al.*, 1980; Leffler & Svanborg-Edén, 1980; Mirelman, 1986). They have evolved to take advantage of the structural variation among glycoconjugates on host cells by using them as receptors at the site of infection. This mechanism allows for microbial specificity, due to the variation in oligosaccharide structure with the species, individual and tissue. Mucosal pathogens, engage epithelial cell glycoconjugates for attachment to the mucosa and to initiate tissue attack. The epithelial cells in the mucosal barrier are highly glycosylated, and the microbial lectins bind oligosaccharide epitopes on glycolipids or glycoproteins. In glycolipids, the oligosaccharides are bound to ceramide, which spans the outer leaflet of the lipid bilayer but in glycoproteins, the oligosaccharides are covalently linked to membrane bound or soluble proteins. Cholera toxin binds to members of the ganglioseries of glycolipids in the intestine and bacterial adhesins in the respiratory tract show similar specificity (Scharfman *et al.*, 1996; van Heyningen & King, 1976). Glycolipids of the globoseries act as receptors for P fimbriae and shiga toxin (Leffler & Svanborg-Edén, 1980; Lingwood *et al.*, 2000). Mannosylated glycoproteins have been identified as receptors for type 1 fimbriae and sialylated structures are recognised by S fimbriae (Aronson *et al.*, 1979; Korhonen *et al.*, 1986; Ofek *et al.*, 1977).

Uropathogenic *Escherichia coli* (*E. coli*) exemplify a large number of mucosal pathogens that bind to glycoconjugate receptors on epithelial cells (Leffler & Svanborg-Edén, 1980; Svanborg Eden, 1980). We have studied P fimbriae and the globoseries of glycosphingolipids (GSLs) as a model of receptor specific interactions between pathogen and host. When these studies were initiated, microbial adherence was thought to rely on non-specific forces like

charge and hydrophobicity, but such mechanisms were not compatible with the host and tissue specificity of disease. Guided by the specificity for urinary tract epithelial cells, we identified the globoseries of GSLs as receptors for P fimbriated *E. coli* (Leffler & Svanborg-Edén, 1980) (Fig. 2). Three main criteria were used to define the receptor specificity. First, the isolated glycolipid was shown to be necessary and sufficient for fimbrial binding. Coating of inert surfaces like silica plates with receptor glycolipids enabled these surfaces to specifically bind P fimbriated bacteria. The binding was later shown to depend on the PapG protein, which is the tip adhesin of P fimbriae (Lindberg *et al.*, 1986; Lindberg *et al.*, 1984; Lund *et al.*, 1987). Second, the attachment of P fimbriated bacteria was inhibited by the soluble receptor glycolipid (Leffler & Svanborg-Edén, 1980). This approach was efficient also *in vivo* as shown by the administration of soluble receptor analogues and the resulting prevention of infection in the murine urinary tract (Svanborg-Edén *et al.*, 1982). Third, genetic variation in receptor expression was shown to influence the attachment to intact human epithelial cells (Marcus *et al.*, 1981). The majority of individuals belong to blood group P1 (75%) or P2 (24%) and express the globoseries of glycolipids on epithelial cells in the bladder, renal pelvis and tubuli but individuals of blood group p lack the globoseries of GSLs, due to an enzyme deficiency that precludes the elongation of the carbohydrate chains (Holgersson *et al.*, 1992; Lomberg *et al.*, 1981). Cells from p individuals were shown not to bind P fimbriated *E. coli*, and extracted GSLs from p individuals lacked receptor activity either after binding to silica plates or when used as soluble inhibitors (Bock *et al.*, 1985; Leffler & Svanborg-Edén, 1980). The epithelial glycolipids structure is further defined by the secretor state (Lomberg *et al.*, 1986; Stapleton *et al.*, 1992).

