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The Multiple Carbohydrate Binding Specificities of *Helicobacter pylori*

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Abstract Persistent colonization of the human stomach by *Helicobacter pylori* is a risk factor for the development of peptic ulcer disease and gastric cancer. Adhesion of microbes to the target tissue is an important determinant for successful initiation, establishment and maintenance of infection, and a variety of different candidate carbohydrate receptors for *H. pylori* have been identified. Here the different the binding specifities, and their potential role in adhesion to human gastric epithelium are described. Finally, recent findings on the roles of sialic acid binding SabA adhesin in interactions with human neutrophils and erythrocytes are discussed.

Keywords Adhesin, Carbohydrate binding, Glycoconjugate receptor, *Helicobacter pylori*, Microbial adhesion

Contents

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Abbreviations

BabA	blood group antigen binding adhesin
HpaA	*H. pylori* adhesin A
HP-NAP	neutrophil-activating protein of *pylori*
SabA	sialic acid binding adhesin.

The glycosphingolipid nomenclature follows the recommendations by the IUPAC-IUB Commission on Biochemical Nomenclature (CBN for Lipids: Eur J Biochem 257:293, 1998).

1 Introduction

Half of the world's population is estimated to be colonized with the gastric pathogen *Helicobacter pylori.* However, although infection with *H. pylori* and the associated chronic gastritis are common, only a small fraction of those infected develops any further consequences such as peptic ulcer or gastric adenocarcinoma [1]. Thus, the infection is by itself not sufficient to cause disease and, since the discovery of *H. pylori* by Warren and Marshall in 1983 [2], this bacterium has been subjected to intense studies aimed at characterizing host and bacterial factors associated with pathological sequelae of the infection. The most well-recognized virulence factors identified to date are the urease, the flagellae, the cytotoxin-associated pathogenicity island (*cag*-PAI), the vacuolating cytotoxin (VacA), and adhesins (reviewed in [3]).

Bacterial adherence to mucosal cells of the target tissue is an important virulence trait of pathogenic bacteria. The majority of known microbial attachment sites on host cells and tissues are glycoconjugates. A number of different approaches have been employed for elucidation of carbohydrate receptors for *H. pylori*, such as, e.g., hemagglutination and hemagglutination-inhibition [4], binding to glycosphingolipids separated on thin-layer plates [5, 6], or in situ analysis of the binding of *H. pylori* to human gastric surface mucosal cells [7]. Thereby, at least nine separate carbohydrate binding specificities have been identified, as summarized in Table 1.

It should, however, be noted that despite this multitude of potential carbohydrate receptors, only two *H. pylori* adhesins have been identified to date, i.e., the Le^b-binding adhesin BabA [16], and the sialic acid-binding adhesin SabA [8]. An additional *H. pylori* carbohydrate binding protein is the soluble neutrophil-activating protein

Table 1 Potential *H. pylori* carbohydrate receptors

1. Sialic acid-terminated glycoconjugates	[4, 8, 9]
2. Sulfate-containing carbohydrates	[5, 10]
3. Ganglioseries glycosphingolipids	[6, 11]
4. Fucosylated blood group antigens	[7]
5. NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAc-terminated glycoconjugates	[12]
6. Lactosylceramide	[6]
7. Galactosylceramide/glucosylceramide	[13]
8. Lactotetraosylceramide	[14]
9. Neolacto-glycolipids	[15]

(HP-NAP), which interacts with NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ-terminated glycoconjugates [12].

In the following the various *H. pylori* carbohydrate binding specificities, and identified bacterial adhesins, are first described, followed by a discussion of their role(s) in adhesion of the bacteria to the two target tissues: human gastric epithelium and human neutrophil granulocytes. Finally, recent findings on the roles of sialic acid binding in interactions of *H. pylori* with human neutrophils and erythrocytes are highlighted.

2 Fucosylated Blood Group Antigens

The recognition of the Le^b blood group antigen (Fucα2Galβ3(Fucα4)GlcNAc) by certain strains of *H. pylori* was reported over a decade ago [7]. A combination of in situ analysis of the binding of *H. pylori* to human gastric surface mucosal cells, blocking of binding by pre-incubation of bacteria with neoglycoconjugates, and binding of *H. pylori* to glycoconjugates immobilized on protein blots, was used to elucidate this binding specificity. Subsequently, the cognate *H. pylori* Le^b-binding adhesin BabA (blood group antigen binding adhesin), a 78-kDa protein belonging to the hop family of *H. pylori* outer membrane proteins, was identified by the retagging technique based on its affinity for Le^b [16]. Two genes, *babA1* and *babA2*, both potentially encoding the BabA adhesin were identified. Only the *babA2* gene gives rise to a functional adhesin, since in *babA1* the initiation codon is missing. However, several Le^b non-binding strains possessing silent *babA* gene sequences may become active by recombination into the partially homologous *babB* locus forming chimeric *babB/A* genes [17]. *H. pylori* strains expressing BabA together with the vacuolating cytotoxin VacA and the cytotoxin associated antigen CagA (triple positive strains) are highly associated with severe gastrointestinal diseases, as peptic ulcer or gastric adenocarcinoma [18, 19].

