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MINI REVIEW

The human gastric colonizer *Helicobacter pylori*: a challenge for host–parasite glycobiology

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The Gram-negative bacterium *Helicobacter pylori* was first described in 1983 and currently represents one of the most active single research topics in biomedicine. It is specific for the human stomach and chronically colonizes a majority of the global population, which results in a symptom-free local inflammation. In 10–20% of carriers, gastroduodenal disease develops, including gastric or duodenal ulcer, and atrophic gastritis, which is a precondition to gastric cancer. A probable long coevolution of microbe and *homo sapiens* in a restricted niche has apparently generated a complex and sophisticated interplay. Access to complete bacterial genome sequences assists in a comparative functional characterization. A dynamic glycosylation of both microbe and host cells is of growing interest to analyze. Several glycoforms of bacterial surface lipopolysaccharides show advanced molecular mimicry of host epitopes and a distinct phase variation. An unusually large family of 32 outer membrane proteins probably reflects the complex interrelationship with the host. The unique diversity found for carbohydrate-binding specificities may be mediated by these surface proteins, of which the Lewis b–binding adhesin is the only known example so far, and these binding activities are subject to phase variation. The host mucosa glycosylation may also vary with different conditions, allowing a modulated crosstalk between microbe and host. The bacterium actively stimulates the host inflammatory response, apparently for nutritional purposes, and there is no evidence for a spontaneous elimination of the microbe. Colonization appears to be preventive for upper stomach and esophageal diseases. Current antibiotic treatment eradicates the microbe and cures ulcer disease. Alternative approaches must, however, be developed for a potential global prevention of disease.

Key words: Helicobacter pylori/microbial adhesion/ lipopolysaccharide/adhesin/disease

Recent discovery with important implications

The first isolation of *Helicobacter pylori* from human stomach less than 20 years ago (Warren and Marshall, 1983; Marshall and Warren, 1984) has led to dramatic changes in clinical gastroenterology and revealed the most sophisticated colonizer of humans. Rational treatment has now replaced lots of milk and antacids which was the standard recommended care for peptic ulcer. The field is currently one of the most active single topics in biomedicine (Doolitle, 1997) with more than 1000 papers published during 1999. Exciting and unexpected characteristics are being described (Blaser, 1996; Dunn *et al.*, 1997; Covacci *et al.*, 1999; Westblom *et al.*, 1999), and a dynamic glycobiology of the microbe–host interface is of rapidly growing relevance for our full understanding of *H.pylori* colonization.

Global occurrence and life-long persistence

More than half of the global human population is colonized in the stomach by *H.pylori*, the human-specific spiral-shaped Gram-negative bacterium that escaped detection so long. In developing countries, 70–90% of the population carry the bacterium, which is acquired before the age of 10 and persists through life (Dunn *et al.*, 1997). In developed countries the prevalence of colonization ranges from 25–50%. However, microbiological and epidemiological evidence indicates that *H.pylori* perhaps once was universal, but has gradually disappeared as industrialization has proceeded. In fact, in developed countries, less than 10% of the children are becoming infected today (Dunn *et al.*, 1997). There is an interesting global distribution of bacterium and human with an overlap of geographically isolated human and *H.pylori* populations (Covacci *et al.*, 1999). This supports the hypothesis that *H.pylori* was established in human stomach before the migrations of anatomically modern humans and followed humans thereafter. The mode of transmission is not clear, but the organism is probably transmitted directly from person to person and the family is therefore the core reservoir. Children chronically maintain the same strain, and subsequent colonization with other strains is rare.

Colonization is mostly nonsymptomatic but may induce serious disease

All colonized individuals develop chronic gastric inflammation, but this condition is usually asymptomatic (Dunn *et al.*, 1997). Apparently, a sophisticated adaptation of the persistent microbe–host interaction exists (Blaser and Kirschner, 1999), where the major nutrient source for the bacterium may result from the inflammation, which is kept by the host at a limiting level to reduce bacterial growth and avoid disease. In 10–20% of infected individuals the end result of colonization can be life-threatening. However, the factors behind this are still unclear, and the relation between bacterial and host parameters

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and clinical outcome is probably the most dynamic area in current *H.pylori* studies. In any population, *H.pylori* causes the majority of both gastric and duodenal ulcers. Carriage is also strongly associated with the risk to develop atrophic gastritis, which is a precursor lesion to gastric cancer, the second leading cause of cancer death in the world. In 1994 the WHO International Agency for Cancer Research declared *H.pylori* a human carcinogen of the highest class. Also, gastric mucosa-associated lymphoid tissue lymphoma, MALT lymphoma, is strongly associated with carriage of *H.pylori*. There are current investigations on the relation of other potential nongastric conditions and colonization with *H.pylori*, including atherosclerosis, allergic and autoimmune diseases, anemia and growth retardation (Covacci *et al.*, 1999; Gasbarrini and Franceschi, 1999).

As the bacterium has been reduced in developed countries, peptic ulcer disease and noncardia gastric cancers have been decreasing. However, diseases such as gastroesophageal reflux, Barret esophagus, and adenocarcinomas of the lower esophagus and gastric cardia have been progressively increasing, raising the question if this is related to the reduction of *H.pylori* infections (Blaser, 1999a,b). Therefore, *H.pylori* colonization may have positive effects, and the microbe may be considered more as a normal gastric probiotic as long as disease does not develop. This has a parallel to other microbes, which may be "amphibiotic" organisms that may be either beneficial or disease-causing, depending on the conditions (Blaser, 1997). A sophisticated supplementation after eradication (see *Current therapy is not suitable for a large-scale prevention*) may be a re-colonization with selected *H.pylori* strains to patients to reduce this risk (Blaser, 1999a). Interestingly, a growing number of novel variants of *Helicobacter* are being described in human and animal gastrointestinal tracts (Wadström and Hänninen, 1999; Westblom *et al.*, 1999). One may note the exciting recent discovery that *H.pylori* produces antibacterial peptides to which *H.pylori* is resistant (Putsep *et al.*, 1999a,b).

