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(54) Title: USE OF OLIGOSACCHARIDE GLYCOSIDES AS INHIBITORS OF BACTERIAL ADHERENCE (57) Abstract <i>Helicobacter pylori</i> has been implicated as a contributing factor in a number of pathological conditions, including acute (type B) gastritis, gastric and duodenal ulcers, gastric adenocarcinoma, and gastric lymphoma. The present invention relates to the use of di- or oligosaccharide glycosides containing at least one terminal L-fucose unit for the preparation of pharmaceutical compositions for the treatment or prophylaxis in humans of conditions involving infection by <i>Helicobacter pylori</i> in the human gastric mucosa, as well as a method of treating such conditions using the di- or oligosaccharide glycosides by administering to a patient in need thereof an effective amount of a di- or oligosaccharide glycoside comprising at least one terminal L-fucose unit.		

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USE OF OLIGOSACCHARIDE GLYCOSIDES AS INHIBITORS OF BACTERIAL ADHERENCE

FIELD OF THE INVENTION

The present invention relates to the use of di- or oligo-
5 saccharide glycosides containing at least one terminal L-
fucose unit for the preparation of pharmaceutical composi-
tions for the treatment or prophylaxis in humans of condi-
tions involving infection by *Helicobacter pylori* in the human
gastric mucosa, as well as a method of treating such condi-
10 tions using the di- or oligosaccharide glycosides.

BACKGROUND OF THE INVENTION

Helicobacter pylori is a microaerophilic spiral shaped orga-
nism (originally assigned to the genus *Campylobacter*) which
is found in the stomach and generally appears to have an
15 exclusive habitat in the human gastrointestinal mucosa. It
has been estimated that this bacterium infects the gastric
mucosa of more than 60% of adult humans by the time they are
60 years old. Moreover, *Helicobacter pylori* has been impli-
cated as a contributing factor in a number of pathological
20 conditions, including acute (type B) gastritis, gastric and
duodenal ulcers, and gastric adenocarcinoma.

Tissue tropism of bacteria is partly governed by the ability
of a bacterial strain to adjust to the local chemical envi-
ronment in its specific habitat. In addition, adherence is a
25 necessary prerequisite for colonization in order to prevent
removal from the new habitat, e.g. through peristalsis in the
gastrointestinal tract. In mammals, bacteria adhere to pro-
teins or glycoconjugates (glycosphingolipids, glycoproteins)
on or in the vicinity of epithelial cell surfaces (mucus),
30 and a number of specific bacterial adhesin-protein and adhe-
sin-carbohydrate interactions have been described in the
literature.

With respect to *Helicobacter pylori*, studies in model systems such as mouse adrenal Y-1 cells (see D. G. Evans, D. J., Jr. Evans, and D. Y. Graham, (1989) *Infect. Immun.* 57, 2272-2278) have suggested that surface-associated flexible fibrillar
5 structures that surround this bacterium function as adhesins or colonization factor antigens to mediate the binding of *Helicobacter pylori* to cellular sialic acid-containing glycoprotein receptors.

SUMMARY OF THE INVENTION

10 Through studies of interactions between *Helicobacter pylori* and human stomach mucosa epithelial cells, it has now surprisingly been found, firstly, that *Helicobacter pylori* adheres or binds only to certain differentiated epithelial cells in the glandular epithelium which is composed of gas-
15 tric units. The gastric units in the glandular epithelium are lined with cells that have differentiated to perform various functions. Thus, the upper pit region of the gastric unit (gastric pit) is lined with mucus-producing surface epithelial cells, whereas the constricted mid-portion of the gas-
20 tric unit (its isthmus) is composed of proliferating and non-proliferating immature cells, mucus neck cells and parietal cells, and the lower portion of the gastric unit (the gland) may contain intrinsic factor- and pepsinogen-producing chief cells, acid-producing parietal cells, mucous neck and gland
25 cells, and a variety of enteroendocrine cell types. The present studies have shown that *Helicobacter pylori* binds solely to the mucus-producing surface epithelial cells in the upper pit region, i.e. the surface mucus cells.

Secondly, the studies have shown that the binding of *Helicobacter pylori* to the mucus-producing surface epithelial
30 cells does not involve interaction between bacterial adhesins and sialic acid-containing receptors but rather involves interaction with carbohydrate structures containing terminal units of L-fucose (also known as 6-deoxy-L-galactose).

Consequently, the present invention concerns in one aspect the use of a di- or oligosaccharide glycoside having at least one terminal L-fucose unit for the preparation of a pharmaceutical composition for the treatment or prophylaxis in humans of conditions involving infection by *Helicobacter pylori* of the human gastric mucosa. Likewise, the invention concerns a method of treating and/or preventing diseases in humans caused by infection by *Helicobacter pylori* by administering to a patient in need thereof an effective amount of a di- or oligosaccharide glycoside comprising at least one terminal L-fucose unit.

DETAILED DESCRIPTION OF THE INVENTION

In the present context, the term "terminal L-fucose unit" is intended to mean that the L-fucose unit or units in question, which is/are bound via the 1-position, is/are not itself/themselves glycosylated by other carbohydrate units or groups. This does not exclude, however, that a carbohydrate unit which is glycosylated by a L-fucose unit is itself glycosylated by other carbohydrate units or groups.

In the present context, the term "oligosaccharide" is intended to mean that the saccharide portion of the glycoside comprises three or more carbohydrate units, such as between three and ten carbohydrate units.

It is preferred that the di- or oligosaccharide glycoside having at least one terminal L-fucose unit used according to the invention is capable of binding to adhesins present on the surface of *Helicobacter pylori*.

At the present time, the most useful model system for determining the ability of the di- or oligosaccharide glycoside having at least one terminal L-fucose unit to inhibit the binding of cells of *Helicobacter pylori* to the gastric epithelium is an *in vitro* histological model involving the

use of histological sections of human gastric tissue containing gastric epithelium.

Consequently, a preferred embodiment of the use or the method of the invention is that in which the di- or oligosaccharide glycoside having a terminal L-fucose unit is one which is capable of inhibiting or substantially reducing the adhesion of cells of *Helicobacter pylori* to epithelial cells in a histological section of human gastric mucosa.

More preferably, the di- or oligosaccharide glycoside employed in the use or the method of the invention is one which, when cells of *Helicobacter pylori* are preincubated with the di- or oligosaccharide glycoside in a concentration of up to 500 $\mu\text{g/ml}$, is capable of inhibiting the adhesion of said bacterial cells to epithelial cells of a histological section of gastric human mucosa by at least 50% compared to the adhesion of corresponding non-preincubated bacterial cells.

In the presently preferred histological model system, the histological section of human gastric mucosa is prepared by fixing a sample of non-diseased gastric human mucosa tissue with formalin, embedding the sample in paraffin, providing an approximately 5 μm section of the embedded sample, placing the section on a glass slide, deparaffinizing the section by washing with xylene and isopropanol, and incubating the section with a buffer consisting of bovine serum albumin and a non-ionic polyoxyethylene sorbitan monolaurate surfactant (such as Tween-20), preferably at a concentration of about 0.2% and 0.05%, respectively, in phosphate-buffered saline, cf. also the example.

For the purpose of determining the degree of adhesion of the *Helicobacter pylori* cells to the section of gastric mucosa, it is advantageous that the bacterial cells are labelled in some manner to enable detection of the bacterial cells at specific sites on the section. The labelling may conceivably

be performed according to any of the well-known methods for labelling live bacterial cells or bacterial cells located on a microscopic sample, such as staining by means of a dye (such as a dye specific to e.g. Gram-negative bacteria) followed by microscopy; labelling with a radioactive isotope or a compound containing such an isotope followed by microautoradiography; treatment (before or after the adhesion to the epithelium) with a labelled (enzyme-labelled, radiolabelled or otherwise) antibody recognizing *H. pylori*, followed by detection or visualization of the adhered antibody by a secondary antibody enzyme conjugate or an ELISA-type detection or by microautoradiography.

However, the presently preferred labelling principle is fluorescence-labelling, in particular labelling by means of fluorescein isothiocyanate. This may advantageously be carried out by treating a suspension of cells of *Helicobacter pylori* in buffer containing sodium chloride and sodium carbonate, preferably at a concentration of about 0.15 M and 0.1 M, respectively, at about pH 9.0 with fluorescein isothiocyanate at a concentration of about 0.1 mg/ml, incubating the bacterial cell suspension for 1 hour at room temperature and separating the bacterial cells by centrifugation followed by washing of the bacterial cells with phosphate buffered saline at about pH 7.5 containing a non-ionic polyoxyethylene sorbitan monolaurate surfactant, preferably at a concentration of about 0.05%.

In order to determine the ability of a di- or oligosaccharide glycoside having at least one terminal L-fucose unit to inhibit the adhesion of *Helicobacter pylori* to human epithelial gastrointestinal cells, the bacteria are typically subjected to preincubation with the glycoside prior to bringing the bacteria into contact with the tissue section to allow them to adhere to the surface of epithelial cells. One useful way of performing the preincubation of the bacterial cells is by adding the di- or oligosaccharide glycoside in a concentration of up to 500 µg/ml to a suspension of the

bacterial cells in a buffer consisting of bovine serum albumin and of a non-ionic polyoxyethylene sorbitan monolaurate surfactant, preferably at a concentration of about 0.2% and 0.05%, respectively, in phosphate-buffered saline at about pH 7.5 for a period of about 2 hours at room temperature, separating the bacterial cells by centrifugation, and washing the cells in the same buffer.

When cells of *Helicobacter pylori* (whether preincubated or not, and whether labelled or not) are to be tested for their ability of adhering to epithelial cells in the human gastrointestinal mucosa, this is typically performed by applying a dilute *Helicobacter pylori* cell suspension containing about 10^6 - 10^8 bacterial cells per millilitre to the histological section, incubating the section with the bacterial cell suspension for 1 hour at room temperature in a humidified chamber, washing the slide in phosphate buffered saline, and establishing the degree of adhesion to the epithelial cells of the section. In the embodiment where the bacteria are fluorescence-labelled, e.g. with fluoresceine isothiocyanate, establishing the degree of adhesion is typically and conveniently carried out by studying the section under a fluorescence microscope and assessing the degree of adhesion from the number of fluorescent bacteria attached to the epithelial surface cells in the section, cf. also the examples, in particular the figures explained therein.

The adhesion testing may be carried out using cells of the *Helicobacter pylori* strains NCTC 11637, NCTC 11638, WV229 or P466 (cf. the example below).