The initial studies were performed using the South American *H. pylori* strain P466, which binds to Le^b (Fucα2Galβ3(Fucα4)GlcNAc-) and H type 1 (Fucα2Galβ3GlcNAc-) blood group determinants, related to the blood group O phenotype [7]. However, investigation of the binding characteristics of a large number of *H. pylori* strains of different geographical origins revealed a different pattern [20]. Of the strains from the South American Amerindian population, 40% bind only to Le^b/H type 1 blood group determinants, which correlates with the unusual predominance of the blood group O phenotype among the Amerindians. In contrast, 95% of the *H. pylori* strains from Europe, Asia and Alaska also recognize the ALe^b (GalNAcα3(Fucα2)Galβ3(Fucα4)GlcNAc-) and BLe^b (Galα3(Fucα2)Galβ3(Fucα4)GlcNAc-) blood group determinants (exemplified in Table 2), demonstrating an adaption of the BabA adhesin to the receptor availability in the local host population.

The BabA adhesins are thus divided into "specialist" BabA adhesins which preferentially bind the Le^b determinant, and "generalist" BabA which also bind the ALe^b and BLe^b determinants, i.e., the "generalist" adhesins tolerate the substitution by an α-linked GalNAc or Gal at the 3-position of the Gal, indicating a variant architecture of the carbohydrate binding site of the BabA adhesin.

Table 2 Comparison of binding of *H. pylori* strains CCUG 17875 and P466 to glycosphingolpids on thin-layer chomatograms

No. trivial name	Glycosphingolipid structure	CCUG 17875	P466
1. H5 type 1	Fucα2Galβ3GlcNAcβ3Galβ4Glcβ1Cer	+[a]	+
2. H5 type 2	Fucα2Galβ4GlcNAcβ3Galβ4Glcβ1Cer	–	–
3. Le[a]-5	Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	–	–
4. X-5	Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	–	–
5. Le[b]-6	Fucα2Galβ3(Fucα4) GlcNAcβ3Galβ4Glcβ1Cer	+++	+++
6. Y-6	Fucα2Galβ4(Fucα3)GlcNAcβ3Galβ4G	–	–
7. B6 type 1	Galα3(Fucα2) Galβ3GlcNAcβ3Galβ4Glcβ1Cer	+	–
8. B6 type 2	Galα3(Fucα2) Galβ4GlcNAcβ3Galβ4Glcβ1Cer	–	–
9. A6 type 1	GalNAcα3(Fucα2) Galβ3GlcNAcβ3Galβ4Glcβ1Cer	+	–
10. B7 type 1	Galα3(Fucα2)Galβ3(Fucα4) GlcNAcβ3Galβ4Glcβ1Cer	+++	–
11. B7 type 2	Galα3(Fucα2)Galβ4(Fucα3) GlcNAcβ3Galβ4Glcβ1Cer	–	–
12. A7 type 1	GalNAcα3(Fucα2)Galβ3(Fucα4) GlcNAcβ3Galβ4Glcβ1Cer	+++	–

[a]Binding is defined as follows: + denotes a weak and +++ a significant darkening on the autoradiogram when 4 μg is applied on the thin-layer plate, while – denotes no binding

3 Lactosylceramide and Ganglioseries Glycosphingolipids

Many *H. pylori* strains, like several other bacteria – both pathogens and commensals [21], also bind to gangliotetraosylceramide (Galβ3GalNAcβ4Galβ4Glcβ1Cer) [11], with a concomitant binding to lactosylceramide (Galβ4Glcβ1Cer), isoglobotriaosylceramide (Galα3Galβ4Glcβ1Cer) and gangliotriaosylceramide (GalNAcβ4Galβ4Glcβ1Cer) [6].

There are conflicting suggestions concerning which part of the gangliotriaosylceramide/gangliotetraosylceramide structures that is recognized by the lactosylceramide-binding bacteria. One alternative is that the lactose saccharide (Galβ4Glcβ) is primarily recognized by the adhesin, and that the extensions specifying the ganglio-series (i.e., the β4-linked GalNAc and Galβ3GalNAc) are tolerated for steric reasons, while other extensions make the lactose epitope inaccessible [21]. The alternate suggestion is that the binding to lactosylceramide and gangliotriaosylceramide/gangliotetraosylceramide represents two separate binding specificities [22].

The gangliotriaosylceramide/gangliotetraosylceramide binding of *H. pylori* was abolished by conversion of the acetamido group of the *N*-acetylgalactosamine to an amine [6]. These results are most compatible with the interpretation that lactosylceramide and gangliotriaosylceramide/gangliotetraosylceramide represent two separate binding specificities. However, binding of *H. pylori* to lactosylceramide is

frequently accompanied by binding to gangliotriaosylceramide and gangliotetraosylceramide, suggesting that the factors controlling the expression of the lactosylceramide-binding adhesin are similar or identical to those which regulate the expression of the gangliotriaosylceramide/gangliotetraosylceramide-binding adhesin.

Lingwood et al. have reported that the gangliotriaosylceramide/gangliotetraosylceramide binding of *H. pylori* also involves a concomitant binding to phosphatidylethanolamine [11], and this glycerolipid was used for affinity isolation of a 63-kDa protein from *H. pylori* with phosphatidylethanolamine and gangliotriaosylceramide/gangliotetraosylceramide binding properties [23]. However, subsequent studies identified this protein as a catalase [24], and thus, most likely, not an adhesin.