Genome sequences with essential information

Complete genome sequences have been determined for two unrelated *H.pylori* strains (Tomb *et al.*, 1997; Alm *et al.*, 1999), and interesting functional consequences have recently been discussed in detail on a comparative basis (Alm and Trust, 1999; Doig *et al.*, 1999; Ge and Taylor, 1999; Marais *et al.*, 1999). The genome size is about one third that of *E.coli* and similar to *H.influenzae*, probably reflecting an adaptation to a restricted ecological niche. The two sequenced strains show relatively conserved genomic organizations; 1406 open readings frames are in common, and only 89 and 117, respectively, are strain-specific. A comparison with complete genome sequences of *E.coli* and *H.influenzae* indicated that as much as 63% of the *H.pylori*-specific gene products are potentially involved in *H.pylori*–host interaction. One provoking feature is that about 1% of the genome encodes a unique family of 32 outer membrane proteins, OMPs, to which three identified adhesins belong (see *The glycobiology of bacterial-host interplay is sophisticated and complex*). The significantly higher percentage of its coding capacity for OMPs compared to other bacteria may correspond to the unique complexity of carbohydrate-binding specificities that has been detected for *H.pylori* (see *Sialic acid binding specificity* and *Other binding specificities*). Other virulence factors of interest for colonization and survival in the human stomach are urease for buffering the gastric acid, flagella to swim through the mucus, vacuolating cytotoxin (VacA), neutrophil-activating protein (Hp-NAP, see *Bacterial contact with neutrophils is not suicidal*), and *cag*-PAI pathogenicity island encoding for the immunodominant CagA protein and for several other important proteins. VacA is a secreted protein that is responsible for the vacuolar degeneration of epithelial cells seen in colonized hosts. The protein is able to form hexameric anion-selective channels in planar lipid bilayers, following a disassembly of water-soluble dodecameric VacA at low pH, and this has been suggested to produce the osmotic imbalance necessary for the target cell vacuolation (Reyrat *et al.*, 1999). VacA specifically interacts with a novel filament-associated protein (de Bernard *et al.*, 2000).

The glycobiology of bacterial–host interplay is sophisticated and complex

Glycobiology is emerging as the central topic of *Helicobacter*–host investigations. One area concerns the microbial surface lipopolysaccharides, LPS, which show molecular mimicry with host antigens and undergo distinct phase variations. Another is microbial recognition of and binding to host glycoconjugates, a situation that is uniquely complex for *H.pylori* among so far analyzed microbes. This provides a potential explanation for the unexpectedly large number of OMPs identified from the genome sequence (see *Genome sequences with essential information*). These proteins may prove to be carbohydrate-binding adhesins of great interest to identify for therapeutic and diagnostic purposes.

Bacterial surface and molecular mimicry of host glycosylation

One distinct feature of several Gram-negative mucosal pathogens is that oligosaccharides of their LPS are identical or show strong structural similarity to human glyco-epitopes, including glycolipid-specific sequences (Preston *et al.*, 1996). This molecular mimicry may have relevance for pathogenicity in several ways. Interestingly, different microbes may develop a separate mimicry apparently depending on the locale of colonization. As a constructive example of this, *H.pylori* will be compared with *Haemophilus influenzae*. *H.influenzae* was the first organism for which a complete genome sequence was obtained (Fleishmann *et al.*, 1995) and since then advanced information has been gained about host mimicry, LPS diversity and phase variation (see Hood *et al.*, 1999; Risberg *et al.*, 1999). This important Gram-negative bacterium exclusively colonizes the human nasopharynx, where it is able to persist for extended periods without causing disease. It is potentially pathogenic, however, and may cause respiratory tract infections and invade to cause systemic disease, such as meningitis. LPS has been shown to carry lactose, globotriaose or p^k antigen, globotetraose or P antigen, and sialyllactose (sequences discussed in the present paper are listed in Table I). Interestingly, these epitopes on human cells are membrane-bound and glycolipid-specific and do not appear on glycoproteins or in secretions (see Karlsson, 1998). A high-frequency phase variation found for the expression of saccharide epitopes is thought to provide an adaptive mechanism for survival in different microenvironments. For example, phosphorylcholine

Table I. Saccharide and glycoconjugate sequences used for discussion

Sequence	Common name
Galβ4Glc	Lactose, Lac
Galβ4GlcβCer	Lactosylceramide
Galβ3GlcNAc	Type 1, Lewis c
Galβ4GlcNAc	Type 2, N-acetyllactosamine, LacNAc
Fucα2Galβ3GlcNAc	H type 1, Lewis d
Fucα2Galβ4GlcNAc	H type 2
Fucα3Fucα4GlcNAc	Difucose sequence
Galα4Galβ4Glc	Globotriaose, p^k antigen
GalNAcβ3Galα4Galβ4Glc	Globotetraose, P antigen
Galα3Galβ3GlcNAc	Linear B blood group type 1
Galα3Galβ4GlcNAc	Linear B blood group type 2
GalNAcα3(Fucα2)Galβ3GlcNAc	A blood group type 1
Galβ4(Fucα3)GlcNAc	Lewis x
Fucα2Galβ4(Fucα3)GlcNAc	Lewis y
Galβ3(Fucα4)GlcNAc	Lewis a
Fucα2Galβ3(Fucα4)GlcNAc	Lewis b
NeuAcα3Galβ4Glc	Sialyllactose
NeuAcα3Galβ4(Fucα3)GlcNAc	Sialyl-Lewis x
GalNAcβ4Galβ4GlcβCer	Gangliotriaosylceramide
Galβ3GalNAcβ4Galβ4GlcβCer	Gangliotetraosylceramide
SO_3-3GalβCer	Sulfatide
SO_3-3Galβ3(Fucα4)GlcNAc	Sulfated Lewis a

is linked to a hexose on the outer core region and located on the cell surface and it mimics host membrane phospholipid. Bacteria containing this modification appear to favor colonization of respiratory epithelia in a rat model, whereas its absence, probably after invasion, may confer resistance to host factors such as C-reactive protein for serum clearance (Weiser *et al.*, 1998). Although there is growing information on various glycoforms and genetic loci involved in phase variations (Hood *et al.*, 1999; Risberg *et al.*, 1999), and basic mechanisms of molecular switches are known (Henderson *et al.*, 1999), the potential local signals for this variation have not yet been identified. The precise meaning of mimicking host blood group P antigens for the nasopharynx colonization by this pathogen remains to be understood.