The conditions involving gastrointestinal infection by *Helicobacter pylori*, the treatment or prophylaxis of which the pharmaceutical composition prepared in the use according to the invention as well as the treatment method according to the invention may be employed for, comprise for example chronic active (type B) gastritis, gastric ulcers, duodenal ulcers, gastric adenocarcinoma, and gastric lymphoma.

In a preferred embodiment, the oligosaccharide contains two terminal fucose units as defined above.

In a preferred embodiment, the di- or oligosaccharide glycoside to be employed is a glycoprotein. Interesting
5 examples of compounds to be employed are human κ -casein, human colostrum IgA, and bovine submaxillary gland mucin.

In a further preferred embodiment, the terminal tetra-saccharide at the non-reducing end of the oligosaccharide chain is Lewis b-tetrasaccharide, i.e.
10 $\text{Fuc}\alpha 1-2\text{Gal}\beta 1-3(\text{Fuc}\alpha 1-4)\text{GlcNAc}\beta 1-$.

The di- or oligosaccharide glycosides may be contained in any appropriate amount in a pharmaceutical composition, and are generally contained in an amount between 0.01% and 99%, or between 0.1% and 99% by weight of the total weight of the
15 composition.

The pharmaceutical compositions containing the active ingredients may suitably be formulated so that they provide doses within these ranges either as single dosage units or as multiple dosage units.

20 The active di- or oligosaccharide glycosides used according to the invention as well as compositions containing the glycosides may typically be administered to a human patient in amounts corresponding to from about 1 mg per day to about 50 g per day, preferably from about 10 mg per day to about 5
25 g per day, of the active glycoside ingredient.

The dosage of a di- or oligosaccharide glycoside depends in general on the choice of administration route, the particular disease to be treated and the severity of same, and also whether the disease is to be treated or prevented, as well as
30 the age and weight of the person to be treated.

The pharmaceutical composition may preferably be administered by the oral route, by the parenteral route or by the rectal route.

The invention is further illustrated by the following non-limiting examples.

EXAMPLE 1

BIOLOGICAL EXPERIMENTS

MATERIALS AND METHODS

Purification of κ -casein from human milk

Human milk was kept frozen at -20°C until used. The milk was thawed and skimmed milk obtained by centrifugation at $15,000 \times g$ for 45 min. The fat layer at the top of the tube was carefully removed while the soluble fraction and the pellet were pooled. The pool was acidified with HCl to pH 4.3 and CaCl_2 was added to a concentration of 60 mM. Following incubation at room temperature for 1 h, the precipitated casein fraction was collected by centrifugation at $18,000 \times g$ for 90 min.

The casein fraction was dissolved in 20 mM ethanolamine, 6 M urea, at pH 9.5. To remove any remaining fat, this aqueous fraction was extracted three times with 4 volumes of hexane. The aqueous phase was thereafter dialysed against distilled water and lyophilized. The lyophilized proteins were dissolved in 50 mM imidazole-HCl, 0.5% SDS, 0.5% 2-mercaptoethanol at pH 7.0 and chromatographed on a Sephadex G-200 column (1.6 x 120 cm, Pharmacia-LKB, Uppsala, Sweden), equilibrated with 50 mM imidazole-HCl, 0.5% SDS, 0.5% 2-mercaptoethanol at pH 7.0 at a temperature of 37°C . κ -Casein-containing fractions which were detected by Schiff staining were pooled, dialysed against 40% methanol in distilled water for 3 h and lyophilized to reduce the volume. The protein was redissolved

in 50 mM imidazole-HCl, 0.5% SDS, 0.5% 2-mercaptoethanol at pH 7.0 and a second time on the Sephadex G-200 column under the same conditions as above. Human κ -casein-containing fractions completely free of β -casein were collected,
5 dialysed against 40% methanol and lyophilized.

Purification of bovine mature milk and colostrum κ -casein

Bovine mature milk and colostrum collected within 24 h of parturition were obtained from Agrisera AB (Tväråbäck, Sweden). Both were defatted and the respective casein fraction was prepared as described above for the purification of
10 human milk with the exception that no delipidation with hexane was necessary. The casein pellet was dissolved in 10 mM imidazole-HCl, 3.3 M urea, 10 mM 2-mercaptoethanol, at pH 7.0 and applied to a Q-Sepharose column (2.6 x 15 cm, Pharmacia LKB, Sweden). After washing, the column was eluted with a
15 linear gradient of 0.1-0.5 M NaCl in the same buffer. Bovine κ -casein-containing fractions were dialysed against water and lyophilized. The identification of bovine caseins was confirmed by chymosin cleavage.

20 *In situ* adherence assay for *H. pylori*

Multiple non-diseased tissue samples of adult human esophagus, stomach, duodenum, colon, kidney, cervix, endometrium and midbrain were obtained from the surgical pathology and autopsy files of the Department of pathology at Washington
25 University. The gastrointestinal tract of 250 g Sprague-Dawley rats, and 6-12 week old FVB/N mice were removed after sacrifice by cervical dislocation and regionally dissected as described in Sweetser, D. A., Birkenmeier, E. H., Hoppe, P. C., McKeel, D. W., and Gordon, J. I., *Genes Devel.* 2 (1988),
30 1318-1332. Stomachs from adult dogs were obtained from the Department of Surgery, St. Louis University Medical Center. All tissues were fixed in 10% formalin or in a solution of picric acid/formaldehyde/glacial acetic acid (15:5:1; Bouin's fluid) and subsequently embedded in paraffin (see Sweetser,

D. A., Birkenmeier, E. H., Hoppe, P. C., McKeel, D. W., and Gordon, J. I., *Genes Devel.* 2 (1988), 1318-1332). Sections with a thickness of 5 μ m placed on glass slides were prepared and used for hematoxylin and eosin staining (to identify the cell types present in gastric units, and to verify that the tissue samples had no pathologic changes) and/or for subsequent adherence, histochemical and/or immunocytochemical assays.

Five previously characterized clinical isolates of *H. pylori* were used. NCTC 11637 and NCTC 11638 are reference strains isolated in 1982 from patients with active chronic gastritis (see Warren, J. R., and Marshall, B., *Lancet* 1 (1983), 1273-1275). Strain WV229 was recovered from a patient with gastric ulcer (see Westblom, T. U., Madan, E., Subik, M. A., Buriex, D. E., and Midkiff, B. R., *Scand. J. Gastroenterol.* 27 (1992), 249-252), while P466 (obtained from The Johns Hopkins University School of Medicine, Baltimore) was obtained from a patient with acute gastritis. Strain MO19 was isolated from an asymptomatic carrier (see Barthel, J. S., Westblom, T. U., Havey, A. D., Gonzalez, F. and Everett, E. D., *Arch. Intern. Med.* 148 (1988), 1149-1151). Strains of *H. pylori* were grown at 37°C on Brucella agar supplemented with 10% bovine blood and 1% IsoVitalex (Becton Dickinson Microbiology Systems, Cockeysville, MD) under microaerophilic conditions (5% O₂, 10% CO₂, 85% N₂) and 98% humidity. Five days after inoculation, about 1 μ l of bacteria were removed with a sterile loop from a plate and were resuspended by gentle pipetting in 1 ml of 0.15 M NaCl/0.1 M sodium carbonate with a pH of 9.0. 10 μ l of a freshly prepared 10 mg/ml solution of fluorescein isothiocyanate (FITC, Sigma Chemical Co.) in dimethylsulfoxide was added to the suspension which was then incubated for 1 h at room temperature in the dark. The bacterial cells were recovered by centrifugation at 3,000 x g for 5 min, and then resuspended in 1 ml of a phosphate buffered saline containing 0.2% bovine serum albumin and 0.05% polyoxyethylene sorbitan monolaurate (blocking buffer 1, BB1) at pH 7.4 by gentle pipetting, and pelleted by centrifugation as above. The wash

- procedure was repeated 3 times. The intensity of FITC-labelling of all bacterial strains was similar as judged by inspection of comparable numbers of organisms by fluorescence microscopy. Aliquots of 100 μ l were taken from the final
- 5 suspensions with an optical density of 1.00 O.D./ml, measured by A_{600nm} , and utilized immediately or stored at -20°C until use. No differences in binding patterns were observed between bacteria labelled and used fresh and bacteria that were frozen and thawed once before use.
- 10 Slide-mounted tissue sections were deparaffinized in xylene and isopropanol (xylene, 10 min; xylene, 3 min; isopropanol, 3 min; isopropanol, 3 min; and isopropanol 3 min) rinsed in water followed by 3 times in PBS and then incubated for 30-60 min in blocking buffer 1 (0.2% bovine serum albumin/0.05%
- 15 polyoxyethylene sorbitan monolaurate in PBS). The FITC-labelled bacterial suspension was diluted 20-fold in blocking buffer 1 and a 200 μ l aliquot was placed on a slide-mounted tissue section and then incubated for 1 h at room temperature in a humidified chamber. The slides with the treated tissue
- 20 sections were subsequently washed 4-6 times with PBS prior to inspection under a fluorescence microscope.

- To analyze the ability of various glycoproteins or free oligosaccharides to inhibit binding, 200 μ l aliquots of suspensions of FITC-labelled bacteria were preincubated for
- 25 2 h with bovine submaxillary mucin (Sigma, final concentration of 500 μ g/ml, dissolved in PBS pH 7), fetuin (Sigma, 100 μ g/ml, dissolved in PBS pH 7), asialofetuin (Sigma, 100 μ g/ml, dissolved in PBS pH 7), human sialyl-lactose (NeuAc- α 2 $\rightarrow$ 6Gal β 1 $\rightarrow$ 4Glc) (Sigma, 5 μ g/ml, dissolved in PBS pH 7),
- 30 bovine sialyl-lactose (NeuAc α 2 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4Glc) (Sigma, 5 μ g/ml, dissolved in PBS pH 7), purified human κ -casein (concentrations of 1000, 500, 250, 50, 10, and 1 μ g/ml, dissolved in PBS pH 7), or purified bovine mature or colostrum κ -casein (concentration of 1,000 μ g/ml, dissolved in PBS pH 7) for 2 h
- 35 at room temperature, human serum IgA (Cappel Research Products, 500 μ g/ml, dissolved in PBS pH 7), or human colostrum

secretory IgA (Cappel Research Products, 15 μ g/ml, dissolved in PBS, pH 7). The bacteria were washed once in blocking buffer 1 before the mixture was added to tissue sections.