Interestingly, by structure similarity searches Fantini et al. recently identified a glycosphingolipid-binding motif in HpaA (*H. pylori* adhesin A; see below) [25]. A synthetic peptide corresponding to this proposed glycosphingolipid-binding motif interacted with lactosylceramide but not with globotriaosylceramide, in line with the glycosphingolipid binding pattern of the whole bacterial cells [6]. The identification of this glycosphingolipid binding motif may allow further dissection of the lactosylceramide binding properties of *H. pylori* and other lactosylceramide-binding bacteria.

The predominant diglycosylceramide of the human gastric epithelium was recently identified as galabiaosylceramide (Galα4Galβ1Cer), a glycosphingolipid not recognized by *H. pylori* bacterial cells [26]. Still, a distinct binding of *H. pylori* in the diglycosylceramide region in one out of seven samples was found upon assaying binding to non-acid glycosphingolipids of human gastric epithelium [6]. By mass spectrometry of the binding-active glycosphingolipid sample dihexosylceramides both sphingosine and phytosphingosine and both hydroxy and non-hydroxy fatty acids were identified. This *H. pylori*-binding non-acid glycosphingolipid fraction most likely contained lactosylceramide, indicating that there are individual differences in the composition of diglycosylceramides in the human gastric epithelium. Galabiosylceramide is present in all individuals, but the relative amount of lactosylceramide varies, and only a few individuals have enough lactosylceramide to allow detection of *H. pylori* binding in the chromatogram binding assay.

4 Sulfated Carbohydrates

In the first report of *H. pylori* glycosphingolipid binding, Saitoh et al. demonstrated binding of the bacteria to the two major acid glycosphingolipids of human stomach, i.e., the GM3 ganglioside (NeuAcα3Galβ4Glcβ1Cer) and sulfatide (SO_3–3Galβ1Cer), by binding of *H. pylori* to glycosphingolipids separated on thin-layer chromatograms [5]. However, the GM3 binding was later in refuted in a study using KATO III cells, where only sulfatide binding was obtained [27]. The heat-shock protein Hsp 70, which can be induced at low pH, has been proposed to be the adhesin involved in the binding of *H. pylori* to sulfatide [28, 29]. The neutrophil-activating protein HP-NAP

is to some extent associated with the bacterial cell surface [10], and may also be involved in binding of *H. pylori* to sulfated carbohydrates, as described below.

In addition, a majority of *H. pylori* strains bind to heparan sulfate with high affinity [30], and a battery of heparan sulfate binding bacterial proteins have been described [14, 31].

5 Lactotetraosylceramide

An *H. pylori*-binding non-acid glycosphingolipid of human meconium was initially isolated, and characterized by mass spectrometry and proton NMR as lactotetraosylceramide (Galβ3GlcNAcβ3Galβ4Glcβ1Cer) [32]. Subsequently, lactotetraosylceramide was also identified in human gastric epithelial cells, in one out of seven samples. Enzymatic removal of the terminal galactose of lactotetraosylceramide abolished the binding, as did hydrazinolysis of the acetamido group of the *N*-acetylglucosamine (Table 3), indicating that the *H. pylori* binding epitope is the Galβ3GlcNAcβ sequence.

The Le^b determinant (Fucα2Galβ3(Fucα4)GlcNAcβ) is based on the type 1 disaccharide unit, which is the terminal part of lactotetraosylceramide. However, *H. pylori* strains devoid of Le^b-binding BabA (e.g., the 26695 strain and the MO19 strain) recognized lactotetraosylceramide, and inactivation of the *bab*A gene had no influence on lactotetraosylceramide binding (Fig. 1). Thus, the binding of *H. pylori* to the Le^b determinant and to lactotetraosylceramide are two separate binding specificities.

The blood group status of the individual with *H. pylori*-binding gastric lactotetraosylceramide was ALe(a+b−)non-secretor, in agreement with the presence of unsubstituted lactotetraosylceramide in this individual. This non-secretor status is interesting in view of the increased prevalence of duodenal ulcer among non-secretors [33]. Non-secretion is not associated with increased susceptibility to infection with *H. pylori* [13]. However, a speculative theory is that the secretor status determines the outcome of the colonization, i.e., that the increased liability of non-secretors to develop peptic ulcer disease is due to the presence of the *H. pylori*-binding lactotetraosylceramide on the gastric epithelium of these individuals.

Table 3 Comparison of lactotetraosylceramide-related binding preferences of *H. pylori*

No. trivial name	Glycosphingolipid structure	Binding
1. Lactotri	GlcNAcβ3Galβ4Glcβ1Cer	–[a]
2. Lactotetra	Galβ3GlcNAcβ3Galβ4Glcβ1Cer	+
3.	$Gal\beta3GlcNH_2\beta3Gal\beta4Glc\beta1Cer$	–
4. Le^a-5	Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	–
5.	Galα3Galβ3GlcNAcβ3Galβ4Glcβ1Cer	–
6.	NeuGcα3Galβ3GlcNAcβ3Galβ4Glcβ1Cer	–
7.	NeuAcα6Galβ3GlcNAcβ3Galβ4Glcβ1Cer	–

[a]Binding is defined as follows: + denotes a binding when 2 μg of the glycosphingolipid is applied on the thin-layer chromatogram, while – denotes no binding even at 4 μg