Compared to *H.influenzae* with its exclusive primary colonization in the human nasopharynx, the gastric colonizer *H.pylori* expresses a different set of mimicking sequences. Careful chemical analyses of *H.pylori* LPS (Aspinall and Monteiro, 1996; Aspinall *et al.*, 1996, 1997, 1999; Monteiro *et al.*, 1998, 2000; Knirel *et al.*, 1999) allow eight or more glycoforms to be listed at present (see Monteiro *et al.*, 2000). Interestingly, the O-chain region apparently lacks lactose (in contrast to *H.influenzae*), but instead carries up to about 40 LacNAc repeats (LacNAc is lacking in *H.influenzae*). The O-chains may terminate with a type 2 or a type 1 disaccharide sequence, which may be substituted with Fuc to produce Lewis x, Lewis y, Lewis a, Lewis b, H type 1 or Lewis d, or with sialic acid to produce sialyl-Lewis x, or remain nonsubstituted as repeated LacNAc (i antigen), or type 1 disaccharide (Lewis c); see Table I. Lewis b and sialyl-Lewis x determinants were detected on LPS by antibody (Wirth *et al.*, 1996) and recently by chemical analysis (Monteiro *et al.*, 2000). All these sequences may be expressed on human cells. However, a difucose and a linear B blood group sequence (Monteiro *et al.*, 2000) have not been detected on human cells. Both sialyl-Lewis x and Lewis b are specifically recognized by bacterial cells (see *Adhesin-mediated adherence*), thus creating potential autoagglutination. A single fucosyltransferase is responsible for Lewis x (α3) and Lewis a (α4) synthesis with a 5-fold preference for the former product (Rasko *et al.*, 2000a). Although the α2Fuc-tranferase exists, the apparent lack of H type 2 was explained by a dependence of Fuc3 on GlcNAc for Fuc2 on Gal to be formed (Appelmelk *et al.*, 1999; Wang *et al.*, 1999a,b). This order of fucosylation is the opposite compared to that found for animal systems, where H type 2, Lewis x, and Lewis y may exist in parallel. However, this is not true for all strains, since *in vitro* studies of some isolates recently documented that Lewis a and Lewis x as well as unfucosylated type 1 and type 2 disaccharides could be fucosylated (Rasko *et al.*, 2000a). *H.pylori* strains thus apparently predominantly express Lewis x and Lewis y, but not H type 2, and to a less degree Lewis a and Lewis b. They maintain a higher level of α3,4Fuc-transferase than of α2Fuc-transferase, and the latter is responsible for conversion of Lewis x to Lewis y. Lewis a and Lewis b determinants which have been considered primate-specific may thus exist in a microbe.

There are probably several more subtle differences in *H.pylori* LPS to be found. In a recent structural study (Aspinall *et al.*, 1999), two strains were compared, of which one did and the other did not stimulate pepsinogen. LPS has been implicated in this activity, which is elevated in about half of the patients with doudenal ulcers, and *H.pylori* colonizes 90% of patients with this disease. The inactive strain was found to carry Galα6GlcNAc residues on the LacNAc-glycan, which the active strain did not, and it was proposed that this substituent could cause a distortion of conformation to make this LPS inactive. This study further strengthens the importance of detailed structural studies to find out the roles of LPS in *H.pylori* pathogenesis.

A rapid phase variation of the expression of Lewis antigens in *H.pylori* has been documented in colonies derived from biopsies of single patients (Wirth *et al.*, 1997), and one bacterial cell population showed a frequency of such a phase variation in the range of 0.2–0.5% (Appelmelk *et al.*, 1998). There is apparently a variation in Lewis antigen expression during the course of colonization in single individuals, as documented from gastric biopsies (Rasko *et al.*, 2000b). The role of the fucosyltransferase level on the mechanism of this phase variation is growing (Appelmelk *et al.*, 1999; Wang *et al.*, 1999a,b). Regulation apparently occurs at multiple levels, including replication, transcription, and translation. The potential signals for this are not known. It has been proposed that the phenotypic diversity in Lewis expression may provide a pool in human subjects for continuous selection of host-adapted populations suitable for persistence (Wirth *et al.*, 1999; Heneghan *et al.*, 2000). Dependence of both host and bacterial strain has been shown for colonization and inflammation in model animals, including nonhuman primates (Dubois *et al.*, 1999) and mice (van Doorn *et al.*, 1999). Of interest in this respect is the

finding in ferrets, which are naturally colonized in the stomach by *Helicobacter mustelae*, of a parallel expression of blood group A type 1 antigen on the bacteria and on gastric cells (Monteiro *et al.*, 1997; Croinin *et al.*, 1998).