Human κ -casein or human colostrum secretory IgA was then
5 washed with PBS using $M_r = 10,000$ cutoff Centricon filters (Amicon). Human κ -casein and human colostrum secretory IgA were also incubated with 100 mU of bovine kidney α -L-fucosidase or *Vibrio cholerae* neuraminidase (Boehringer-Mannheim, Germany) for 2 h at 37°C prior to incubation with bacteria.
10 κ -Casein incubated in phosphate buffered saline at pH 7.4 served as control. Glycosidase treated samples and the control were all incubated at 80°C for 20 min to inactivate the enzymes before the κ -casein was incubated with bacteria. IgA incubated in PBS served as control. Glycosidase treated
15 samples and the control were all incubated at 80°C for 20 min to inactivate the enzymes before the IgA was incubated with bacteria.

To further characterize the receptor active domain of human κ -casein and of human colostrum secretory IgA, respectively,
20 periodate oxidation was performed as described below.

Two other experiments were conducted to ascertain the nature of the bacterial receptor in tissue sections. Firstly, deparaffinized sections were treated with 200 mU proteinase K which is a serine protease that is able to catalyse the
25 hydrolysis of the peptide bond to the COOH-site of aliphatic and/or aromatic amino acids (see Ebeling et al. *Eur. J. Biochem.* 4 (1974), 91-97) from *Trichiratum albus* (Boehringer-Mannheim, Germany) prepared as a 1 mg/ml stock solution in PBS for 2 h at 37°C. They were subsequently washed three
30 times in PBS and treated with blocking buffer 1, and a suspension of FITC-labelled *H. pylori* strain P466 or WV229 and was then overlaid as described above. Secondly, deparaffinized sections were treated with 10 mM sodium metaperiodate/50 mM sodium acetate at a pH of 4.5 and for 10 min
35 at 0°C to selectively cleave carbon atoms number 8 and 9 of

the unsubstituted side chain of terminal sialic acids (see Manzi, A. E., Dell, A., Azadi, P., and Varki, A., *J. Biol. Chem.* 265 (1990), 8094-8107), or with 10 mM sodium meta-periodate/50 mM sodium acetate at a pH of 4.5 for 1 h at room temperature to cleave carbon-carbon bonds between vicinal hydroxyl groups in most carbohydrates with a free hydroxyl group in the 3 position (thereby breaking up the ring structure of the monosaccharide and thus destroying the carbohydrate epitope while leaving peptides intact, see Woodward, M. P., Young, W. W Jr., and Bloodgood, R. A., *J. Immunol. Meth.* 78 (1985), 143-153. Control sections were incubated with 50 mM sodium acetate buffer alone. After two PBS washes, the sections were reduced by adding 50 mM sodium borohydride prepared in PBS with a pH of 7.6. After several additional washes with PBS, suspensions of FITC-labelled strains WV229, P466, or MO19 were applied and the slides were processed as described above.

Control experiments were used to establish that the antigenicity of proteins present in gastric epithelial cell lineages was preserved under the "harsher" periodate oxidation at pH 4.5. Thus, periodate-treated sections of rat stomach were incubated with a rabbit polyclonal antisera (10 µg protein/ml, 4°C, overnight) raised against intrinsic factor (IF) and the antigen-antibody complexes subsequently detected by Texas Red conjugated donkey anti-rabbit IgG (see Roth, K. A., Cohn, S. M., Rubin, D. C., Trahair, J. F., Neutra, M. R. and Gordon, J. I., *Am. J. Physiol. (Gastrointest. Liver Physiol.)* 263 (1992), G186-G187, and Lee, E. Y., Seetharam, B., Alpers, D. H. and DeSchryver-Kecsckemeti, K., *Gastroenterol.* 97 (1989), 1171-1180 for the staining protocol used and details about the specificity of this sera). The intensity of IF staining of chief cells in periodate-treated sections was similar to the intensity of staining of chief cells in sections that had been incubated with buffer alone while the binding of the α -L-fucose specific *Ulex europaeus* type 1 lectin to surface mucous cells was completely abolished under the same conditions.

Immunohistochemical studies

The cellular distribution of sialylated oligosaccharides was examined in deparaffinized sections of human stomach containing zymogenic glands by incubation with (i) fluorescein-,
5 rhodamine- (LIST Biological Laboratories Inc.), or peroxidase- (Sigma) conjugated cholera toxin B subunit which recognizes internally positioned NeuAc α 2 $\rightarrow$ 3 residues linked to galactose (see Holmgren, J., Lönnroth, I., Månsson, J.-E., and Svennerholm, L, *Proc. Natl. Acad. Sci. USA* 72 (1975),
10 2520-2524, final concentration of 5 μ g protein/ml, dissolved in blocking buffer 1), (ii) digoxigenin (DIG)-conjugated *Sambucus nigra* lectin (SNA) which recognizes NeuAc- α 2 $\rightarrow$ 6Gal/GalNAc (see Knibbs, R. N., Goldstein, I. J., Ratcliffe, R. M., and Shibuya, N., *J. Biol. Chem.* 266 (1991),
15 83-88, 10 μ g protein/ml, dissolved in blocking buffer 1), and DIG-conjugated *Maackia amurensis* lectin (MAA) which reacts with NeuAc α 2 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4GlcNAc (see Knibbs, R. N., Goldstein, I. J., Ratcliffe, R. M., and Shibuya, N., *J. Biol. Chem.* 266 (1991), 83-88, 10 μ g protein/ml, dissolved in blocking buffer
20 1). DIG-conjugated lectins were detected with peroxidase-conjugated monoclonal mouse anti-DIG antibody (500 mU peroxidase/ml, dissolved in blocking buffer 1) by washing the tissue three times in PBS, overlaying the secondary antibody on the tissue for 1 h at room temperature, followed by standard
25 H₂O₂/DAB detection.

Fucosylated blood group antigens were detected by first treating the sections with mouse monoclonal antibodies directed against the histo-blood group antigens A, B, and H (Dakopatts A/S, Glostrup, Denmark) (final concentration of
30 1 μ g protein/ml, dissolved in blocking buffer 1), or Le^a and Le^b (Immucor, Norcross, GA) (final concentrations 10 μ g protein/ml, dissolved in blocking buffer 1). Antigen-antibody complexes were detected by visualizing them with a second overlay of FITC-, TRITC- or HRP-conjugated (fluorescein-,
35 rhodamine- or peroxidase-conjugated) rabbit anti-mouse immunoglobulins (Dakopatts A/S, Glostrup, Denmark) (final concen-

tration 30 μ g protein/ml, dissolved in blocking buffer 1) and studying the sections under a fluorescence microscope. In addition, the distribution of fucosylated glycoconjugates was assessed in a similar manner by treating and visualizing sections with FITC-conjugated *Ulex europaeus* type 1 lectin (UEA1, 5 μ g protein/ml, dissolved in blocking buffer 1) which recognizes and binds to α -L-fucose groups.

RESULTS AND DISCUSSION

Analysis of cell lineage-specific binding of *H. pylori* using an *in situ* adherence assay

Five clinical isolates of *Helicobacter pylori* were labelled with fluorescein isothiocyanate followed by 5 days of growth on rich agar under microaerophilic conditions and then overlaid on sections of formalin-fixed human stomach. Strains NCTC 11637, NCTC 11638, WV229, and P466 which all were recovered from patients with dyspeptic syndrome bound to surface mucous cells situated in the upper pit and luminal surface (see Figure 2A,B). Remarkably, mucous neck cells located in the upper portions of the glandular segment of the gastric units were negative indicating that these bacteria were able to distinguish between two differentiated mucus-producing cell lineages present in the stomach (see Ota, H., Katsuyama, T., Ishii, K., Nakayama, J., Shiozawa, T., and Tsukahara, Y., *Histochem. J.* 23 (1991), 22-28). No binding to parietal or chief cells was noted in zymogenic glands. Strain MO19, the only isolate tested which was recovered from an asymptomatic "healthy" carrier, did not bind at a detectable level to any human gastric epithelial cell lineages (see Figure 2C).

Strains WV229 and P466 did not adhere to the squamous epithelium of human esophagus but did adhere under the reaction conditions employed to esophageal submucosal glands and their ducts, and to duodenal villus-associated enterocytes (see Figure 2D). A weak binding to enterocytes situated in the

colonic homolog of small intestinal villi - the surface epithelial cell cuff which surrounds each crypt orifice (see Figure 2E) was also observed. The intensity of staining and hence the density of adherent organisms was considerably less down through the cephalocaudal axis of the intestine than it was in the stomach epithelium (gradient down through the intestine). Control experiments indicated that *Helicobacter pylori* P466 and WV229 did not bind to any epithelial cell populations represented in proximal or distal nephrons, in the cervix, or in the endometrium. Surveys of the central nervous system including the midbrain also failed to produce a signal above background.

The *in situ* adherence assay was used to ascertain whether the cell lineage-specific and *Helicobacter pylori* strain-specific patterns of binding observed in the human gut occurred in other mammalian species. Strains WV229, P466 and M019 grown and labelled with FITC as described above, were incubated with sections of rat and mouse gastrointestinal tracts as well as with stomach from dogs. Strain M019 did not bind in detectable amount to tissue sections prepared from any of these three species. As in the human stomach, strains WV229 and P466 bound to the gastric pit region in the rat but not to any other differentiated epithelial cell population located in the zymogenic or pure mucous zones (see Figure 2F). Unlike in similar human preparations, the stratified squamous epithelium of the rat esophagus and forestomach produced positive results in the *in situ* adherence assay, while the small intestinal epithelium was negative with the exception of Brunner's glands. The forestomach of the mouse showed high-density binding with adherent bacteria, while the remainder of the stomach, i.e. the zymogenic, mucoparietal and pure mucous zones (see Lee, E. R., Trasler, J., Dwivedi, S., and leBlond, C. P., *Am. J. Anat.* **164** (1982), 187-207), and the epithelial and mesenchymal components of the intestine did not yield a signal with FITC-labelled strains WV229 and P466. Finally, no binding of either of the two strains was observed in the dog stomach.

Biochemical studies of the interaction between *Helicobacter pylori* and gastric epithelial cell lineages

To characterize the nature of the interactions between *Helicobacter pylori* and the surface mucous cells, the ability of
5 a series of compounds to inhibit binding of FITC-labelled strains P466 and WV229 to sections of human stomach and proximal small intestine was tested.