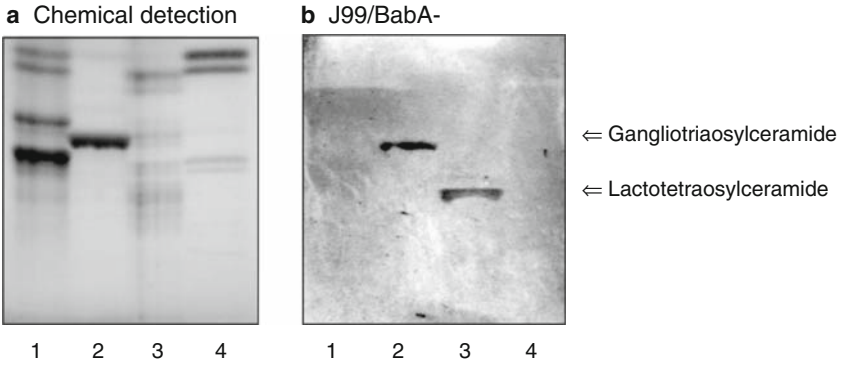

Fig. 1 Binding of a mutant *Helicobacter pylori* strain with deletion of the BabA adhesin (J99/BabA-) to glycosphingolipids on thin-layer chromatograms. Thin-layer chromatogram with separated glycosphingolipids after chemical detection with anisaldehyde (**a**), and autoradiogram after binding of ^{35}S-labeled *H. pylori* strain J99/BabA- (**b**). The lanes were: Lane 1, non-acid glycosphingolipids of human blood group O erythrocytes, 40 μg; Lane 2, non-acid glycosphingolipids of guinea pig erythrocytes, 40 μg; Lane 3, non-acid glycosphingolipids of human meconium, 40 μg; Lane 4, non-acid glycosphingolipids of human neutrophils, 40 μg. The *arrows* denote the relative mobilities of gangliotriaosylceramide (present in lane 2) and lactotetraosylceramide (present in lane 3)

The lactotetraosylceramide binding property was present in the majority (88%) of *H. pylori* isolates tested, and thus a conserved property of this gastric pathogen, indicating that it is an important virulence factor. However, further definition of the role of the lactotetraosylceramide binding capacity in colonization and disease development must await the identification of the corresponding adhesin.

6 Galactosylceramide and Glucosylceramide

Abul-Milh et al. have reported that *H. pylori* bacterial cells bind to galactosylceramide with sphingosine and both hydroxy and non-hydroxy fatty acids [15]. They also found that when the chromatogram binding assay was performed under microaerobic conditions, intended to keep the bacteria viable, *H. pylori* bound to glucosylceramide with sphingosine and hydroxy fatty acids. The galactosylceramide/glucosylceramide adhesin(s) has not yet been identified.

7 Neolacto Glycosphingolipids

A majority of *H. pylori* strains also binds to the neolacto (Galβ4GlcNAcβ) core structure, with a preferential binding to carbohydrate chains with repetitive neolacto elements [34]. Enzymatic and chemical degradation of sialylneolactohexaosylceramide (NeuGcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer) showed that the preferred binding epitope in this context is the GlcNAcβ3Galβ4GlcNAcβ sequence.

The terminal β3-linked GlcNAc can be exchanged for GalNAcβ3, GalNAcα3 or Galα3 without loss of *H. pylori* binding. Calculated minimum energy conformations demonstrated topographical similarities in the spatial orientation of these trisaccharides, despite the different terminal substituents and anomeric configuration, which makes it reasonable that they may be accommodated within the same binding site. However, the adhesin mediating neolacto binding still awaits identification.

8 Sialic Acid-Terminated Glycoconjugates

Binding of *H. pylori* to sialylated glycoconjugates was initially identified by Evans et al. by hemagglutination studies [4], and a potential sialyllactose-binding protein, denoted HpaA (*H. pylori* adhesin A), was later characterized [35]. However, subsequent studies showed that HpaA is a lipoprotein, that deletion of the *hpaA* gene did not eliminate cell binding or hemagglutination, and that the HpaA protein was either located in the cytosol [36] or on the flagellar sheath [37]. The role of HpaA as a sialic acid binding adhesin has therefore been questioned.

A sialic acid dependent binding of *H. pylori* to the extracellular matrix protein laminin, mediated by a 25 kDa outer membrane protein, has also been described [38].

8.1 The Sialic Acid Binding Adhesin (SabA)

Following the identification of the BabA adhesin it was observed that deletion of *babA* did not abolish all *H. pylori* binding to human gastric tissue sections [8]. A further observation made was that this *babA* deletion strain bound to gangliosides of human neutrophils and human adenocarcinomas. Subsequently, a novel high affinity receptor for *H. pylori* was isolated from a human gall bladder adenocarcinoma and characterized as sialyl-dimeric-Le^x (NeuAcα3Galβ4(Fucα3) GlcNAcβ3Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer). The sialic acid binding adhesin of *H. pylori* (SabA) was identified by re-tagging and proteomics-based mass spectrometry techniques using sialyl-Le^x-BSA as probe. SabA, like BabA, belongs to the hop family of *H. pylori* outer membrane protein. The *sabA* gene is frequent among *H. pylori* isolates, but the expression of the SabA adhesin is highly subjected to phase-variation [8, 39].

The structural requirements for SabA-mediated *H. pylori* ganglioside binding was investigated by using a library of variant gangliosides [9]. The wild type SabA-expressing bacteria bound to *N*-acetyllactosamine-based gangliosides with terminal α3-linked NeuAc, while gangliosides of the ganglioseries, or *N*-acetyllactosamine-based gangliosides with terminal NeuAcα6, NeuGcα3, or NeuAcα8NeuAcα3 were not recognized (summarized in Table 4).