Potential autoimmunity mediated by Lewis antigens has not been possible to document as a basis for disease (Faller *et al.*, 1998; Kamiya *et al.*, 1999). This may mean that the mimicry of host glycosylation is optimally balanced to normally avoid pathological immune response. What does it mean that *H.pylori* and *H.influenzae*, which both may colonize persistently in their respective niches, develop distinctly different LPS structures? Maybe this is a sophisticated reflection of host mucosa structure and ecology. On the host side, there may also develop a potential adaptation to the almost inherited bacterial colonization. According to a recent exciting hypothesis glycan diversification in complex multicellular organisms is driven by evolutionary selection pressures mainly from exogenous microbes that recognize glycans (Gagneux and Varki, 1999). Whether *H.pylori* has exerted any selective pressure through the years on stomach glycosylation is practically impossible to investigate. During the long coevolution of *H.pylori* and humans (Blaser, 1999b; Covacci *et al.*, 1999) it is not unlikely that signals in both directions have contributed to a "normal" optimized glycosylation of both microbe and stomach. Such glycosylation of stomach microniches may then be essential for the balanced non-disease state, and any change in glycosylation may disturb this balance to induce disease (see *Modulation of host glycosylation*).

Bacterial contact with neutrophils is not suicidal

H.pylori-induced gastritis is typically associated with a strong infiltration of the colonized stomach mucosa by neutrophils and mononuclear inflammatory cells, and there is a correlation between mucosal damage and neutrophil infiltration (Marshall and Warren and Marshall, 1983; Westblom *et al.*, 1999). However, LPS from *H.pylori* is known to have 1000-fold less ability to stimulate IL-8 production than LPS from *E.coli* (Kirkland *et al.*, 1997). In epithelial cell culture, adherent *H.pylori* are apparently able to stimulate IL-8 production through the ceramide-mediated pathway (Masamune *et al.*, 1999). A detailed mapping is being done of proinflammatory factors, including activator proteins and stress-response kinases, that potentially mediate epithelial cell degeneration following contact with *H.pylori* containing the *cag* pathogenicity island (Naumann *et al.*, 1999). The bacterial protein CagA may be translocated into gastric epithelial cells and phosphorylated on tyrosine (Segal *et al.*, 1999; Odenbreit *et al.*, 2000; Stein *et al.*, 2000). However, proinflammatory activation of neutrophils is not associated with *cag*A genotypes (Hansen *et al.*, 1999). Water-soluble bacterial surface proteins activate neutrophils and upregulate expression of chemokines (Kim *et al.*, 2000). A protein capable of promoting neutrophil adhesion to endothelial cells was identified in *H.pylori* extracts and shown to mediate induction of neutrophil CD11b/CD18 adhesion molecules, which interact with ICAM-1 (Yoshida *et al.*, 1993). This neutrophil-activating protein, Hp-NAP, has recently been characterized in detail including molecular modeling (Tonello *et al.*, 1999). The 17 kDa subunit forms a dodecameric structure with a hollow central core and may bind up to 500 iron atoms per oligomer; it is resistant to thermal and chemical denaturation, which is similar to the case for the related ferritin family of proteins.

Hp-NAP is a carbohydrate-binding protein (Teneberg *et al.*, 1997; Namavar *et al.*, 1998). A recombinant Hp-NAP was shown to bind to distinct gangliosides of human neutrophils having as a common feature repeated non-substituted LacNAc (Teneberg *et al.*, 1997). There was also binding to sulfatide, but this glycolipid was absent from neutrophils. An extracted bacterial surface-located protein, identified as Hp-NAP, was claimed to bind sulfated oligosaccharide structures of human high-molecular-weight salivary mucin, such as sulfated Lewis a, SO_3-3Gal, and SO_3-6GlcNAc (Namavar *et al.*, 1998). In a recent study various glycoconjugates were isolated from neutrophils and tested for bacterial cell binding by overlay assays (Miller-Podraza *et al.*, 1999). An apparently high-affinity sialic acid–dependent binding was detected for gangliosides, polyglycosylceramides and glycoproteins, and this was the only binding specificity found. It has been shown that *H.pylori* cells, upon direct contact with neutrophils, induce a rapid oxidative burst followed by a slower phagocytosis (Mooney *et al.*, 1991; Rautelin *et al.*, 1993). However, the bacteria are not necessarily killed, in contrast to other microbes. Instead, as discussed above, *H.pylori* may satisfy part of its nutritional requirements from degradation products of the inflammatory reaction (Blaser, 1996, 1997; Blaser and Kirschner, 1999). Therefore, *H.pylori* not only resists inflammation, but actively recruits neutrophils to induce the inflammation, which may be beneficial for the microbe. It remains to be shown if the sialic acid–dependent *H.pylori*–neutrophil interactions mediate the biological responses.

Direct microbe–host attachment mediated by carbohydrate

In addition to the sialic acid–dependent recognition of neutrophil glycoconjugates, there are several binding specificities detected for bacterial cells. The majority of *H.pylori* cells reside in the stomach mucus where they divide, and only a small fraction is found adhered to gastric epithelial cells. The tropism in human gastric epithelium is distinct. By use of an *in situ* adherence assay and *H.pylori* strains labeled with fluorescein isothiocyanate (FITC), four out of five strains, including gastric-ulcer and acute-gastritis isolates, attached exclusively to surface mucous cells present in the pit region of gastric units, but not to mucous neck, parietal, or chief cell lineages present in the glandular domains of the units (Falk *et al.*, 1993). Naturally, *H.pylori* colonizes only primates. Only in one case has a carbohydrate-binding protein been convincingly identified on *H.pylori* cells, the Lewis b adhesin (Ilver *et al.*, 1998) (see also Hp-NAP discussed in *Bacterial contact with neutrophils is not suicidal*).

Adhesin-mediated adherence

Only three adhesins of *H.pylori* have been convincingly identified, AlpA and AlpB adhesins necessary for adherence to gastric tissue (Odenbreit *et al.*, 1999) and the BabA adhesin (Ilver *et al.*, 1998). No host receptors are yet known for the AlpA and AlpB adhesins, but the BabA adhesin recognizes the Lewis b blood group antigen (Borén *et al.*, 1993). BabA is encoded by *babA2* in strain CCGU 17875 and is composed of 721 aa residues. A second gene, *babA1*, is identical to *babA2* except for lack of an insertion of 10 bp present in *babA2*. The truncated BabA product of *babA1* was unable to bind Lewis b.