Inhibition of glycosphingolipid expression

A schematic of glycosphingolipid (GSL) biosynthesis is shown in (Fig. 3). Ceramide is formed by the condensation of palmitoyl-CoA with serine to generate 3-ketodihydrosphingosine in a reaction catalysed by serine palmitoyl-transferase. The fatty acids are coupled to sphinganine through fatty acyl CoA sphinganine forming ceramide (Radin, 1984). Ceramide can either form sphingomyelin by coupling of phosphoryl-choline to the primary hydroxyl group of ceramide or GSLs by the action of a ceramide specific glucosyltransferase. Most GSLs are generated by the action of N-acylsphingosine glucosyltransferase which couples glucose (from UDP-glucose) to glucosyl-ceramide (Glc-Cer) (Platt & Butters, 1995; Shayman & Radin, 1991).

Several types of chemical GSL biosynthesis inhibitors have been developed. Inhibition of ceramide biosynthesis may be achieved using Fumonisins but the active components are toxic and carcinogenic. The Fumonisins are produced by *Fusarium verticillioides*, a common fungal contaminant of maize (Marasas *et al.*, 2004; Norred *et al.*, 1992). Ceramide glycosylation may be blocked either at the UDP-binding site on ceramide or at modifying sites on the enzyme itself (Inokuchi & Radin, 1987; Inokuchi *et al.*, 1989; Platt & Butters, 1995). Two main classes of glucosyltransferase inhibitors have been described, the PDMP-series and the *N*-alkylated imino sugars. The cationic lipid D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) is a ceramide mimetic (Inokuchi & Radin, 1987; Radin *et al.*, 1993). PDMP has been shown to reduce cell growth *in vitro*, in the kidneys of mice and in cancer cells and to block proliferative responses to lymphocyte mitogens (Inokuchi *et al.*, 1987; Inokuchi *et al.*, 1989). The MDCK cell line showed increased levels of free ceramide and diglycerides after GSL depletion by PDMP (Shayman & Radin, 1991). The second generation of PDMP derivatives such as the P4 compound can be tolerated *in vivo* in the mouse (Abe *et al.*, 2000).

The imino-sugar deoxynojirimycin (DNJ) occurs naturally in plants, fungi and bacteria, and is better tolerated than other glycosylation inhibitors (Platt & Butters, 2004). It probably forms a part of a chemical defence strategy against other competing organisms in the same ecological niche. A derivative of DNJ, *N*-butyldeoxynojirimycin (*N*B-DNJ) blocks the *N*-linked oligosaccharide processing enzymes α-glucosidases I and II and inhibits the activity of ceramide glucosyltransferase, resulting in the inhibition of GSL biosynthesis (Platt & Butters, 1995; Platt, 1994)

Receptor depletion and lipid storage disease

*N*B-DNJ is an orally available compound and has been used successfully as a therapeutic agent in a GSL lysosomal storage disease, Gaucher disease. This is a genetic disorder characterised by deficient glucocerebrosidase activity leading to the accumulation of Glc-Cer, particularly in macrophages (Platt & Butters, 2004; Platt, 1998, 1997). The patients suffer severe hypertrophy of the spleen and liver, haematological abnormalities and bone disease. In addition, these patients have an unexpectedly high incidence of leukemia and other B-cell disorders. Using a murine macrophage cell line, an *in vitro* model was created by inhibiting glucocerebrosidase (with conduritol beta epoxide) to mimic the defect in Gaucher disease. After *N*B-DNJ treatment of the WEHI 3B cells, the Glc-Cer content decreased in a dose-dependent way, suggesting that the compound might have potential as a therapeutic (Platt *et al.*, 1994). Healthy mice treated with the glucose analogue *N*B-DNJ exhibited 70% peripheral GSL depletion (Jeyakumar *et al.*, 1999; Platt *et al.*, 1997). Clinical trials have recently been completed in type 1 Gaucher patients and are currently in progress in late onset Tay-Sachs disease, Niemann-Pick type C1 and type 3 Gaucher disease (Cox *et al.*, 2000; Elstein *et al.*, 2004). *N*B-DNJ was administered during a 12-month period to patients suffering from non-