Table 4 Comparison of ganglioside binding of SabA-expressing *H. pylori* strains and *H. pylori* neutrophil-activating protein HP-NAP

No. trivial name	Structure	SabA+ *H. pylori*	HP-NAP
1. NeuAc-GM3	NeuAcα3Galβ4Glcβ1Cer	–[a]	–
2. NeuAc-GM1	Galβ3GalNAcβ4(NeuAcα3)Galβ4Glcβ1Cer	–	–
3. NeuAc-GD1a	NeuAcα3Galβ3GalNAcβ4 (NeuAcα3)Galβ4Glcβ1Cer	–	–
4. NeuAc-GD1b	Galβ3GalNAcβ4(NeuAcα8 NeuAcα3)Galβ4Glcβ1Cer	–	–
5. NeuAc-GT1b	NeuAcα3Galβ3GalNAcβ4 (NeuAcα8NeuAcα3)Galβ4Glcβ1Cer	–	–
6. NeuAcα3SPG	NeuAcα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+	–
7. NeuAcα6SPG	NeuAcα6Galβ4GlcNAcβ3Galβ4Glcβ1Cer	–	–
8. NeuGcα3SPG	NeuGcα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	–	–
9. NeuAc-DPG	NeuAcα8NeuAcα3Galβ4Glc NAcβ3Galβ4Glcβ1Cer	–	–
10. NeuAcα3-Le[a]	NeuAcα3Galβ3(Fucα4) GlcNAcβ3Galβ4Glcβ1Cer	–/+[b]	–
11. NeuAcα3-Le[x]	NeuAcα3Galβ4(Fucα3) GlcNAcβ3Galβ4Glcβ1Cer	+	–
12. NeuAcα3-nLc$_6$	NeuAcα3Galβ4GlcNAcβ3Galβ 4GlcNAcβ3Galβ4Glcβ1Cer	+	+
13. NeuGcα3-nLc$_6$	NeuGcα3Galβ4GlcNAcβ3Galβ 4GlcNAcβ3Galβ4Glcβ1Cer	–	+
14. NeuAcα3-nLc$_8$	NeuAcα3Galβ4GlcNAcβ3Galβ4Glc NAcβ3Galβ4GlcNAcβ 3Galβ4Glcβ1Cer	+	+
15. NeuGcα3-nLc$_8$	NeuGcα3Galβ4GlcNAcβ 3Galβ4Glc NAcβ3Galβ4GlcNAcβ 3Galβ4Glcβ1Cer	–	+
16. VIM-2	NeuAcα3Galβ4GlcNAcβ 3Galβ4(Fucα3) GlcNAcβ3Galβ4Glcβ1Cer	+	–
17. NeuAcα3-dimer-Le[x]	NeuAcα3Galβ4(Fucα3) GlcNAcβ3Galβ4 (Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	+	–
18. NeuAc-G-10	NeuAcα3Galβ4GlcNAcβ6(NeuAcα3 Galβ4GlcNAcβ3) Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+	–
19. NeuGc-G-10	NeuGcα3Galβ4GlcNAcβ 6(NeuGcα3Galβ4GlcNAcβ3) Galβ4GlcNAcβ3Galβ4Glcβ1Cer	–	–
20.	NeuGcα3Galβ4GlcNAcβ 6(NeuGcα3Galβ4GlcNAcβ3) Galβ4GlcNAcβ3Galβ4Glcβ1Cer	–	–
21. G9-B	Galα3(Fucα2)Galβ4GlcNAcβ 6(NeuAcα3Galβ4GlcNAcβ3) Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+	–

[a]Binding is defined as follows: + denotes a binding when 2 μg of the glycosphingolipid is applied on the thin-layer chromatogram, while – denotes no binding even at 4 μg

[b]Binding to NeuAcα3-Le[a] (No. 10) is variable, i.e., dependent on the *H. pylori* strain used [39, 63]

Several studies have shown that the minimal epitope for SabA-mediated *H. pylori* binding to gangliosides is NeuAcα3Gal [4, 40, 41]. However, comparative binding studies [39] showed that an increased binding affinity is obtained by:

1. Increased length of *N*-acetyllactosamine core chain: NeuAcα3nLc8 (NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer) >> NeuAcα3nLc6 (NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer) > NeuAcα3SPG (NeuAcα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer). This effect is most likely due to an improved accessibility of NeuAcα3Gal epitope when presented on a longer core chain.
2. Branches of the carbohydrate chain: NeuAc-G-10 (NeuAcα3Galβ4GlcNAcβ6(NeuAcα3Galβ4GlcNAcβ3)Galβ4GlcNAcβ3Galβ4Glcβ1Cer) >> NeuAcα3nLc6), giving a divalent presentation of the binding epitope.
3. Fucose substitution of the *N*-acetyllactosamine core chain: NeuAcα3-dimeric-Le^x (NeuAcα3Galβ4(Fucα3)GlcNAcβ3Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer) > VIM-2 (NeuAcα3Galβ4GlcNAcβ3Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer) >> NeuAcα3nLc6). The fucose residues of NeuAcα3-dimeric-Le^x and the VIM-2 ganglioside may either interact with the carbohydrate binding site of the SabA adhesin, or affect the conformation of the ganglioside providing an optimal presentation of the NeuAcα3Gal head group.