AlpA, AlpB and BabA all belong to the family of 32 OMPs (Tomb *et al.*, 1997). Six out of six wild-type strains expressed AlpA and AlpB but only 34% of tested strains expressed Lewis b binding. Members of the remaining OMPs may prove to be adhesins recognizing some of the other carbohydrate-binding specificities listed below. The OMPs share extensive sequence homology in the N- and C-terminal domains. The C-terminal portion of AlpA and AlpB was predicted to form a porin-like β-barrel in the outer membrane of *H.pylori*, consisting of 14 transmembrane amphipathic β-strands (Odenbreit *et al.*, 1999).

The fine specificity of BabA binding has been studied using various assays and substances (Borén *et al.*, 1993; Borén *et al.*, 1994). Of a panel of fucosylated neoglycoproteins the Lewis b derivative gave 93% reduction of binding in the *in situ* biopsy assay, while the H type 1 derivative reduced binding to 52%. However, in the form of glycoproteins, Lewis a, Lewis x, Lewis y, and H type 2 determinants were inactive as receptors. Similar results were obtained in solid-phase binding on western blots. However, for free oligosaccharides in solution Lewis b, H type 1, and Lewis y inhibited binding equally well, indicating that Fucα2Gal is the minimal requirement, rather than Lewis b, and that a Fuc branch on GlcNAc may stabilize the epitope presentation for better binding. These data were confirmed by inhibition with synthetic saccharides linked up to polymer (Eklind *et al.*, 1996). The *babA2* gene is of clinical relevance, and its presence is significantly associated with duodenal ulcer and adenocarcinoma, and it may therefore be a useful marker to identify those individuals at higher risks for disease (Gerhard *et al.*, 1999).

Sialic acid binding specificity

The first binding specificity detected for *H.pylori* was sialic acid-dependent, as shown by hemagglutination studies (Evans *et al.*, 1988). The gene encoding the adhesin protein on *H.pylori* was reportedly identified in 1993, and an antibody to the protein was raised that bound to bacterial cell bodies (Evans *et al.*, 1993). Later, however, the cytoplasmic localization and lipoprotein nature of this protein was shown, but knock-out of the gene did not change adhesion properties (O'Toole *et al.*, 1995). The lipoprotein character was confirmed by a separate investigation which repeated the cloning and expression, and a carefully defined antibody and immunogold labeling was used to localize the protein to the flagellar sheath, rather than intracellularly or to the cell body. Adhesion characteristics were unaffected by gene inactivation (Jones *et al.*, 1997). Therefore, an adhesin recognizing sialic acid still awaits identification.

The initial conclusion of a NeuAcα3Gal-based specificity (Evans *et al.*, 1988) was later confirmed (Hirmo *et al.*, 1996; Johansson and Karlsson, 1998). Apparently, there is a strict requirement for an intact NeuAc, since various chemical modifications eliminate binding (Miller-Podraza *et al.*, 1998). A type 2 core is essential (H.Miller-Podraza *et al.*, unpublished observations). This binding is expressed only when bacterial cells are grown on agar and is lost after growth in broth. However, a second sialic acid–dependent binding specificity remained after growth in broth and strict selectivity was proposed (Miller-Podraza *et al.*, 1996, 1997). Only polyglycosylceramides were positive, but traditional gangliosides or sialylated glycoproteins were inactive. These complex glycolipids with about 15 to 45 monosaccharides linked to ceramide are highly heterogeneous with incompletely branched, N-acetyllactosamine-based chains terminating with 1–2 NeuAc per molecule (Karlsson *et al.*, 1999). The binding is completely lost after mild periodate oxidation, but further details of the binding epitope are still lacking, except that NeuAc is probably 3-linked (Johansson *et al.*, 1999). These two specificities differ in detailed binding pattern from the binding of Hp-NAP to sialyl conjugates (see *Bacterial contact with neutrophils is not suicidal*). The extracellular matrix protein laminin has been shown to bind *H.pylori* in a sialic-dependent way, and a bacterial 25 kDa outer membrane protein was proposed to mediate this binding (Valkonen *et al.*, 1997).

Other binding specificities

On thin-layer plates, several glycolipids have been shown to bind *H.pylori*, including sulfatide, which is abundant in human gastric epithelium (Saitoh *et al.*, 1991; Kamisago *et al.*, 1996), gangliotria- and gangliotetraosylceramide (Gold *et al.*, 1993), lactosylceramide (Ångström *et al.*, 1998), and some other glycolipids (S.Teneberg *et al.,* unpublished observations). Heparan sulfate shows a strong binding by *H.pylori* (Ascencio *et al.*, 1993), and heparin-binding bacterial proteins have been detected (Utt and Wadström, 1997). Evidence has been presented that blood group sequences H type 2, Lewis a and Lewis b mediate binding to cultured epithelial cells, based on blocking of binding by monoclonal antibodies, and by binding biotinylated blood group antigens (Alkout *et al.*, 1997). Affinity adsorption of bacterial extracts on blood group particles resulted in a major protein of 61 kDa. Binding to phosphatidylethanolamine has also been reported (Lingwood *et al.*, 1992). The relevance for colonization or pathogenicity has not been shown for any of these specificities.