neuronopathic type 1 Gaucher disease. The treatment was efficient as shown by a decrease in cell surface GM1 on leucocytes and a decrease in the volume of liver and spleen (Cox *et al.*, 2000). Importantly, the treatment was well tolerated by the patients and sustained improvement was seen over 36 months of therapy (Elstein *et al.,* 2004). The drug NB-DNJ (miglustat/Zavesca) was approved for clinical use in type 1 Gaucher disease in 2003. As Gaucher disease does not involve the glycolipids, which are receptors for P fimbriae, the UTI susceptibility would not be expected to change in those patients. The UTI frequency in patients who receive *N*B-DNJ treatment has not been examined, however.

Effects of GSL inhibitors on bacterial attachment and the epithelial response *in vitro*

Based on the receptor specificity of P fimbriae, we examined if GSL inhibitors might prevent attachment and attenuate the disease process. We first used PDMP to inhibit GSL expression in A498 kidney epithelial cells *in vitro*. The receptor expression was reduced, as was the attachment of P fimbriated bacteria (Svensson *et al.*, 1994). PDMP treatment was limited to *in vitro* experiments, however, due to *in vivo* toxicity.

*N*B-DNJ and related compounds were subsequently used to block the expression of GSL receptors *in vitro*. *N*B-DNJ blocked the glycosylation of ceramide in kidney epithelial cells, as shown by thin layer chromatography of GSL extracts from the *N*B-DNJ treated cells. Total glycolipid extracts from cells grown in the presence or absence of *N*B-DNJ were separated by TLC and over-layed with ^{35}S labelled P fimbriated *E. coli,* to detect receptor active bands (Svensson *et al.,* 2003). There was a significant decrease in the globoseries of GSL as shown by autoradiography.

The consequence for bacterial attachment was examined using clinical *E. coli* isolates and *N*B-DNJ treatment reduced their binding. The specificity for P fimbriae was confirmed using a panel of recombinant strains that expressed either P or type 1 fimbriae, and the inhibition was shown to be specific for P fimbriated *E. coli*. Type 1 fimbriae bind to mannose expressing glycoproteins, which increased after *N*B-DNJ treatment, causing an increase in attachment of type 1 fimbriated *E. coli* to the treated epithelial cells. We conclude that receptor depletion by *N*B-DNJ treatment of epithelial cells can be achieved *in vitro,* and that this treatment reduces the attachment of P fimbriated *E. coli.*

_N_B-DNJ and GSL receptor depletion *in vivo*

*N*B-DNJ treatment was subsequently used to study the effect of receptor depletion *in vivo* in the murine experimental UTI model (Svensson *et al.*, 2003). The model mimics important aspects of disease pathogenesis in man. Bacteria are introduced into the bladder through a soft polyethylene catheter, bacteriuria is established, and the innate host response is triggered if the bacteria express virulence-associated lectins like P fimbriae. Non-adherent bacteria may establish bacteriuria, but do not break the inertia of the mucosal barrier or cause inflammation (Bergsten *et al.*, 2004; Fischer *et al.*, 2005).

In vivo experiment were performed in 8 –12 weeks old female C3H/HeN mice. Mice were fed *N*B-DNJ (5mg/mouse/day) for two weeks prior to experimental UTI and a reduction of tissue glycolipid expression was observed. There was a marked reduction in tissue levels of the globoseries of GSL in treated mice as compared to the untreated controls (Svensson *et al.*, 2003). The effect of receptor depletion on bacterial persistence was quantified by bacterial counts in urine and tissues, before and at defined time point after bacterial installation. The number of P fimbriated bacteria was reduced in the kidneys and bladders of the *N*B-DNJ

treated mice at all times, with the maximum difference after 6 hours ($p < 0.028$). Type 1 fimbriated *E. coli*, in contrast, showed higher bacterial counts in *N*B-DNJ treated compared to control mice, consistent with the increased attachment of this strain. The results demonstrate that GSL depletion by *N*B-DNJ treatment attenuates the virulence of P fimbriated *E. coli*.