When calf serum fetuin, asialofetuin, soluble sialyl-lactose prepared from bovine milk (in which 85% of the sialic acid is
10 linked to galactose via an $\alpha 2 \rightarrow 3$ linkage and 15% is linked via an $\alpha 2 \rightarrow 6$ linkage, i.e. NeuAc $\alpha 2 \rightarrow 3/6$ Gal $\beta 1 \rightarrow 4$ Glc) or human milk sialyl-lactose (15% NeuAc $\alpha 2 \rightarrow 3$; 85% NeuAc $\alpha 2 \rightarrow 6$) were preincubated with the above-mentioned two *Helicobacter pylori* strains, no subsequent reduction in adherence was noted as judged by
15 the *in situ* assay. These findings, i.e. sialic acid independent binding, are in agreement with the work of Fauchère, J.-L., and Blaser, M. J. in *Microb. Pathog.* 9 (1990), 427-439.

However, preincubation of the bacteria with 0.5% bovine submaxillary gland mucin which is a rich source of both
20 fucosylated and sialylated carbohydrates (see Savage, A. V., D'Arcy, S. M. T., and Donoghue, C. M., *Biochem. J.* 279 (1991), 95-103 and 33), completely inhibited binding of bacteria to surface mucous cells (see Figure 1A,B) and to duodenal, villus-associated, epithelial cells. Human κ -casein
25 and human colostrum secretory IgA were also potent inhibitors: 250 μ g/ml of human κ -casein (see Figure 3A) and 15 μ g/ml of human colostrum secretory IgA, respectively, fully inhibited adherence of *Helicobacter pylori*.

Human κ -casein oligosaccharides are fucosylated via an $\alpha 1 \rightarrow 4$ -
30 linkage to N-acetylglucosamine and differs in this respect from bovine κ -casein. Human colostrum secretory IgA carries a highly varied set of N- and O-linked oligosaccharides.

Thus, it is therefore interesting that only the human κ -casein and human colostrum secretory IgA were able to prevent the cell-lineage specific attachment of *Helicobacter pylori* to human gastric surface mucous cells. Several observations suggested that this inhibition of *Helicobacter pylori* binding by human colostrum secretory IgA was not mediated by a bacterial Fc-receptor or by a classical immunoglobulin recognition effect, involving the hypovvariable of the Fab fragments. Thus, it was observed that (i) inhibition was abolished when the human κ -casein or human colostrum secretory IgA was pretreated with meta-periodate, (ii) preincubation at 85°C for 30 min in PBS did not affect the inhibitory activity of the two glycoproteins, and (iii) treatment of human κ -casein (see Figure 3C) or human colostrum secretory IgA with α -L-fucosidase markedly reduced inhibitory activity while neuraminidase treatment had a lesser effect on the inhibitory activity of human κ -casein and no detectable effect on the inhibitory activity of human colostrum secretory IgA.

In comparison, κ -casein from bovine mature milk and colostrum (see Figure 3B) did not inhibit binding of *Helicobacter pylori* at concentrations as high as 1000 μ g/ml, and human serum IgA did not inhibit binding at concentrations as high as 100 μ g/ml. Pretreatment of sections of human stomach with proteinase K also produced a marked decrease in the binding of the two *Helicobacter pylori* strains P466 and WV229.

Taken together, these results strongly indicate that binding is mediated by a fucosylated rather than a sialylated glycoprotein receptor expressed on surface mucous cells.

The indication that binding of *Helicobacter pylori* adhesin(s) to the surface mucous cell population of human stomach does not depend upon sialic acid epitopes in a cellular receptor is further supported by the results of two additional experiments.

Firstly, the distribution of sialic acid-containing complex carbohydrates in the human gastric mucosa does not correlate with the cellular patterns of adherence observed in the *in situ* assay. Determination of this distribution was carried out in the experiments using *Maackia amurensis* agglutinin (MAA) which is specific for NeuAc α 2 $\rightarrow$ 3Gal β 1 $\rightarrow$ 4GlcNAc epitopes (on sialylated N/O-glycans and glycosphingolipids) (see Knibbs, R. N., Goldstein, I. J., Ratcliffe, R. M., and Shibuya, N., *J. Biol. Chem.* **266** (1991), 83-88), and *Sambucus nigra* agglutinin which recognizes NeuAc α 2 $\rightarrow$ 6Gal/GalNAc structures (see Shibuya, N., Goldstein, I. J., Broekaert, W. F., Nsimba-Lubaki, M., Peeters, B., and Peumans, W. J., *J. Biol. Chem.* **262** (1987), 1596-1602). It turned out that binding of the MAA and SNA lectins was confined to the submucosal compartment of the human stomach. Thus, they did not react with members of any gastric epithelial cell lineage (see Figure 1C,D).

Similarly, the cholera toxin B subunit recognizes internally linked sialic acid in glycoproteins and glycosphingolipids, specifically GalNAc β 1 $\rightarrow$ 4(NeuAc α 2 $\rightarrow$ 3)Gal- β -structures (see Holmgren, J., Lönnroth, I., Mansson, J.-E., and Svennerholm, L, *Proc. Natl. Acad. Sci. USA* **72** (1975), 2520-2524). It turned out that this lectin did not bind to surface mucous cells but rather that binding was confined to mucous neck cells located in the upper glandular domains of gastric units (see Figure 1E). Control experiments with dog stomach demonstrated (i) that MAA binds to surface mucous cells, (ii) that SNA binds to surface mucous and parietal cells, and (iii) that cholera toxin B subunit does not bind to any gastric epithelial cell lineage in this species. This suggests that these lectins can be used to define fundamental differences in the differentiation program of the surface mucous cell lineage between dogs and humans - differences which may account for their distinct abilities to bind *Helicobacter pylori*.

Secondly, when tissue sections were incubated with 10 mM meta-periodate/sodium acetate at pH 5.5 and at 0°C, selective cleavage of carbons 8 and 9 of the unsubstituted side chain of terminal sialic acids can be achieved (see Manzi, A. E., Dell, A., Azadi, P. and Varki, A., *J. Biol. Chem.* **265** (1990), 8094-8107), e.g. as was evident by the loss of SNA binding to surface mucous cells in the dog stomach. Using these periodate oxidation conditions, there was no reduction in adherence of any of the binding strains to human stomach or small intestinal epithelial cell populations as compared to control sections that had been treated with sodium acetate buffer alone.

Other observations support a role for fucosylated epitopes in the glycoprotein(s) that mediate binding of *Helicobacter pylori* to surface mucous epithelial cells in the human stomach. Bacterial binding was reduced in sections of human stomach containing zymogenic glands that had been preincubated with monoclonal antibodies specific for either of the three blood group antigens (e.g. see Figure 1F-H). *Ulex europaeus* type 1 agglutinin (UEA1) which is specific for α -L-fucose also bound to the same cells which contained a receptor for *Helicobacter pylori* adhesin(s) (see Figure 1I). The "mild" meta-periodate oxidation reaction conditions employed above had no effect on UEA1 binding (see Figure 1J). This treatment also had no effect on *Helicobacter pylori* binding (see Figure 1K,L). Harsher cleavage conditions (i.e. reducing the pH to 4.5, increasing the incubation time to 1 h; and raising the incubation temperature to 20°C) were required to ablate UEA1 binding and the binding of the monoclonal blood group H antibody to human surface mucous cells. These conditions also resulted in loss of cholera toxin B subunit binding and loss of adherence of *Helicobacter pylori* strains WV229 and P466.

The above described multilabel immunohistochemical studies indicated that receptor sites for FITC-labelled strains P466 and WV229 are co-expressed in members of this epithelial cell

lineage together with the fucosylated histo-blood group antigens H, B and Le^b (e.g. see Figure 1F-H).

Together, these results strongly imply that the binding of *Helicobacter pylori* to UEA1-positive, fucosylated blood group antigen-positive, surface mucous cells in the human stomach is not dependent on terminal non-substituted sialic acid residues. Furthermore, the distribution of MAA and SNA lectin and cholera toxin B subunit binding sites plus the failure of fetuin and soluble sialyl-lactose to inhibit binding argue strongly against involvement of the $\alpha 2 \rightarrow 3$ linked sialic acid residues that has been invoked in previous studies (see Evans, D. G., Evans, D. J. Jr., and Graham, D. Y., *Infect. Immun.* **57** (1989), 2272-2278, Evans, D. G., Evans, D. J. Jr., Moulds, J. J., and Graham, D. Y., *Infect. Immun.* **56** (1988), 2896-2906, and Saitoh, T., Natomi, H., Zhao, W., Okuzumi, K., Sugano, K., Iwamoto, M., and Nagai, Y., *FEBS Lett.* **282** (1991), 385-387); studies which employed cells that are not known targets for the organisms *in vivo* (see Evans, D. G., Evans, D. J. Jr., and Graham, D. Y., *Infect. Immun.* **57** (1989), 2272-2278, and Evans, D. G., Evans, D. J. Jr., Moulds, J. J., and Graham, D. Y., *Infect. Immun.* **56** (1988), 2896-2906).

Figure 1 shows the characterization of a putative *Helicobacter pylori* adhesin receptor as a glycoprotein that lacks sialyl-lactose.

Fig. 1A and 1B: Sections of human stomach containing gastric units with zymogenic glands were incubated with FITC-labelled *Helicobacter pylori* strain WV229 (1A) or with bacteria that had been treated with a 0.5% solution of bovine submaxillary gland mucin (1B). The mucin preparation produces marked reductions in binding.

Fig. 1C and 1D: Section of human stomach containing members of the surface mucous, mucous neck, parietal and chief epithelial cell lineages were incubated with

digoxigenin-labelled *Maackia amurensis* agglutinin (MAA, 1C) or *Sambucus nigra* agglutinin (SNA, 1D). DIG-conjugated lectins were detected with a peroxidase-conjugated monoclonal mouse anti-DIG antibody preparation. None of the epithelial cell lineages produces a signal above background when incubated with these lectins that recognize sialic acid-containing carbohydrate epitopes. Control experiments employing the monoclonal antibody alone produced no staining (data not shown).

Fig. 1E: Adjacent section of human stomach incubated with rhodamine-conjugated cholera toxin B subunit. Mucus-producing cells located in the isthmus or upper portion of the glandular domain of gastric units react with this toxin which recognizes internally linked sialic acids in glycoproteins and glycosphingolipids. The subunit does not bind to surface mucous cells located in the pit.