LPS–host cell and potential carbohydrate–carbohydrate interactions

In view of the growing evidence for selective carbohydrate–carbohydrate interactions of biological importance (for a recent review, see Spillman and Burger, 2000), it may be appropriate to include some comments concerning a potential relevance of this type of interaction for *H.pylori* in human stomach. There is one paper reporting *H.pylori* cell binding to laminin, apparently mediated by LPS (Valkonen *et al.*, 1994). Laminin is a basement membrane glycoprotein that may become exposed after disruption of the epithelial cell layer, potentially caused by damage or rapid cell turnover. The conclusion after using various parts of modified LPS as inhibitors was that a phosphorylated structure in the core oligosaccharide mediated interaction with a hemagglutinating strain, and a nonphosporylated structure mediated interaction with a poorly hemagglutinating strain. No further details are available, and the binding site on laminin, whether peptide or saccharide, remains to be identified. Thus, there are two separate interactions of *H.pylori* with laminin, one mediated by LPS and the other by a protein recognizing sialic acid (see *The sialic acid binding specificity*). Recently, convincing evidence was provided that LPS Lewis x is involved in targeting *H.pylori* to human gastric mucosal epithelial cells and cells lining gastric pits, probably a saccharide–protein interaction (Edwards *et al.*, 2000). Targeting with Lewis x–conjugated beads was indistinguishable from that of bacterial cells. For other bacteria there is evidence for LPS involvement in adher-

ence (Jacques, 1996) and direct binding to glycolipids. *Pseudomonas aeruginosa* cells and extracted LPS bound gangliotetraosylceramide on thin-layer chromatograms, but not other glycolipids (Gupta *et al.*, 1994). For *Actinobacillus pleuropneumoniae*, which causes pleuropneumonia of pigs, evidence suggests that LPS has a role in adherence to pig tracheal tissue (Bélanger *et al.*, 1990). Recently this was studied in detail by use of defined glycolipids separated on thin-layer plates (Abul-Milh *et al.*, 1999). Extracted LPS and intact cells showed similar binding to glycolipids, and among many tested glycolipids, GlcβCer, GalβCer, sulfatide, lactosylceramide, gangliotriaosylceramide, and gangliotetraosylceramide were positive. Binding to the former four glycolipids, but not to the latter two, was inhibited by preincubation of bacteria with monoclonal antibodies to the O antigen part of LPS, indicating that different parts of LPS may be involved in interaction with glycolipids.

Therefore, *H.pylori* LPS should be carefully tested for a potential binding to sulfatide, lactosylceramide, gangliotriaosylceramide, and gangliotetraosylceramide. The latter two glycolipids have, however, not been detected in human stomach (S.Teneberg *et al.*, unpublished observations). Also a potential Lewis x–Lewis x interaction should be tested (Spillman and Burger, 2000). In the case of *H.influenzae* (see *Bacterial surface and mimicry of host glycosylation*), both lactose and blood group P sequences should be tested for potential carbohydrate-carbohydrate binding (Spillman and Burger, 2000).

Dynamic microniche ecology with effects on interplay

Our assumption in the last few decades that specific microbe–host protein–carbohydrate interactions are essential first-contact steps for infections is based on well-established cases for several bacterial toxins and viruses. This concept is presently being subjected to promising clinical trials (Zopf and Roth, 1996; McAuliffe and Hindsgaul, 1997; Karlsson, 1998; Laver *et al.*, 1999). However, for more complex microbes including bacteria, fungi, and eukaryotic parasites, convincing information on the relevance for pathogenesis of such interactions is mostly lacking. Only in a few cases results have been obtained in animal infection models proving that also bacteria carry carbohydrate-binding specificities of critical importance for disease to develop (Karlsson, 1998). The relation of *H.pylori* to the host is revealing a growing sophistication, which probably will require much more work to allow development of rational medical approaches. A long coevolution with humans has provided *H.pylori* with a qualified setup of essential characteristics, with some variation between isolated human populations. In a shorter perspective, these properties may adapt and undergo phase variation during the life-long colonization in the individual host, depending on host geno- and phenotype including disease and modified glycosylation.

Variable expression of carbohydrate-binding specificities

Individual type-collection strains do not exhibit all specificities summarized above. Usually a majority are expressed, although with an apparent phase variation of expression during standard cultivation (see Karlsson, 1998). However, individual specificity, such as the sialic acid binding after cultivation on agar, has been shown to be more consistently expressed in fresh clinical isolates, in contrast to strains with large passage numbers (Simon *et al.*, 1997). Two different sialic acid–dependent specificities were obtained from separate cultivation conditions. Of the two types of strains, CCUG 17874 and CCUG 17875, the first recognizes sialic acid but not Lewis b, and the second recognizes Lewis b but not sialic acid. However, a surprising result from CCUG 17875 was recently obtained indicating a sophisticated phase variation and interdependence in expression (T.Borén *et al.*, unpublished observations). After inactivation of the *babA* genes the Lewis b binding was lost as expected (Ilver *et al.*, 1998). However, the lost Lewis b binding was apparently replaced by the two sialic acid-dependent binding mechanisms, with a binding pattern identical to that of strain CCUG 17874 when tested against various glycoconjugates. In the case of sulfatide binding, this was shown to depend on stress conditions to be expressed (Huesca *et al.*, 1996). An overlay assay on thin-layer plates showed that this binding appeared first after brief treatment of bacteria at low pH. The effect could be linked to surface-located heat shock proteins and was prevented by inhibitors of protein synthesis.

These scattered data may indicate a capacity of *H.pylori* for regulated phase variation in carbohydrate-binding specificities, possibly as a reaction to specific as yet unidentified signals in the different microniches that *H.pylori* cells are known to occupy.