Effects of *N*B-DNJ on *E. coli* induced mucosal inflammation.

Attaching, uro-pathogenic *E. coli* trigger the innate host response in the urinary tract mucosa and the epithelial cells amplify the response by secretion of cytokines and other mediators and by recruitment of inflammatory cells (Agace *et al.*, 1992; Agace *et al.*, 1993; Godaly *et al.*, 1998; Hedges *et al.*, 1990; Hedges *et al.*, 1996). The resulting mucosal inflammation is a cause of symptomatic disease, but also essential to clear the bacteria from the tissues. P fimbriated *E. coli* trigger a mucosal response both in mice and human patients (Hagberg *et al.*, 1983; Wullt *et al.*, 2001). Human inoculation studies have demonstrated that attachment through the PapG tip adhesin enables the mucosa to recognise bacteria in the lumen and to activate mucosal inflammation (Bergsten *et al.*, 2004). P fimbriae thus fulfil the molecular Koch's postulated as independent virulence factors (Bergsten *et al.*, 2004). The host response is further controlled through Toll like receptor 4 (TLR4) signalling (Fischer *et al.*, 2005; Frendeus *et al.*, 2001). The glycosphingolipid receptors for P fimbriae recruit TLR4 for transmembrane signalling and P fimbriae influence the adaptor proteins, which are involved downstream (Fischer *et al.*, 2005). Strains carried by patients with asymptomatic bacteriuria rarely express P fimbriae, suggesting that it is possible to persist in the urinary tract without engaging the GSL receptors.

The effect of GSL depletion on the innate host response to P fimbriated *E. coli* was first examined *in vitro,* in PDMP treated human kidney cells. PDMP treatment lowered the cytokine response of epithelial cell lines to uropathogenic *E. coli* strains or recombinant P fimbriated strains but had no

effect on the type 1 stimulated IL-6 response (Svensson *et al.*, 1994). The innate response to infection was subsequently examined *in vivo*. *N*B-DNJ treated and control mice were subjected to experimental infection with P fimbriated *E. coli* and the urine neutrophil numbers were used to quantify the innate response to infection. The epithelial chemo-attractants recruit inflammatory cells from the circulation to the mucosa and especially the neutrophils are essential to remove bacteria from the urinary tract (Haraoka *et al.*, 1999; Shahin *et al.*, 1987). *N*B-DNJ treated mice showed a drastic reduction in urine neutrophil numbers following infection with P fimbriated *E. coli*. This effect was P fimbriae dependent as shown by comparison with an isogenic strain lacking P fimbriae. The vector control strain did not trigger the innate host response, and there was no difference between *N*B-DNJ treated and control mice. The chemoattractant MIP-2 is a homologue of IL-8 and is involved in neutrophil-epithelial cell interactions in the urinary tract (Hang *et al.*, 1999). The secretion of MIP-2 into the urine was monitored in treated and control mice and a significant reduction of the MIP-2 response to P fimbriated *E. coli* was observed. In contrast, *N*B-DNJ treatment had no effect on the PMN or MIP-2 responses to the type 1 fimbriated strain (Svensson *et al.*, 2003). These *in vivo* results confirmed that GSL expression is important for the innate host response to P fimbriated *E. coli.*

Other approaches to the prevention of attachment

Many approaches have been taken to prevent disease by inhibition of attachment (Fig. 1). These include antibodies to the bacterial adhesins, which occupy the bacterial binding site and thus prevent the fimbriae from binding to the cellular receptor. The *V. cholerae* model was the first to show that antibodies could be used, and this was confirmed for Streptococci in the oral cavity, for uropathogenic *E. coli*, and for a variety of intestinal pathogens (Evans *et al.*, 1984; Freter, 1969; Svanborg-Edén *et al.*, 1976; Svanborg-Edén & Svennerholm, 1978; Williams & Gibbons, 1972). Several veterinary vaccines were developed to prevent the attachment of entero-pathogens bearing the K88 or K99 adhesins, and were used quite extensively. Vaccines

based solely on adhesins and the prevention of attachment have not been developed for human use, even though some currently used vaccines have effects on bacterial colonisation. For example, antibodies to the capsular polysaccharides of *Haemophilus influenzae* were found to protect both against the systemic and the mucosal phase of infection (Takala *et al.*, 1993). Recently, a type 1 fimbrial vaccine was tested in patients with urinary tract infection but the results have not yet been published.