Fig. 1F-1H: Sections of human stomach were incubated with FITC-labelled *Helicobacter pylori* strain P466 (1F) and a mouse monoclonal antibody directed against the fucosylated blood group antigen H (visualized with rhodamine-conjugated rabbit anti-mouse IgG in Fig. 1G). Fig. 1H is a double exposure showing that surface mucous cells co-express the bacterial adhesin receptor and the blood group antigen. A marked reduction in adherence of bacteria to surface mucous cells was noted (1F) when compared to sections not treated with this monoclonal antibody (e.g. Fig. 1A). Similar reductions in binding were obtained using mouse monoclonal antibodies directed against fucosylated blood group antigens B and Le^b (data not shown). Non-immune mouse IgG failed to produce this effect (data not shown).

Fig. 1I: A section of human stomach was incubated with FITC-conjugated *Ulex europaeus* agglutinin (UEA1) which recognized α -L-fucose. The lectin binds to surface mucous cells.

Fig. 1J: Pretreatment of a section of human stomach with sodium meta-periodate, pH 5.5 at 0°C for 10 min produces no appreciable reduction in binding of UEA1.

5 Fig. 1K and 1L: The binding of *Helicobacter pylori* to pit mucous cells (1K) was also unaffected by the sodium meta-periodate pretreatment (1L).

Figure 2 shows the *in situ* assay for binding of *Helicobacter pylori* to gut epithelial cell lineages.

10 Fig. 2A: Section of human stomach stained with hematoxylin and eosin showing the uppermost portion of gastric units in the zymogenic zone. Surface mucous (M) and parietal (P) cells are indicated.

15 Fig. 2B and 2C: Sections of stomach of an adult human were incubated with FITC-labelled *Helicobacter pylori* strains WV229 (2B) and MO19 (2C) after the bacteria had been grown on blood agar for 5 days under microaerophilic conditions. Strain WV229, which was recovered from a patient with gastric ulcer disease, is associated with surface mucous cells located in the upper pit of gastric unit and their associated luminal surfaces. Strain MO19, 20 which was isolated from a healthy carrier, is not bound to any cell lineage.

25 Fig. 2D and 2E: Incubation of strain WV229 with section of duodenum (2D) and colon (2E) from an adult human. The FITC-labelled bacterial preparation used for staining sections of stomach also stains villus-associated epithelial cells (including enterocytes) and very weakly colonocytes located in the upper portion of colonic crypts and their surface epithelial cuffs. Less differentiated, proliferating and non-proliferating, cells 30 located in small and large intestinal crypts did not contain adherent bacteria. Cell populations located in the mesenchymal compartment were also negative. Incuba-

tion of section of human stomach, intestine and colon with strains NCTC 11637, NCTC 11638 and P466 produced results comparable to those shown in Fig. 2B, 2D and 2E (data not shown).

- 5 Fig. 2F: A section from the zymogenic zone of stomach from an adult Sprague-Dawley rat was incubated with FITC-labelled strain WV299 grown for 5 days on blood agar under microaerophilic conditions. *Helicobacter pylori* associated with rat surface mucous cells but not with
10 cell lineages associated with the isthmus or glandular domains of rat gastric units.

Figure 3 shows the evaluation of the inhibitory activity of various κ -caseins on the attachment of *Helicobacter pylori* to the human gastric mucosa *in situ*.

- 15 Fig. 3A: *Helicobacter pylori* strain P466 preincubated with human κ -casein (250 $\mu\text{g/ml}$) prior to overlay on section of human stomach.

- Fig. 3B: *Helicobacter pylori* strain P466 preincubated with bovine colostrum κ -casein (1000 $\mu\text{g/ml}$) prior to
20 overlay on section of human stomach.

Fig. 3C: Pretreatment of human κ -casein with α -L-fucosidase prior to overlay on section of human stomach. Overlays were done on adjacent sections from the same paraffin embedded tissue.

25 EXAMPLE 2

SDS-PAGE of glycoproteins

The labelling of the bacteria was performed as described in Example 1 and the bacterial strains used were the *H. pylori* strain P466. The *in situ* adherence assay for *H. pylori* and

the immunohistochemical studies were carried out by the procedures described in Example 1.

SDS-PAGE of human colostrum samples, goat and cow milk, ammonium sulphate precipitated fractions of human colostrum
5 and neoglycoconjugates was performed with 4-20% or 12% pre-cast gels (Bio-Rad, Melville, NY.). Proteins were denatured for 3 min at 100°C in SDS-2-mercaptoethanol. Gels were stained with Coomassie brilliant blue and the molecular weight standards were obtained from Sigma, St. Louis, MO.

10 Immunoblot analysis

Proteins which were separated on SDS-PAGEs were transferred by a semidry Multiphore Nova Blot system onto nitrocellulose for 1.5 hour. Proteins were stained with Ponceau S solution (Sigma, St. Louis, MO) and blots were then incubated for 1-3
15 hours in blocking buffer 2 (BB2) consisting of 1% Tris buffered saline (TBS), 1% Blocking reagent (Boehringer Mannheim, Indianapolis, IN.), 1mM MnCl₂, 1mM MgCl₂, 1mM CaCl₂, 0.05% polyoxyethylene sorbitan monolaurate, 0.1% NaN₃. The following blood group-specific monoclonal antibodies were
20 used: Anti-Le^a (CBM-LA1) and anti-Le^b (CBM-LB1) from Immucor, Norcross, GA; anti-Le^x (630/7H1), anti-Le^y (672/7E3), anti-Le^{b,y} (64/4D8), and anti-H-2 (92FR A2) from Accurat Chemical & Scientific Corporation, Westbury, NY.; anti-type 1 chain (LNT) (K21), anti-H-1 (17-206), and anti-Le^b (T218)
25 from Signet Laboratories, Dedham, MA. The following lectins were used: Biotin-labelled *Ulex europaeus* type 1, *Lotus tetragonolobus*, and *Anguilla anguilla* recognizing the H-antigen (Sigma, St. Louis, MO); digoxigenin (DIG)-labelled *Aleuria aurantia* recognizing branched Fucα1-6GlcNAc in glycocon-
30 jugates (Boehringer Mannheim). Blots were incubated with antibodies or lectins in concentrations of 5-10 µg/ml in BB2 for 6 hours followed by 7 washes in TBS. Monoclonal antibodies were detected by alkaline phosphatase (AP)-conjugated goat anti-mouse antibodies (Boehringer Mannheim). Digoxigenin
35 or biotin labelled lectins were detected with AP-conjugated

sheep anti-DIG or anti-biotin Fab fragments (Boehringer Mannheim), respectively. Blots were washed 5x in TBS, once in 0.1M Tris-HCl pH 9.5, 0.1M NaCl, 5 mM MgCl₂ (APBIII) and developed with BCIP/NBT.

5 Biotin labelling of lectins

Anguilla anguilla lectin was biotin labelled using a biotin-NHS kit from Calbiochem Immunochemicals, La Jolla, CA. The degree of biotinylation (n=7) was determined by the shift in molecular weight on SDS-PAGE.

10 Size fractionation of human secretory IgA-preparation

500 µg of human secretory IgA from pooled human colostrum (Cappel Laboratories, West Chester, PA.) was applied to a Superose 6HR 10/30 eluting with 0.2 ml/min in PBS, pH 6.8. Samples of 400 µl were collected and 20 µl aliquots of every UV-absorbing fraction were run on SDS-PAGE. The fractions of the major peak and the minor peak corresponding to secretory IgA and 120-150 kDa glycoproteins, respectively, were pooled separately and assayed for bacterial adherence inhibition properties (see below) in the *in situ* adherence assay.

20 PNGase F digestion of human secretory IgA-preparation

50 µg of secretory IgA was incubated in 100 µl 0.1M phosphate buffer, pH 8.0, 10 mM β-MSH, 20 mM EDTA and 6 units of PNGase F (Boehringer Mannheim) (native conditions) or 50 µg of secretory IgA was first incubated in 0.2% SDS, 10 mM β-MSH for 3 min at 100°C and then diluted with 0.1M phosphate buffer pH, 8.0 to 100 µl (denatured conditions). n-Octylglucoside (0.5%) was added to the denatured sample to reduce the interference of the PNGase F enzyme with SDS. Finally 6 units of PNGase F were added. Incubations were carried out at 37°C and samples were removed after 0 hour, 1.5 hour, 4 hours, 7 hours, and 18 hours and analyzed on SDS-PAGE and immunoblots.

Preparation of human colostrum and milk samples

Human colostrum samples were from Children's Hospital, St. Louis, MO. Colostrum, goat and cow milk were delipidated and cellular debris was removed by centrifugation at 20 krpm in a Sorvall SS-34 rotor for 30 min before the material was used for inhibition experiments or analysis on SDS-PAGE.

Glycoconjugate inhibition studies

The ability of glycoconjugates to inhibit the bacterial adherence to human stomach *in situ* was analyzed by preincubation of 200 μ l of labelled bacterial suspension in BB1 for 1.5 hour at room temperature with the glycoconjugates listed below. The bacteria were washed once, resuspended in 200 μ l of BB1, and added to the sections. Adherence was compared to sections incubated with non-inhibited bacteria. The following glycoconjugates were used where (n) represents the number of carbohydrate chains chemically attached to the neoglycoprotein as determined by the total carbohydrate anthron assay: Secretory IgA, lacto-N-tetraose (LNT)-human serum albumin (HSA) (n=26), lacto-N-neotetraose (LNnT) HSA (n=26), lacto-N-fucopentaose I (H-1)-HSA (n=35), lacto-N-fucopentaose II (Lea)-HSA (n=30), lacto-N-fucopentaose III (Le^x)-HSA (n=30), lacto-N-difucohexaose I (Le^b)-HSA (n=32), Le^y-tetrasaccharide (Le^y)-HSA (n=26), lacto-N-neofucopentaose I (H-2)-bovine serum albumin (BSA) (n=29), GlcNAc β 1-4(Fuc α 1-6)GlcNAc β -BSA (all from IsoSep AB, Tullinge, Sweden). The H1-HSA was also obtained from Accurate Chemical & Scientific Corporation. All oligosaccharides for neoglycoconjugate preparations were purified by HPLC and structurally identified and characterized and shown by the manufacturer to be more than 95% pure with NMR-spectroscopy. The identity of the neoglycoproteins was verified by immunoblots using monoclonal antibody K21 recognizing LNT (non-fucosylated precursor chain), anti-H-1 (17-206) (the H-antigen on a lactoseries type 1 chain), anti-H-2 (92FR A2) (the H-antigen on a lactoseries type 2 chain), anti-Le^a (CBM-LA1) (monofucosylated type 1 chain),

(anti-Le^b CBM-LB1) (difucosylated type 1 chain), anti-Le^b (T218) (a clone with less cross-reactivity in our hands), anti-Le^x (630/7H1) (monofucosylated type 2 chain), anti-Le^y (672/7E3) (difucosylated type 2 chain), anti-Le^{b,y} (64/4D8) (recognizing difucosylated type 1 and 2 chains) and the H-antigen specific lectins *Ulex europaeus* type 1 and *Anguilla anguilla*.