8.2 H. Pylori Neutrophil-Activating Protein (HP-NAP)

The *H. pylori* neutrophil-activating protein HP-NAP was first isolated from water extracts of bacterial cells, and by PCR analyses the gene for this protein was found in all strains analyzed [42]. The name neutrophil-activating derives from the capacity of HP-NAP to promote production of reactive oxygen radicals in human neutrophil granulocytes, and upregulate CD11b/CD18 on the neutrophil surface leading to increased adhesion of the neutrophils to endothelial cells. In addition, HP-NAP is an iron-binding protein [43], and has a role in protection of bacterial DNA against oxidative stress [44].

The potential carbohydrate recognition by HP-NAP was investigated by binding to glycosphingolipid fractions from various sources on thin-layer chromatograms [12]. A distinct binding to two compounds in the acid fraction of human neutrophil granulocytes (the target cells of HP-NAP) was thereby obtained, and the binding-active gangliosides were subsequently identified as NeuAcα3neolactohexaosylceramide (NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer) and NeuAcα 3neolactooctaosylceramide (NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer) (Table 4).

Other HP-NAP binding compounds, not found in the human target white cells, were NeuGcα3neolactohexaosylceramide (NeuGcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer), NeuGcα3neolactooctaosylceramide (NeuGcα3Gal

β4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer), and sulfate-containing glycosphingolipids, as sulfatide (SO_3–3Galβ1Cer) and sulfated gangliotetraosylceramide (SO_3–3Galβ3GalNAcβ4Galβ4Glcβ1Cer). The interaction with sulfated glycosphingolipids was dependent on the sulfate group, since no binding to the corresponding non-sulfated compounds (galactosylceramide and gangliotetraosylceramide, respectively) occurred. Binding of HP-NAP to sulfated oligosaccharides, as SO_3–3Gal, SO_3–3GlcNAc and sulfated Lewis^a of human high-molecular weight salivary mucins, was subsequently demonstrated by Namavar et al. [45].

To delineate further the structural requirements for HP-NAP carbohydrate binding, NeuGcα3neolactohexaosylceramide has been chemically and enzymatically modified (Fig. 2) (ST, unpublished data). First, the terminal sialic acid is necessary for the interaction, since no binding to the de-sialylated compound was obtained (lane 6). Binding was also lost upon converting C(1) of NeuGc into an alcohol (lane 2). However, the C(1)-amide and C(1)-benzylamide were binding-active (lanes 3 and 4), indicating that the hydrogen binding capacity of a carboxyl or amide group at C(1) is necessary for binding to occur.

The partial de-N-acetylation (lane 5) gave rise to at least two band migrating below NeuGca3neolactohexaosylceramide, corresponding to variants with modified sialic acid and/or *N*-acetylglucosamines [46]. HP-NAP bound only to the remaining unmodified NeuGcα3neolactohexaosylceramide, indicating a role for the *N*-acetylglucosamine(s) in the interaction.

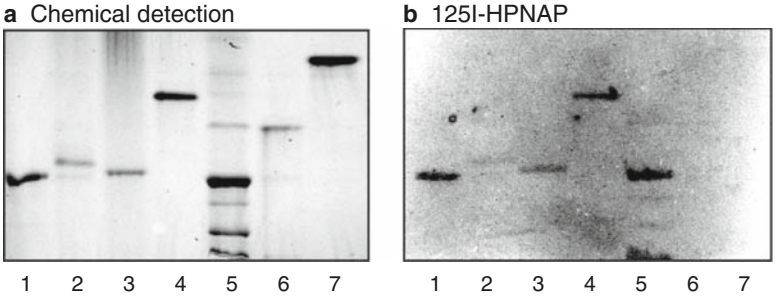

Fig. 2 Binding of ^{125}I-labeled neutrophil-activating protein from *Helicobacter pylori* (HP-NAP) to derivatized NeuGcα3neolactohexaosylceramide (NeuGcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer) on thin-layer chromatograms. Glycosphingolipids detected with anisaldehyde (**a**) and autoradiogram obtained after binding of ^{125}I-labeled neutrophil-activating protein HP-NAP (**b**). Lanes: Lane 1, No. 1 NeuGcα3neolactohexaosylceramide (NeuGcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer), 2 μg; Lane 2, alcohol produced from No. 1 (R–COOH → R–CH_2OH), 2 μg; Lane 3, primary amide produced from No. 1 (R–COOH → R–$CONH_2$), 2 μg; Lane 4, benzylamide produced from No. 1 (R–COOH → R–CONH–$CH_2C_6H_5$), 2 μg; Lane 5, products from de-N-acetylation of No. 1, 2 μg; Lane 6, de-sialylated No. 1 (Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer), 2 μg; Lane 7, reference globoside (GalNAcβ3Galα4Galβ4Glcβ1Cer), 2 μg

HP-NAP is to date the only *H. pylori* carbohydrate binding protein that has been subjected to structural studies, although not in complex with carbohydrates. The neutrophil-activating protein forms a dodecameric structure arranged in a nearly spherical shell with a central iron-containing cavity [47]. The structure of HP-NAP has similarities to dodecameric bacterial ferritins (Dps-like family). However, unlike the Dps proteins, a large number of positively charged residues are found on the surface of HP-NAP.

9 Potential Roles of the Carbohydrate Binding Specificities in Adhesion to Target Cells

9.1 The Human Gastric Epithelium

H. pylori has a very narrow host and tissue range and has only been found in connection with gastric epithelium from humans and monkeys [48]. In the *H. pylori*-infected stomach the majority of the bacteria are found within the gastric mucus layer, while approximately 20% of the *H. pylori* population are found attached to the gastric epithelial cells.