Soluble receptor analogues inhibit attachment by occupying the adhesin, thus preventing the bacteria from binding to the cell bound receptors. Early studies showed that mannose and derivatives of mannose inhibit the attachment of type 1 fimbriated *E. coli* (Duguid & Gillies, 1957; Ofek *et al.*, 1977). *In vivo* experiments showed that injecting methyl α-D-mannopyranoside into the bladder of the mouse blocked the colonization by type 1 fimbriated *E. coli* (Aronson *et al.*, 1979). After the identification of the globoseries of GSL as receptors for P fimbriae, we showed that the soluble receptor inhibited attachment and reduced the persistence of fimbriated bacteria *in vivo* in the urinary tract (Svanborg-Edén *et al.*, 1982). Shiga toxin binds the same family of GSL receptors and receptor analogues have been used to prevent the action of this toxin *in vivo* (Lingwood *et al.*, 2000).

Conclusion

This review summarises information on GSL biosynthesis inhibitors and urinary tract infection. *N*-alkylated imino sugars are orally available compounds, which inhibit the first step in the GSL biosynthetic pathway and have been used successfully to treat a GSL lysosomal storage disease (type 1 Gaucher). The drug *N*B-DNJ has undergone extensive animal and human testing, and offers a potential means of evaluating GSL depletion as an anti-microbial strategy. The strategy is to partially inhibit GSL biosynthesis in order to

attenuate the infectivity of pathogens that rely on GSL receptors for their virulence. Using P fimbriated uro-pathogenic *E. coli* as an example, this study demonstrated that inhibitors of GSL synthesis offer an alternative to antibiotic treatment. The GSL inhibitor reduced receptor expression, leading to a reduction in the adherence of P fimbriated *E. coli* to epithelial cells. The GSLs were also required as recognition receptors for the innate host response and receptor depletion reduced the epithelial chemokine response. Finally, *N*B-DNJ treatment caused a reduction in receptor expression in kidney tissue and prevented colonization by P fimbriated *E. coli in vivo.* The results suggest that *N*B-DNJ or similar compounds should be tested as prophylactic agents in patients with recurrent infections due to P fimbriated *E. coli.* The compounds might also prove useful against other pathogens that rely on glycosphingolipid-specific recognition mechanisms.

Figure Legends

Figure 1

Schematic of approaches to blocking bacterial attachment *in vivo*.

A. Attachment is essential for tissue attack. Non-adherent comensal strains do not provoke the innate host response.

B. Anti-adhesive antibodies prevent attachment by combining with the fimbrial adhesins.

C. Soluble receptor analogues occupy the adhesins and competitively inhibit attachment to the cell bound receptor.

D. Pharmacologic inhibitors of glycosylation reduce the density of receptors on epithelial surfaces, thus preventing attachment.

Figure 2

P fimbriae receptor GSLs contain a single oligosaccharide chain linked to ceramide. At least eleven Galα1-4Galβ containing glycolipids have been found and some are antigens in the P-blood group system.

Figure 3

Glycosphingolipid biosynthetic pathway.