Oligosaccharide inhibition studies

The ability of glycoconjugates to inhibit bacterial adherence *in situ* was analyzed by preincubating labelled bacteria for 3.5 hours at room temperature with the following oligosaccharides diluted in BB1: Lacto-N-fucopentaose I (H-1), lacto-N-fucopentaose II (Le^a), lacto-N-difucohexaose I (Le^b), lacto-N-fucopentaose III (Le^x), lacto-difucotetraose, 2'-fucosyl-lactose, 3-fucosyllactose (all from IsoSep AB), H-disaccharide (Accurate Chemical & Scientific Corporation), Fuc α 1-4-GlcNAc β 0-TMSE (Symbicom AB, Umeå, Sweden), and L-fucose (Sigma, St. Louis, MO).

Antibody inhibitions of bacterial binding in the *in situ* adherence assay

Two-fold serial dilutions of anti-Le^b and anti-H-2 monoclonal antibodies were applied to separate sections of human gastric mucosa in BB1 and incubated at room temperature overnight. Sections were washed 5x in PBS and bound antibodies were detected with FITC-conjugated rabbit antimouse immunoglobulins diluted 1:100 in BB1 and incubated for 1 hour at room temperature. Antibody dilutions resulting in similar fluorescent intensity were chosen for the bacterial inhibition experiments (1:2000 and 1:5000, respectively). Sections were preincubated with the monoclonal antibodies as above, followed by bacterial overlay as described above.

Bacterial Western blot overlay analysis

SDS-PAGE was performed as described above, with 20 μ g each of unfractionated human colostrum, goat milk, cow milk or 1 μ g each of the different neoglycoconjugates. After transfer to
5 nitrocellulose, the filters were incubated with BB2 overnight, and washed 2x in TBS. Bacterial suspension (0.1 OD₆₀₀) was added (0.2 ml/cm² in BB2) and incubated overnight in room temperature in rolling bottles. Filters were washed 6x5 min in TBS and rabbit antiserum was added 1:100 and incubated for
10 1 hour. Blots were washed 5x in TBS. Bacterial bindings were then incubated with either AP-conjugated antibody diluted 1:2000x for 1 hour, washed 5x in BB2, washed once in APBIII and developed with BCIP/NBT or incubated with gold-labelled antibody, washed 5x in BB2, washed 4x in ddH₂O, and developed
15 with silver enhancement IntenSE BL (Amersham, Arlington Heights, IL.).

Glycolipid preparations

Immunostainings and bacterial HPTLC overlay analyses

Monoclonal antibodies anti-Le^a (CBM-LA1) and anti-Le^b
20 (CBM-LB1) were used to detect the presence of the corresponding antigens in the glycolipids with secondary antibodies and development as described in Materials and Methods in the section entitled Immunoblot Analysis.

RESULTS AND DISCUSSION

25 Analysis of fucosylated blood group antigens in human secretory IgA and serum IgA by lectins and monoclonal antibodies

Immunoblots using the H-antigen recognizing lectins *Ulex europaeus* type 1, and *Anguilla anguilla* revealed the presence
30 of H-antigens in the heavy and light chains of both secretory IgA and serum IgA. *Aleuria aurantia* recognizing branched Fuc α 1-6GlcNAc in glycoconjugates also identified common fuco-

5 sylated structures. These observations taken together indicate that terminal α -1-2 linked fucose presented in the H-antigen or α -1.6 linked fucose does not by itself constitute the *H. pylori* receptor. Monoclonal antibodies against the Lewis blood group antigens Le^a, Le^b, Le^x and Le^y recognized the presence of Le^x and Le^y in the heavy chains of both IgA types while Le^b was restricted to the secretory component of secretory IgA. Le^a could not be found in either of the two IgA types but was detected in a 120-150 kD glycoprotein co-purified with the secretory IgA. This glycoprotein in addition presents the Le^b antigen of equal intensity to the secretory component corresponding to a 10-fold higher Le^b/protein ratio.

Size fractionation of secretory IgA preparation

15 Human secretory IgA was applied to a FPLC-Superose 6 column and the dominating secretory IgA component was separated from the minor 120-150 kD glycoproteins. The peaks were identified by SDS-PAGE and pooled separately. The purified fractions were then analyzed for *H. pylori* inhibitory properties. The two components did not mediate adherence inhibitory activity related to their respective protein content but rather reflected the Le^b content, since 15 μ g/ml of secretory IgA was needed for an efficient bacterial inhibition as described previously (PNAS) compared to 2 μ g/ml of the 120-150 kD protein.

Analysis of the distribution of the Le^b antigen among N- and O-linked carbohydrate chains

30 The unfractionated secretory IgA preparation was digested by the N-chain releasing enzyme PNGase F in denatured and native conditions. During native conditions the digestion was very limited and only a minor part of the carbohydrate content was removed, while after SDS-denaturation the oligosaccharide chains of the secretory component were readily released as visualized by a decrease of molecular weight in SDS-PAGE and

the absence of Le^b antigens as detected by immunoblots. Neither the Le^a nor the Le^b content of the 120-150 kD glycoprotein was, however, affected by the PNGase F treatment indicating the presentation of these blood group antigens in
5 O-linked carbohydrate chains on this protein.

Inhibition of *H. pylori* adherence by human colostrum samples

Human delipidated colostrum samples were screened for Lewis blood group activity using Le^a and Le^b monoclonal antibodies on SDS-PAGE transferred immunoblots. One Le^a-Le^b⁺ individual
10 (A) expressing low levels of Le^a and high levels of Le^b colostrum glycoproteins was found. In addition, one Le^a⁺Le^b⁻ individual (B) with high Le^a levels and undetectable Le^b level was identified. Both these individuals expressed the H-antigen and Fuc α 1-6GlcNAc as recognized by *Ulex europaeus*
15 type 1 and *Aleuria aurantia* lectins, respectively. Titration of the inhibitory levels of colostrum protein showed that 10 μ g/ml of protein of the Le^a-Le^b⁺ colostrum proteins totally abolished bacterial adherence (Fig). In contrast, 100 μ g/ml of the Le^a⁺Le^b⁻ colostrum proteins resulted in only a minor
20 reduction in bacterial binding.

Inhibition of *H. pylori* adherence by goat and cow milk antigens

No Le^a or Le^b antigen activities could be detected in delipidated goat and cow milk. Neither of these non-human milk
25 samples exhibited inhibitory activities in concentrations of 100 μ g/ml.

Analysis of adherence inhibitory properties of fucosylated neoglycoproteins

The following neoglycoproteins having natural HPLC-purified
30 or synthesized carbohydrate chains characterized by NMR-spectroscopy chemically attached to serum albumin were obtained: Lacto-N-tetraose (LNT)-HSA, lacto-N-neotetraose

(LNnT)-HSA, lacto-N-fucopentaose I (H-1)-HSA, lacto-N-fucopentaose II (Le^a)-HSA, lacto-N-fucopentaose III (Le^x)-HSA, lacto-N-difucohexaose I (Le^b)-HSA, Le^y-tetrasaccharide (Le^y)-HSA, lacto-N-neofucopentaose I (H-2)-BSA,

5 GlcNAc β 1-4(Fuc α 1-6)GlcNAc β -BSA. The identity of the neoglycoproteins were verified by immunoblots using monoclonal antibodies. The results confirmed a high degree of purity of the oligosaccharide chains of the neoglycoproteins since all antibodies except the anti-Le^b monoclonal antibodies selectively stained the corresponding neoglycoconjugate. The

10 anti-Le^b (CBM-LB1) clone cross-reacted with the H-1-HSA and to a lesser extent with the Le^y-HSA. The anti-Le^b (T218) clone which recognizes the Le^b-HSA with identical sensitivity did not cross-react with the Le^y-HSA but recognized the

15 H-1-HSA weakly. Consequently, this indicates that the reactivity with the Le^y-HSA is a true cross-reactivity of the CBM-LB1 clone. This was also confirmed by information from IsoSep AB since the Le^y-oligosaccharide was chemically synthesized and therefore could possibly not be contaminated by

20 Le^b. The remaining reactivity with the H-1-HSA conjugate could nevertheless be interpreted as a low level of contamination (less than 5%) of Le^b-oligosaccharide.

For the analysis of adherence inhibitory properties of the fucosylated neoglycoproteins, bacteria were preincubated as

25 described above with 20 μ g/ml of neoglycoconjugates, and the inhibitory activity was compared to 20 μ g/ml of the previously described secretory IgA. In addition to secretory IgA, the Le^b neoglycoprotein was the only glycoconjugate that could totally eliminate *H. pylori* binding at this concentration

30 although a reduction in binding could be observed with the H-1-neoglycoconjugate. Nevertheless, this could be interpreted as a reduction due to low level of Le^b contamination of the H-1-neoglycoconjugate as discussed previously. Increasing the concentrations of the glycoconjugates to 100 μ g/ml

35 did not result in any inhibition by H-2-BSA, Le^a-HSA, Le^x-HSA, Le^y-HSA or GlcNAc β 14(Fuc α 1-6)GlcNAc β -BSA (data not shown). The levels of Le^b-glycoconjugate and secretory IgA

which resulted in almost complete reduction of bacterial binding was estimated to 5 $\mu\text{g/ml}$ and 20 $\mu\text{g/ml}$, respectively, although partial inhibitory effects could be demonstrated at concentrations below 1 $\mu\text{g/ml}$ for the former.

5 Analysis of adherence inhibitory properties of fucosylated oligosaccharides

For the analyses of bacterial inhibition of the fucosylated oligosaccharides, bacteria were preincubated with series of 0.25 mM, 2.5 mM and 25 mM concentrations of the H-1, Le^a,
10 Le^b, Le^x, and Le^y oligosaccharides. The H-1, Le^b, and Le^y oligosaccharides reduced bacterial binding at a concentration of 2.5 mM and almost eliminated binding at a concentration of 25 mM. Le^a and Le^x oligosaccharides did not exhibit binding inhibition at a concentration of 25 mM. The H-disaccharide
15 and 2'-fucosyllactose reduced bacterial binding by 50-75% at a concentration of 20 mM. The Fuc α 1-4GlcNAc β 0-TMSE and 3-fucosyllactose preparations reduced bacterial binding at a concentration of 50 mM. The monosaccharide α -L-fucose was inactive even at a concentration of 175 mM.