Modulation of host glycosylation

For potential interaction sites, including mucus, gastric epithelial cells, neutrophils, and basement membrane, it is important to study not only the nonsymptomatic chronic gastritis state, but also conditions which may result from disturbances or disease. Concerning mucus, it is not known if there are mucus epitopes of importance being recognized by *H.pylori* in normal or diseased stomach. The glycosylation pattern of human gastric mucins varies in different gastric compartments and are reversibly changed by *H.pylori* colonization (Ota *et al.*, 1998). Regarding epithelial cells, the *in situ* adherence assay based on biopsy material from human stomach is highly informative (Falk *et al.*, 1993). The distinct binding to surface mucous cells of the pit region of gastric units was inhibited by Lewis b conjugates, but no evidence was obtained for interaction with sialyl conjugates. Instead sialic acid–binding plant lectins were shown to recognize sites in the submucosal compartment. Although the FITC labeling may modify the bacterial properties, and the extraction step of biopsy treatment may remove glycolipids (see discussion by Simon *et al.*, 1997), the results show that Fuc-epitopes mediate *H.pylori* attachment to normal mucosa and that sialic acid may not be essential. Our group has indirectly confirmed this by chemical analyses of mucosa scrapings, which practically lack a sialic acid-dependent binding to glycolipids or glycoproteins (S.Teneberg *et al.*, unpublished observations). In contrast, neutrophils, which are numerous in inflamed mucosa, are rich in sialylated glycoconjugates (Miller-Podraza *et al.*, 1999), which might indicate that neutrophils are the major potential targets for the sialic acid–dependent binding, at least in the nonsymptomatic situation.

Interestingly, the pathological state may show a pattern of glycosylation of potential importance for colonization of *H.pylori* and disease progression. A transgenic mouse model was developed where the Lewis fucosyltransferase was trans-

fected. This resulted in expression of Lewis b in the surface mucous cells of the gastric pit, similar to the human situation (Falk *et al.*, 1995). *In vitro*, clinical isolates bound to these cells of the transgenic mouse but not of their normal littermates. In a more recent study, the same group used a transgenic mouse model deficient in parietal cells (Syder *et al.*, 1999). This situation amplifies presumptive gastric epithelial stem cells and their immediate committed daughters, which were shown to express sialylated conjugates, including sialyl-Lewis x. These sites mediated attachment of *H.pylori* which resulted in enhanced cellular and humoral immune responses. The authors proposed that similar cellular modulations may be the basis of tumorigenesis in patients with chronic atrophic gastritis. A high salt diet in a mouse model induced gastric hyperplasia and parietal cell loss and enhanced *H.pylori* colonization (Fox *et al.*, 1999). In a separate study the *H.pylori* mutant with inactivated Lewis b binding and induced sialic acid binding (see *Variable expression of carbohydrate-binding specificities*) was used to detect sialyl-Lewis x binding in inflamed human and Rhesus monkey gastric mucosa (T.Borén *et al.*, unpublished observations). In this way a Lewis b–independent and sialyl/Lewis x–dependent binding was found in the gastric pit region, the same location as found in the original study using the wild-type strain expressing Lewis b binding (Falk *et al.*, 1993). There was a positive correlation between adherence and the level of an inflammatory response, and in the monkey a challenge with *H.pylori* established a chronic inflammation that promoted sialyl–Lewis x binding. However, such a relation was not found for the Lewis b binding. Of 91 tested clinical isolates of *H.pylori*, 40 were positive binders of sialyl–Lewis x. Interestingly, also the transgenic mouse expressing Lewis b in the gastric pit cells, but not the normal mouse, expressed sialyl-Lewis x binding, indicating that the increased fucosylation from transfection also affected sialyl conjugates. These two studies therefore document a modulation of host glycosylation after genetic manipulation and bacterial colonization, respectively, with distinct consequences for bacterial interaction. Of interest is that dimeric sialyl–Lewis x expression in human gastric carcinoma correlates with a poor outcome (Amado *et al.*, 1998), and it is known that human gastric tumors show aberrant glycosylation and may be highly sialylated (Hakomori, 1996).

Therefore, when the persistent and nonsymptomatic *H.pylori* colonization may come out of balance for still unknown reasons, distinct changes in host glycosylation may appear, which may affect adhesin expression to strengthen an association or switch target cell. One may note that intimate adhesion has clear effects on the outcome. For the transgenic mouse model expressing Lewis b in gastric pit cells, it was shown that a persistent colonization with *H.pylori* did not differ in bacterial density from the normal mouse (Guruge *et al.*, 1998). However, only the transgenic mouse produced autoantibodies to Lewis x antigens, chronic gastritis, and parietal cell loss, probably a result of close attachment to epithelial cells.

Current therapy is not suitable for a large-scale prevention

The discovery of a bacterium behind peptic ulcer disease invited rational therapy (Dunn *et al.*, 1997). A combination of antibiotics with proton pump inhibitors not only eradicates *H.pylori* and cures this disease with practically no recurrency, but may also reverse progress of the MALT lymphoma (Dunn *et al.*, 1997; Westblom *et al.*, 1999). However, although antibiotics are still a good and necessary solution for individual treatment of disease, resistant strains are growing in number, prohibiting the use on whole populations for eradication and prevention of disease. When more information is available, new pharmaceuticals may be designed that may interfere with colonization and other mechanisms. Existing alternative approaches are vaccination or anti-adhesive glycoconjugates. As *H.pylori* disappears, due to eradication or natural decline (see *Colonization is mostly non-symptomatic but may induce serious disease*), ulceration and cancer rates will fall, but more proximal diseases of esophagus will probably increase, as some *H.pylori* strains appear to be protective (Blaser, 1999b). Therefore clinical approaches in the future may have to consider parameters in addition to simply elimination or prevention of colonization, possibly including a recolonization with suitable strains (Blaser, 1999a).

Naturally acquired human immune responses against *H.pylori* are currently being mapped as a basis for developing therapeutic and prophylactic vaccines (Zevering *et al.*, 1999). Current vaccination approaches (Blanchard *et al.*, 1999) use various models including transgenic and knock-out animals (Nedrud, 1999). A recent study on infected humans (Michetti *et al.*, 1999) used *H.pylori* urease mixed with *E.coli* heat-labile enterotoxin for immunization. This composition was well tolerated and immunogenic and a decrease in the density of bacteria was observed. In a commentary (Czinn and Nedrud, 1999), however, the evaluation was that a useful vaccine is probably far away due to still very weak effects. An expression library of *H.pylori* genes was screened with sera from infected humans and from immunized rabbits and a number of immunogenic proteins were detected (Lazowska *et al.*, 2000).