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Fig. 1

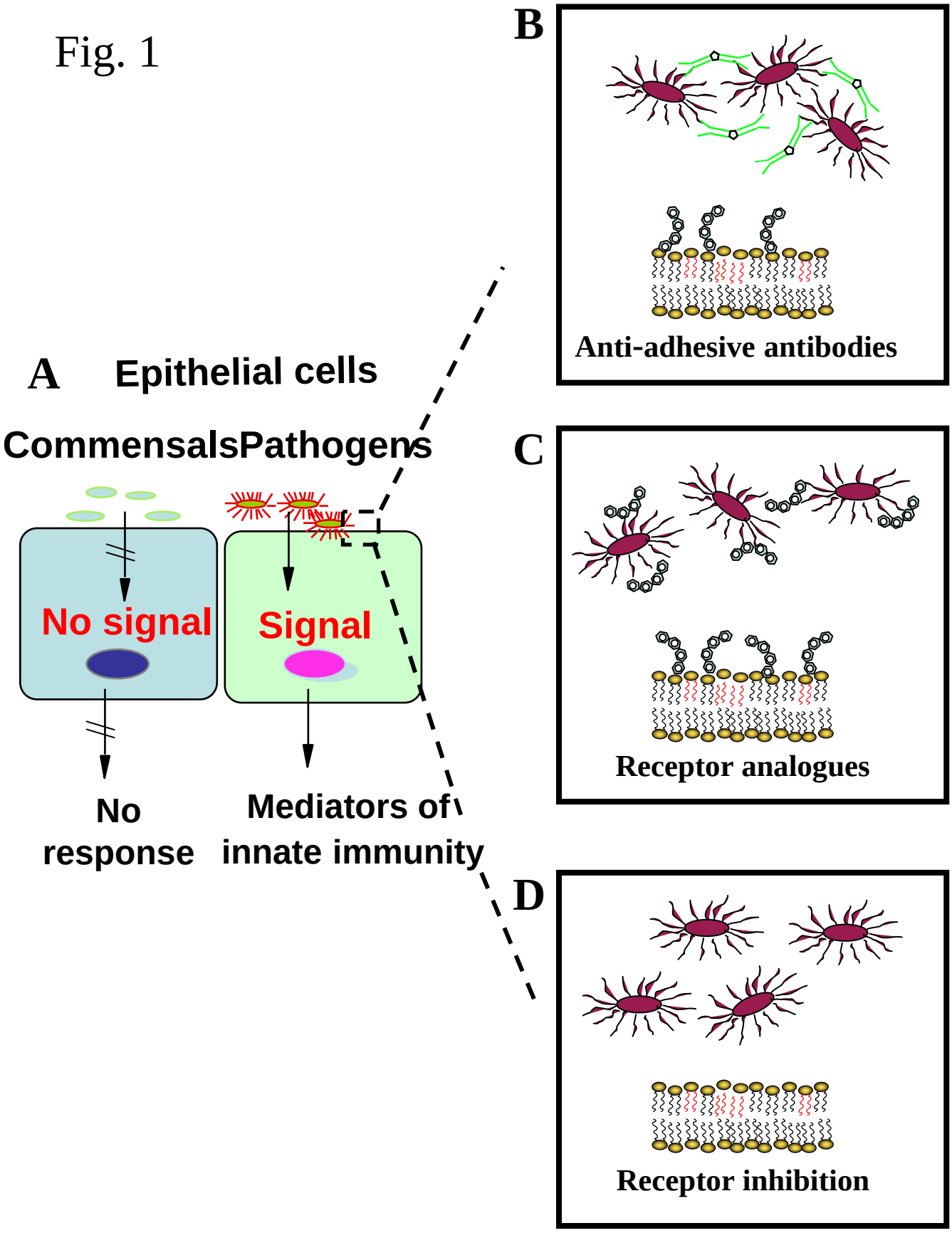

A
Epithelial cells
Commensals
Pathogens
No signal
Signal
No response
Mediators of innate immunity
B
Anti-adhesive antibodies
C
Receptor analogues
D
Receptor inhibition