20 Western blot overlays of *H. pylori* to milk proteins

Human colostrum, goat milk, and cow milk were separated on SDS-PAGE and transferred on to nitrocellulose and the filters were incubated with bacteria. Binding to the immobilized glycoproteins was detected to a 90 kD protein in case of
25 human colostrum while the goat and cow milk samples did not show any binding.

Western blot overlays of *H. pylori* to neoglycoproteins

The series of neoglycoproteins were run on SDS-PAGE and transferred on to nitrocellulose. The filters were incubated
30 with bacteria and binding to the immobilized glycoproteins was detected with gold-conjugated antibodies. The gold-conjugated antibody signal was amplified by silver enhancement.

Specific binding of *H. pylori* to the Le^b-HSA and weak binding to the H-1-HSA could be demonstrated.

HPTLC overlays of *H. pylori* to glycolipids

The panel of glycolipids was chromatographed on HPTLC-plates, incubated with bacteria and binding to the immobilized glycolipids was detected with AP-conjugated antibodies. No binding of *H. pylori* could be detected to any glycolipid even after extensive development of the chromatogram. Le^a and Le^b antigens were detected in the respective glycolipids by the corresponding monoclonal antibodies.

To do: overlays of mabs and bacteria to all 5 colostrum samples for comparison and to purified secretory IgA and the 50% and 80% AmSo4 fractions (8 samples + Le^b conjugate and a mw-reference).

- 15 Attachment of *Helicobacter pylori* to human gastric epithelium using an *in situ* adherence assay was shown in Example 1 to be inhibited by human colostrum secretory IgA, a molecule carrying a highly variable set of N- and O-linked oligosaccharides while serum IgA was devoid of such inhibitory properties.
- 20 This inhibitory activity of secretory IgA could be markedly reduced by α -L-fucosidase treatment of the secretory IgA. The efforts to delineate the fucosidase sensitive receptor structure for *H. pylori* have focused on the distribution of fucose residues in the secretory IgA molecule. Immunoblots with
- 25 monoclonal antibodies and lectins revealed that both IgA types have fucosylated carbohydrate structures such as the H-antigen, branched Fuc α 1-6GlcNAc, and the Le^x- and Le^y-blood group antigens in common. These observations indicate that terminal α -1,2-linked fucose as presented in the H-antigen,
- 30 branched α -1,6-linked fucose, or blood group antigens on type-2 chains (containing Gal β 1,3GlcNAc) do not by themselves constitute the *H. pylori* receptor. The Le^b-blood group antigen was found to be a carbohydrate structure restricted to the secretory component of secretory IgA. Free secretory com-

ponent has previously been described to present the Le^x-antigen and GlcNAc β 1-4(Fuc α 1-6)GlcNAc β (Mizoguchi/Kobata, 1982) and unusual fucosylated carbohydrate structures such as Fuc α 1-3Fuc and Gal β 14(Fuc α 1-6)GlcNAc (Purkayastha/Lamm, 1979)
5 The Le^a-blood group antigen, however, could not be found in either of the two IgA types, but was detected in a 120-150 kD glycoprotein co-purified with the secretory IgA.

Size-fractionation chromatography of the secretory IgA preparation separated the secretory IgA from the 120-150 kD proteins. This high molecular weight glycoprotein in addition
10 presents the Le^b-antigen of equal intensity to the secretory component, corresponding to a 10-fold higher Le^b to protein ratio.

Subsequent *in situ* adherence assays of the separated protein
15 fractions revealed the 120-150 kD protein as an efficient inhibitor of *H. pylori* binding with an inhibitory titre reflecting the Le^b-antigen content.

This observation indicated the Le^b-antigen as the only fucosylated carbohydrate structure unique for both the secretory
20 IgA molecule, as well as for fractions with *H. pylori* adherence inhibitory activity.

The carbohydrate chains of the secretory component have been reported to be exclusively N-linked. Subsequent analysis of the distribution of the Le^b antigens of the secretory IgA and
25 the 120-150 kD proteins revealed that these antigens can be presented on both N- and O-linked carbohydrate chains, since only the Le^b-presenting carbohydrate chains of the secretory component were released by PNGase F even after SDS-denaturation of the glycoprotein for maximum processivity of the
30 glycosidase. This indicates that the Le^b-blood group antigen can be presented on a multitude of complex carbohydrate chains in glycoproteins, in addition to glycolipids, and variations in receptor presentation can probably affect their

efficiencies as functional receptors in the human gastric epithelium (Gl, PNAS).

As a natural source of Le^b-positive and Le^b-negative glycoproteins, human colostrum samples were analyzed for bacterial adherence inhibitory properties. Colostrum with glycoproteins rich in Le^a-antigen but devoid of Le^b-antigen, as demonstrated by immunoblots, poorly inhibited bacterial binding while colostrum rich in Le^b-antigen and low in Le^a-antigen was a powerful inhibitor of bacterial adherence in the *in situ* adherence assay. For comparison, pooled goat milk and cow milk were both negative for bacterial inhibition in the *in situ* adherence assay, correlating to their lack of Le^a- and Le^b-antigens, as demonstrated by immunoblots.

In order to analyze the fine-detailed inhibitory properties of glycoproteins carrying defined fucosylated structures, a library of neoglycoproteins was analyzed for their inhibitory properties in the *in situ* adherence assay. The neoglycoprotein conjugate exclusively carrying the Le^b-blood group antigen was found to be the only structure which, at a concentration of 1 µg/ml, could interfere with bacterial adherence, while the Le^a-, Le^x- and Le^y-neoglycoproteins at a concentration of 100 µg/ml did not display any inhibitory activities. This indicates the presence of further fucose residues in addition to terminal fucose presented on a type 1 chain as important properties of a receptor analogue for *H. pylori*. There could also be an alternative explanation, where the receptor specificity for *H. pylori* would be the intact difucosylated saccharide chain, but since the Le^y-(difucosylated type 2 chain) is synthesized as a tetrasaccharide, the GlcNAcβ1-3Galβ1-4Glc sequence is missing compared to an intact type 2 chain, which might affect the binding properties in the *H. pylori* interaction. This extremely high degree of specificity could be dictated by the presentation of the oligosaccharides in the glycoconjugates and in order to investigate this, a panel of free fucosylated oligosaccharides were analyzed for adherence inhibitory properties in the

in situ adherence assay. Interestingly, *H. pylori* accepts both the monofucosylated H-1- and the difucosylated Le^b- and Le^y- oligosaccharides as efficient inhibitors of bacterial adherence in concentrations of 20 mM. The relaxation in

5 receptor specificity for free oligosaccharides compared to the glycoconjugates indicates that due to steric freedom the importance of the branched fucose residue is diminished. This observation is in analogy to the behaviour of lectins, where the sugar specificities are often less discriminate for free

10 oligosaccharides compared to glycoconjugates. The type 2 chain derived 2-fucosylamine and the Fuc α 1-2Gal (H)-disaccharide were also efficient, although in slightly higher concentrations. This might point to the preference of type 1 chains compared to type 2 chains and/or further indicate that

15 the length of the carbohydrate chain could be an important parameter as discussed above. The branched fucose residue in the difucosylated Le^y- might also contribute to the receptor-adhesin interaction somewhat, since the 2fucosyllactose seems slightly less receptor active. The Fuc α 1-4GlcNAc β 0-TMSE

20 oligosaccharide chain surprisingly inhibits at somewhat higher concentrations, emphasizing the importance of a terminal fucose residue, although the specific linkage might not be crucial and subsequently the monofucosylated 3-fucosyllactose (short Le^x) with a Fuc α 1-3-terminal reduces bacterial

25 binding at high concentrations. This indicates that in free oligosaccharides, the terminal Fuc α 1-2Gal-linkage preferable on an full-length type 1 chain is the most efficient structure of a receptor analogue for *H. pylori*, but this configuration can be minimized to the H-disaccharide while still

30 retaining inhibitory activity.

Since the Le^b-antigen can be presented in a multitude of glycoconjugates of more or less complex structure, it was of great interest to see whether monoclonal antibodies against the Le^b-epitope could inhibit bacterial adherence to the

35 human gastric epithelium. As an appropriate negative control, monoclonal antibodies recognizing the H-antigen on type 2 chains were used. By adding similar quantities of monoclonal

antibodies to the sections of gastric epithelium, as detected by FITC-conjugated anti-mouse antibodies, a substantial reduction in binding could be seen when the section had been pre-incubated with the Le^b-monoclonal antibody in comparison to the H-2 monoclonal antibodies before bacterial overlay. This indicates that the Le^b-antigen could also be the *H. pylori* receptor structure of the host target tissue in addition to the receptor structure of natural soluble receptor analogues or clearance factors, such as colostrum. The interaction of *H. pylori* with soluble Le^b-containing glycoproteins could nevertheless be skewed when it comes to immobilized receptors presented on cell surfaces. In order to investigate the fine-detailed specificity of the interaction with immobilized glycoreceptors, two solid phase assay systems were used: bacterial overlay to neoglycoproteins separated on Western blots and bacterial overlays to glycolipids separated on HPTLC-plates. The bacterial Western blot overlays demonstrated a specific interaction with the Le^b-neoglycoprotein and in addition some weak binding to the H-antigen on type 1 chains (LNF1).

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CLAIMS

1. Use of a di- or oligosaccharide glycoside comprising at least one terminal L-fucose unit for the preparation of a pharmaceutical composition for the treatment or prophylaxis
5 in humans of conditions involving infection by *Helicobacter pylori* of the human gastric mucosa.
2. Use according to claim 1 in which the glycoside is capable of binding to adhesins present on the surface of *H. pylori*.
3. Use according to claim 1 or 2 in which the di- or oligo-
10 saccharide glycoside having at least one terminal L-fucose unit is capable of inhibiting or substantially reducing the adhesion of cells of *Helicobacter pylori* to epithelial cells of a histological section of human gastric mucosa.
4. Use according to any of claims 1-3 in which the di- or
15 oligosaccharide glycoside having at least one terminal L-fucose unit is capable of inhibiting the adhesion of cells of *Helicobacter pylori*, said bacterial cells being preincubated with the glycoside at a concentration of up to 500 $\mu\text{g/ml}$, to epithelial cells of a histological section of human gastric
20 mucosa by at least 50% compared to the adhesion of corresponding non-preincubated bacterial cells.
5. Use according to any of claims 3 or 4 in which the histological section of human gastric mucosa is prepared by fixing a sample of non-diseased human gastric mucosa tissue
25 with formalin, embedding the sample in paraffin, providing an approximately 5 μm section of the embedded sample, placing the section on a glass slide, deparaffinizing the section by washing with xylene and isopropanol, and incubating the section with a buffer consisting of bovine serum albumin and
30 of a non-ionic polyoxyethylene sorbitan monolaurate surfactant, preferably in a concentration of about 0.2% and 0.05%, respectively, in phosphate-buffered saline.