Low-abundance adhesins and knowledge of adhesin–host interactions may be used both for vaccination against the adhesin and for design of receptor saccharide analogues based on structural chemistry. Vaccination with mannose-binding FimH adhesin of uropathogenic *E.coli* protects against bladder infection in mice (Langermann *et al.*, 1997) and cynomolgus monkeys (Langermann *et al.*, 2000). Blocking of influenza virus neuraminidase with sialic acid analogues is currently subject to clinical trials (Wade, 1997; Laver *et al.*, 1999), and new promising approaches of receptor analogue design are being optimized (Davis and Wareham, 1999; Sears and Wong, 1999). Natural glycoconjugates are of interest especially if resorption is not necessary (Zopf and Roth, 1996; McAuliffe and Hindsgaul, 1997). Sialyllactose efficiently inhibited *H.pylori* adhesion to human gastrointestinal epithelial cell lines (Simon *et al.*, 1997). Sialyllactose coupled up multivalently to albumin was about 1000 times more effective than free saccharide. An addendum in proof of this paper noted that free saccharide administered orally for 28 days to infected humans caused a significant reduction of *H.pylori* as assayed by the urea breath test. In a separate study on 12 Rhesus monkeys the same trisaccharide was used either alone or in combination with established chemotherapy (Mysore *et al.*, 1999). The conclusion was that antiadhesive treatment is safe and may cure or decrease colonization, but that addition of a proton pump inhibitor or bismuth subsalicylate did not

improve cure rate. Although the *H.pylori* case reveals a diversity of interactions, and there is dynamics in host glycosylation, an optimally designed multivalent glycoconjugate based on only one expressed binding specificity may agglutinate the bacterial cells and therefore prevent them from host cell interaction. Thus, it may not be necessary to use a more expensive broad spectrum of saccharide analogues to inhibit colonization.

Outlook

The present knowledge of the glycosylated surfaces and communication between *H.pylori* and human is creating a list of remaining problems to be solved. Glycoforms of bacterial LPS have to be characterized in further detail to find potential relations to host microniches and their ecology, including potential carbohydrate–carbohydrate interactions. What are the signals for phase variation of LPS and adhesins and do they differ in nonsymptomatic and sick carriers? Additional basic studies using the full potential of bacterial genome sequences must interact with critical clinical investigations. The dynamics of host glycosylation that may be disturbed in disease is an important topic. However, in contrast to bacterial cells, which may be scaled up for chemical analysis, there are growing difficulties in obtaining relevant human material. New microtechniques based on biopsy samples have to be developed further, both for chemical analysis and for various binding assays and specific reagents. This is especially important, since there are probably individual variations in health and disease that are necessary to map. Additional regulatory factors of the cross-talk between microbe and host must be traced.

Most provocative is the family of 32 OMPs that include the three identified adhesins. It is essential to identify the potential recognition by OMPs of host glycoconjugates. A new promising general method was recently developed for the functional expression of genes encoding adherence-associated OMPs (Fischer *et al.*, 1999). This technique makes it possible to manipulate genes to define amino acid sequences critical for adhesin–receptor interaction. This requires improved assays to read-off the potential effects. New aspects of bacterial invasion of gastric epithelium as a way to establish persistent colonization have to be considered (Su *et al.*, 1999). Starting from the other side, the long list of carbohydrate-binding specificities that have been detected may be used to identify corresponding adhesins by new highly sensitive proteomics approaches. This was documented for BabA, which exists in only about 500 copies per bacterial cell (Larsson *et al.*, 2000). A receptor-active glycoconjugate probe is tagged with a photoreactive crosslinker to allow coupling to the adhesin, and with biotin for affinity isolation of the product. This tagging technique was earlier applied for the cloning of BabA (Ilver *et al.*, 1998). Such low-abundant and conserved proteins may prove to be suitable components of vaccine formulas (see example for *E.coli* above); a reason why they normally escape immune detection on the microbe may be their low surface density (Wizemann *et al.*, 1999). Purified adhesins will also be helpful as specific reagents in the mapping of potential binding sites in normal and pathological biopsy samples of host tissues. The unique list of carbohydrate-binding specificities has to be critically evaluated and supplemented with detailed analyses of target glycoconjugates. Determining binding sites in complex mucins may be simplified by coupling of released saccharides to generate neoglycolipids, which are easier to detect as molecular species and simpler to identify by chemical analysis than the intact glycoproteins (Chai *et al.*, 1999).

Recently, the first crystal structure was obtained for a bacterial adhesin, the mannose-binding FimH adhesin of uropathogenic *E.coli* (Choudhury *et al.*, 1999), which has also been used for vaccination (see *Current therapy is not suitable for a large-scale prevention*). Such information is a prerequisite for structural drug design of saccharide analogues to potentially interfere with colonization. Therefore, it is important to express defined adhesins of *H.pylori* in sufficient amounts for crystallization. This may be assisted by computational genomics for the prediction of both structure and function of coded proteins, which recently was applied on *H.pylori* for the first time (Pawlowski *et al.*, 1999).

On the clinical side a major question remains as to why disease develops in a minority of individuals with persistent *H.pylori* colonization. To answer this will require additional epidemiological and other studies. Transgenic mouse infection models have already been helpful (Falk *et al.*, 1995; Guruge *et al.*, 1998; Syder *et al.*, 1999). However, animal models will remain imperfect as long as they are not completely humanized, including glycosylation; therefore, they may provide information only on selected, though essential, aspects.

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