Fig 3

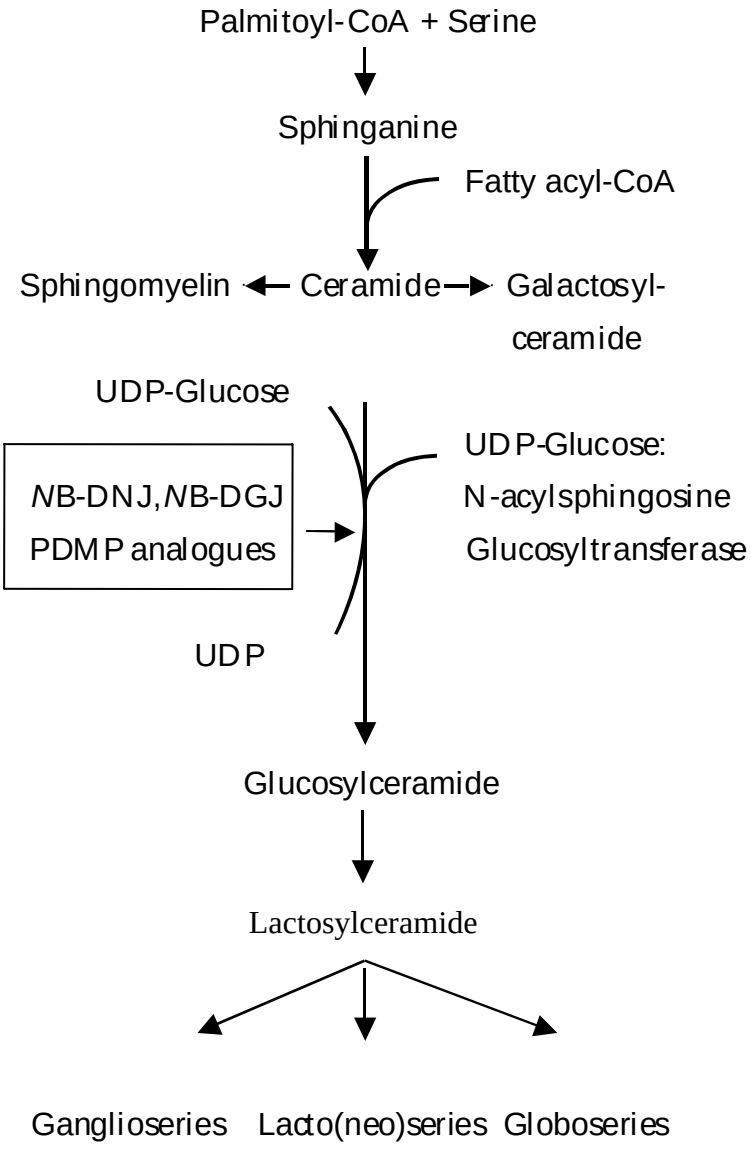
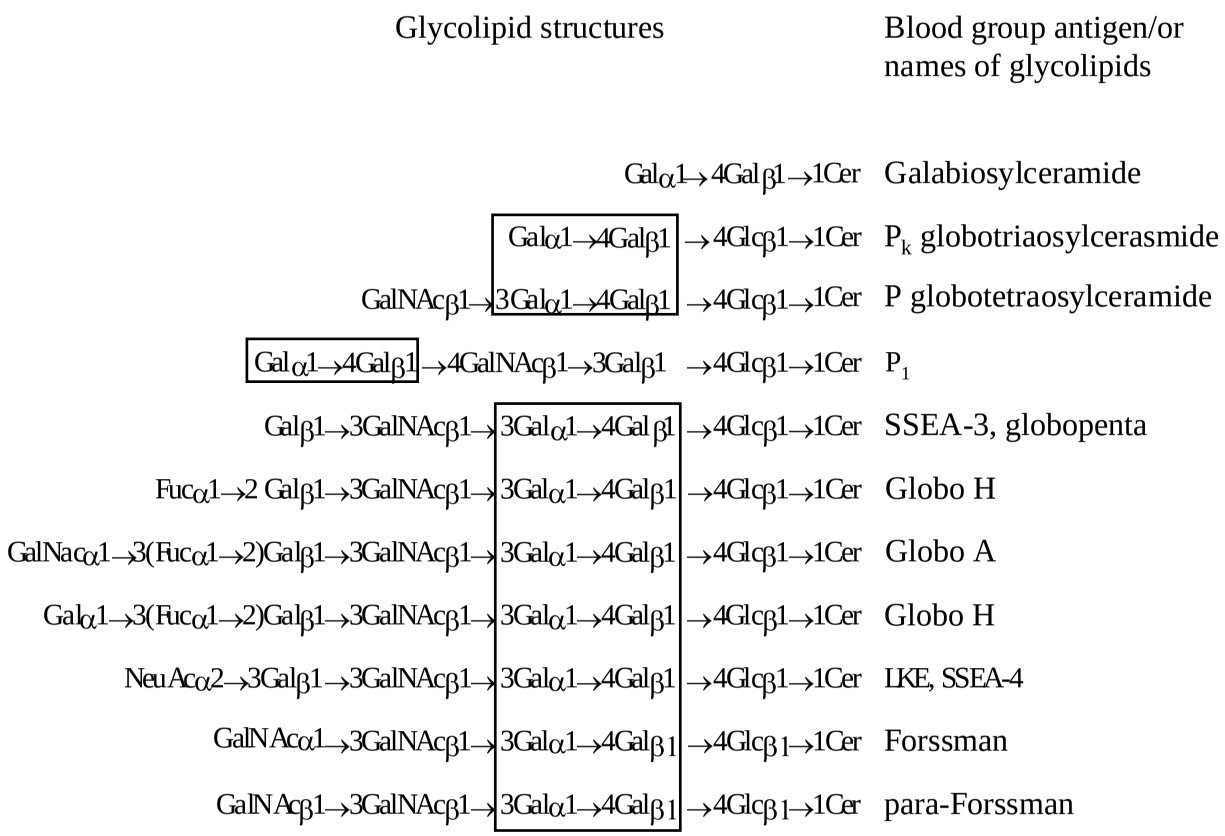