While some of the putative *H. pylori* carbohydrate receptor sequences are found in a variety of human cells, some have hitherto not been identified in human tissues. An example of the latter group is isoglobotriaosylceramide, which has been characterized in, e.g., dogs, cats, and rats, but not in humans. Similarly, gangliotriaosylceramide or gangliotetraosylceramide have not been chemically identified in peripheral human tissues.

In contrast, galactosylceramide, glucosylceramide, sulfatide and lactosylceramide are almost ubiquitous glycosphingolipids, and expressed in a large variety of cells [49]. This broad distribution makes these glycosphingolipids unlikely as determinants of target tissue specificity. However, once the tissue specific binding has been established, a second-step binding to these relatively short glycosphingolipids may confer a more membrane-close attachment, and lead to increased concentrations of toxins and other effector molecules at the cell membrane.

Neolacto-containing glycosphingolipids are also found in several human tissues, such as erythrocytes [49], granulocytes [50–52], placenta [53] and semen [54], and the terminal neolacto sequence is also a common core structure in carbohydrate chains of glycoproteins [55]. However, the *H. pylori*-binding neolacto epitope has not been found in human gastric epithelial cells, and although polylactosamine chains are present in both non-acid glycosphingolipids and in glycoproteins of human neutrophil granulocytes no binding of the bacteria to these compounds occurs, most likely due to steric hindrance from branches [34]. Further studies are thus needed to establish the function for *H. pylori* neolacto binding.

Lactotetraosylceramide, on the other hand, has only been chemically identified in the human gastrointestinal tract, i.e., in the gastric epithelium [32], in human meconium [56], and in the small intestine of one individual after surgery for peptic ulcer disease [57].

The Le^b/ALe^b/BLe^b determinants are also expressed in the human gastrointestinal tract [58, and references therein], and in the human stomach present on the surface mucous cells [7], and on the human gastric mucin MUC5AC [59], both of which are recognized by BabA-expressing strains in a Le^b-dependent manner.

Thus, both the Le^b epitope and lactotetraosylceramide are human specific and present in the gastric epithelial cells, and are candidate determinants of target tissue tropism.

The normal human gastric epithelium has a low content of sialylated glycoconjugates [60]. However, upon inflammation a shift in glycosylation occurs with an upregulated expression of *H. pylori*-binding sialyl-Le^x-containing glycoconjugates [8]. Induction of sialyl-Le^x-containing glycoconjugates in the gastric epithelium also occurs in experimental *H. pylori*-infection of Rhesus monkeys. This glycosylation shift may be relevant for the maintenance of a chronic infection by mediating the adhesion of bacteria to the epithelium in the already inflamed stomach.

9.2 Role of SabA in Activation of Human Neutrophils

As described above, two sialic acid-binding proteins of *H. pylori* were identified, i.e., HP-NAP and the SabA adhesin. Since both bind to NeuAcα3-neolactohexaosylceramide and NeuAca3neolactooctaosylceramide, their binding patterns are overlapping (Table 4). Their relative roles in ganglioside binding were investigated by using knock-out mutant strains deleted of the sialic acid binding adhesin SabA, or the NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ-binding neutrophil activating protein HPNAP [9]. No ganglioside binding was obtained with the mutant strain with deletion of the *sabA* gene, while the HP-NAP knock-out strain had a ganglioside binding capacity identical to the parent wild type strain. Thus the binding of *H. pylori* bacterial cells to gangliosides is mediated by the SabA adhesin only.

Upon *H. pylori* infection, inflammatory infiltrates consisting mainly of neutrophils and monocytes are found in the gastric mucosa, and certain *H. pylori* strains nonopsonized stimulate neutrophils to production of reactive oxygen species causing oxidative damage of the gastric epithelium [61]. Several of the *H. pylori* binding gangliosides, as sialylα3-neolactotetraosylceramide, sialylα3-neolactohexaosylceramide, sialylα3-neolactooctaosylceramide, the VIM-2 ganglioside and sialyl-dimeric-Le^x glycosphingolipid, are found in human neutrophils [51, 52], suggesting that the ganglioside binding capacity might be involved in neutrophil activation. Indeed, the *H. pylori*-induced neutrophil oxidative burst reaction was partly inhibited by preincubation with sialylated oligosaccharides [62]. Thus, nonopsonic *H. pylori*-induced activation of human neutrophils occurs by lectinophagocytosis, i.e., recognition of sialylated glycoconjugates on the neutrophil cell surface by a bacterial adhesin leads to the phagocytosis and the oxidative burst reaction.

The relative contributions of SabA and HP-NAP in neutrophil activation was investigated using a battery of *H. pylori* deletion strains [63].