Palmitoyl-CoA + Serine
Sphinganine
Fatty acyl-CoA
Sphingomyelin
Ceramide
Galactosyl-ceramide
UDP-Glucose
NB-DNJ, NB-DGJ
PDMP analogues
UDP-Glucose:
N-acylsphingosine
Glucosyltransferase
UDP
Glucosylceramide
Lactosylceramide
Ganglioseries
Lacto(neo)series
Globoseries

Fig 2


Glycolipid structures
Blood group antigen/or names of glycolipids
Galα1→4Galβ1→1Cer
Galabiosylceramide
Galα1→4Galβ1 →4Glcβ1→1Cer
P$_k$ globotriaosylcerasmide
GalNAcβ1→3Galα1→4Galβ1 →4Glcβ1→1Cer
P globotetraosylceramide
Galα1→4Galβ1→4GalNAcβ1→3Galβ1 →4Glcβ1→1Cer
P$_1$
Galβ1→3GalNAcβ1→3Galα1→4Galβ1 →4Glcβ1→1Cer
SSEA-3, globopenta
Fucα1→2 Galβ1→3GalNAcβ1→3Galα1→4Galβ1 →4Glcβ1→1Cer
Globo H
GalNacα1→3(Fucα1→2)Galβ1→3GalNAcβ1→3Galα1→4Galβ1 →4Glcβ1→1Cer
Globo A
Galα1→3(Fucα1→2)Galβ1→3GalNAcβ1→3Galα1→4Galβ1 →4Glcβ1→1Cer
Globo H
NeuAcα2→3Galβ1→3GalNAcβ1→3Galα1→4Galβ1 →4Glcβ1→1Cer
LKE, SSEA-4
GalNAcα1→3GalNAcβ1→3Galα1→4Galβ1 →4Glcβ1→1Cer
Forssman
GalNAcβ1→3GalNAcβ1→3Galα1→4Galβ1 →4Glcβ1→1Cer
para-Forssman