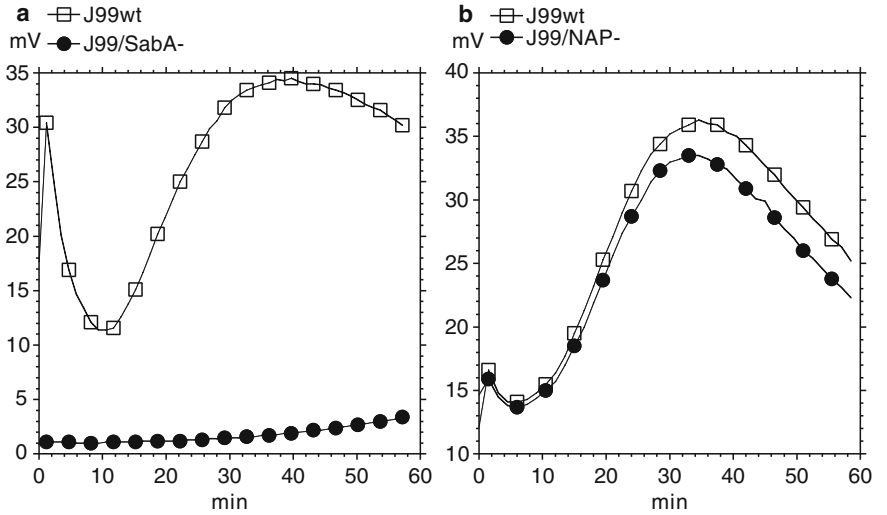

Fig. 3 Luminol-enhanced chemiluminescence of human neutrophils challenged by nonopsonized wild type *Helicobacter pylori* strain J99 (J99 wt), and its isogenic mutants with deletions of the SabA adhesin (J99/SabA-) and HPNAP (J99/NAP-)

Mutant and wild type strains lacking SabA had no neutrophil-activating capacity (Fig. 3a), while the absence of the neutrophil-activating protein HP-NAP (Fig. 3b), or the Le^b-binding BabA had no effect on the neutrophil activation. Thus, SabA-mediated binding of *H. pylori* bacterial cells to sialylated neutrophil receptors plays an important initial role in the adherence and phagocytosis of the bacteria and the induction of the oxidative burst. The events following binding of *H. pylori* to sialylated neutrophil cell surface glycoconjugates leading to NADPH oxidase activation involve a G-protein-linked signaling pathway and downstream activation of PI3-kinase, as shown by experiments using inhibitors of intracellular signaling.

9.3 SabA: The Sialic Acid Dependent Hemagglutinin

The *H. pylori* sialic acid binding was initially discovered by hemagglutination studies in 1988 [4]. Still, the sialic binding hemagglutinin has remained elusive throughout the years. However, recently hemagglutination studies using *sabA* deletion mutants demonstrated that SabA is the sialic acid-dependent hemagglutinin of *H. pylori* [64]. A polymorphic binding of clinical isolates so sialylated glycans was found with variant preferential binding to sialyl-dimeric-Le^x (NeuAcα3Galβ4(Fucα3) GlcNAcβ3Galb4(Fucα3)GlcNAcβ), sialyl-Le^a (NeuAcα3Galβ3(Fucα4)GlcNAcβ) and sialyl-*N*-acetyllactosamine (NeuAcα3Galβ4GlcNAcβ). Protease treatment of the erythrocytes showed that *H. pylori*-mediated hemagglutination mainly occurs through interactions with erythrocyte glycosphingolipids.

Interestingly, in situ hybridization demonstrated *H. pylori* bacterial cells associated with erythrocytes in capillaries of the gastric mucosa in infected humans and in Rhesus monkeys, suggesting that the bacteria can reach the gastric mucosal capillaries, attach to the erythrocytes, and may ultimately disseminate into the circulation.

10 Concluding Remarks

The carbohydrate binding of *H. pylori* is still a complex situation with many postulated receptors. The picture is further confounded by the use of different strains, phase-variation, and use of different methods. The complex carbohydrate recognition pattern identified might partly be a result of the intense research efforts directed towards *H. pylori*. A parallel case is the multiple adhesive mechanisms described for *Pseudomonas aeruginosa*, another bacterium subjected to intense studies [65, and references therein].

The major part of the potential *H. pylori* carbohydrate receptors are orphans in the sense that no corresponding adhesin has yet been identified. An unusually high proportion (1%) of identified open reading frames of the genome sequences of *H. pylori* strains 26695 and J99 is predicted to encode outer membrane proteins (OMPs) [66], which may represent hitherto unidentified adhesins. Characterization of the adhesins corresponding to the orphan carbohydrate receptors, along with studies with deletion mutants, are urgently needed in order to establish the biological role(s) of these candidate carbohydrate receptors.

H. pylori infection tends to persist for life despite the gastric inflammatory response. This persistent infection requires a balanced host-bacterial interaction, with continuous adaption of bacterial binding properties to match the dynamic changes of glycosylation of the gastric epithelium. This may be achieved by phase variation as described for SabA [8], or by recombination events as described for BabA [17].

While patients with higher densities of Le^b in the gastric epithelium have higher *H. pylori* loads [67, 68], the presence of sialyl-Le^x is correlated with higher colonization density in patients lacking gastric Le^b [69]. Thus, the sialic acid binding capacity of *H. pylori* may have multiple roles. First, it mediates adhesion of bacteria to the gastric epithelium, and the binding capacity is augmented by the inflammation-induced up-regulation of sialylated glycoconjugates in the gastric epithelium. Second, binding of *H. pylori* to sialylated neutrophil receptors leads to neutrophil activation to an oxidative burst reaction, with production of reactive oxygen metabolites, and release of biologically active enzymes, giving rise to further tissue damage. Third, the sialic acid dependent hemagglutinating capacity of *H. pylori* may be related to the intriguing finding of the bacteria associated with erythrocytes in capillaries of the human gastric mucosa. These findings merit further investigations in view of the suggested association of *H. pylori* infections and extra-gastrointestinal diseases, such as, e.g., coronary heart disease [70].

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