6. Use according to any of claims 3-5 in which the cells of *Helicobacter pylori* are labelled, preferably fluorochrome-labelled, in particular labelled with fluorescein isothiocyanate.

5 7. Use according to claim 6 in which the bacterial cells are labelled by treating a suspension of bacterial cells in buffer containing sodium chloride and sodium carbonate, preferably at a concentration of about 0.15 M and 0.1 M, respectively, at about pH 9.0 with fluorescein isothiocyanate
10 at a concentration of about 0.1 mg/ml, incubating for 1 hour at room temperature and separating the bacterial cells by centrifugation followed by washing the bacterial cells with phosphate buffered saline containing of a non-ionic polyoxyethylene sorbitan monolaurate surfactant, preferably at a
15 concentration of about 0.05%.

8. Use according to any of claims 4-7 in which the preincubation of the bacterial cells with the glycoside is carried out by adding the glycoside in a concentration of up to 500 $\mu\text{g/ml}$ to a suspension of the bacterial cells in a buffer
20 consisting of bovine serum albumin and of a non-ionic polyoxyethylene sorbitan monolaurate surfactant, preferably at a concentration of about 0.2% and 0.05%, respectively, in phosphate-buffered saline preferably for a period of about 2 hours at room temperature, separating the cells by cen-
25 trifugation, and washing the bacterial cells in the same buffer.

9. Use according to any of claims 4-8 in which the adhesion of preincubated or non-preincubated bacterial cells is determined by applying a dilute bacterial cell suspension containing about 10^6 - 10^8 bacterial cells per millilitre to the
30 histological section, incubating the section with the bacterial cell suspension for 1 hour at room temperature in a humidified chamber, washing the slide in phosphate-buffered saline, and establishing the degree of adhesion to the
35 epithelial cells of the section.

10. Use according to claim 9 in which the bacterial cells used are prepared as described in claim 7, and the degree of adhesion to the epithelial cells of the section is established by inspection under a fluorescence microscope.
- 5 11. Use according to any of claims 1-10 in which the bacterial cells used are selected from cells of the strains *Helicobacter pylori* NCTC 11637, NCTC 11638, WV229 and P466.
12. Use according to any of claims 1-11 in which the conditions involving gastrointestinal infection by *Helicobacter*
10 *pylori* comprise chronic active (type B) gastritis, gastric ulcers, duodenal ulcers, gastric adenocarcinoma, and gastric lymphoma.
13. Use according to any of claims 1-12 in which the di- or oligosaccharide glycoside is a glycoprotein.
- 15 14. Use according to any of claims 1-13 in which the glycoprotein is selected from human κ -casein, human colostrum IgA, and bovine submaxillary gland mucin.
15. Use according to any of claims 1-13 in which the oligosaccharide glycoside has two terminal L-fucose units.
- 20 16. Use according to claim 15 in which the terminal tetrasaccharide of the non-reducing end of the saccharide chain of the oligosaccharide glycoside is Lewis b-tetrasaccharide, $\text{Fuc}\alpha 1-2\text{Gal}\beta 1-3(\text{Fuc}\alpha 1-4)\text{GlcNAc}\beta 1-$.
17. A method of treating and/or preventing diseases in humans
25 caused by infection by *Helicobacter pylori* of human gastric mucosa, said method comprising administering to a human patient in need thereof an effective amount of a di- or oligosaccharide glycoside comprising at least one terminal L-fucose unit.

18. A method according to claim 17 in which the di- or oligosaccharide glycoside comprising at least one terminal L-fucose unit is capable of binding to adhesins present on the surface of *Helicobacter pylori*.
- 5 19. A method according to claim 17 or 18 in which the di- or oligosaccharide glycoside having at least one terminal L-fucose unit is capable of inhibiting or substantially reducing the adhesion of cells of *Helicobacter pylori* to epithelial cells of a histological section of human gastric
10 mucosa.
20. A method according to any of claims 17-19 in which the di-or oligosaccharide glycoside having at least one terminal L-fucose unit is capable of inhibiting the adhesion of cells of *Helicobacter pylori*, said bacterial cells being preincu-
15 bated with the glycoside at a concentration of up to 500 $\mu\text{g/ml}$, to epithelial cells of a histological section of human gastric mucosa by at least 50% compared to the adhesion of corresponding non-preincubated bacterial cells.
21. A method according to any of claims 19 or 20 in which the
20 histological section of human gastric mucosa is prepared by fixing a sample of non-diseased human gastric mucosa tissue with formalin, embedding the sample in paraffin, providing an approximately 5 μm section of the embedded sample, placing the section on a glass slide, deparaffinizing the section by
25 washing with xylene and isopropanol, and incubating the section with a buffer consisting of bovine serum albumin and of a non-ionic polyoxyethylene sorbitan monolaurate surfactant, preferably at a concentration of about 0.2% and 0.05%, respectively, in phosphate-buffered saline.
- 30 22. A method according to any of claims 19-21 in which the bacterial cells of *Helicobacter pylori* are labelled, preferably fluorochrome-labelled, in particular labelled with fluorescein isothiocyanate.

23. A method according to claim 22 in which the bacterial cells are labelled by treating a suspension of bacterial cells in buffer containing sodium chloride and sodium carbonate, preferably at a concentration of about 0.15 M and 0.1 M, respectively, of about pH 9.0 with fluorescein isothiocyanate at a concentration of about 0.1 mg/ml, incubating for 1 hour at room temperature and separating the bacterial cells by centrifugation followed by washing the bacterial cells with phosphate buffered saline containing of a non-ionic polyoxyethylene sorbitan monolaurate surfactant, preferably at a concentration of about 0.05%.

24. A method according to any of claims 20-23 in which the preincubation of the bacterial cells with the glycoside is carried out by adding the glycoside in a concentration of up to 500 μ g/ml to a suspension of the cells in a buffer consisting of bovine serum albumin and of a non-ionic polyoxyethylene sorbitan monolaurate surfactant, preferably at a concentration of about 0.2% and 0.05%, respectively, in phosphate-buffered saline for a period of about 2 hours at room temperature, separating the bacterial cells by centrifugation, and washing the bacterial cells in the same buffer.

25. A method according to any of claims 20-24 in which the adhesion of preincubated or non-preincubated bacterial cells is determined by applying a dilute bacterial cell suspension containing about 10^6 - 10^8 bacterial cells per millilitre to the histological section, incubating the section with the bacterial cell suspension for 1 hour at room temperature in a humidified chamber, washing the slide in phosphate-buffered saline, and establishing the degree of adhesion to the epithelial cells of the section.

26. A method according to claim 25 in which the bacterial cells used are prepared as described in claim 23, and the degree of adhesion to the epithelial cells of the section is established by inspection under a fluorescence microscope.

27. A method according to any of claims 17-26 in which the bacterial cells used are selected from cells of the strains *Helicobacter pylori* NCTC 11637, NCTC 11638, WV229 and P466.
28. A method according to any of claims 17-27 in which the
5 conditions involving gastrointestinal infection by *Helicobacter pylori* comprise chronic active (type B) gastritis, gastric ulcers, duodenal ulcers, gastric adenocarcinoma, and gastric lymphoma.
29. A method according to any of claims 17-28 in which the
10 di- or oligosaccharide glycoside is a glycoprotein.
30. A method according to any of claims 17-29 in which the glycoprotein is selected from human κ -casein, human colostrum IgA, and bovine submaxillary gland mucin.
31. A method according to any of claims 17-29 in which the
15 oligosaccharide glycoside has two terminal L-fucose units.
32. A method according to claim 31 in which the terminal tetrasaccharide of the non-reducing end of the saccharide chain of the oligosaccharide glycoside is Lewis b-tetrasaccharide, $\text{Fuc}\alpha 1-2\text{Gal}\beta 1-3(\text{Fuc}\alpha 1-4)\text{GlcNAc}\beta 1-$.

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Fig. 1A

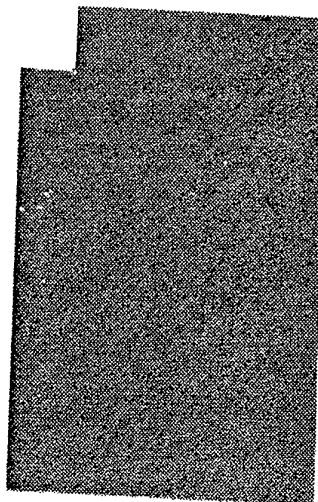


Fig. 1B

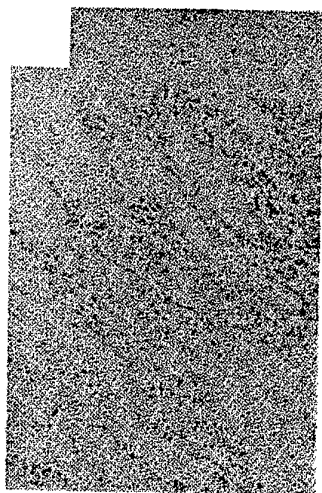


Fig. 1C

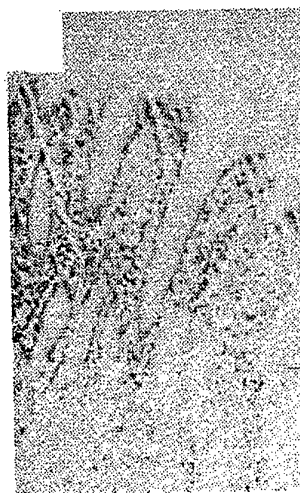


Fig. 1D

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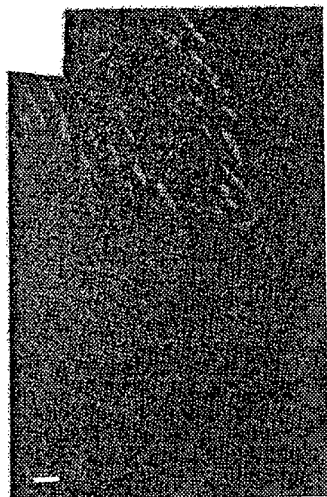


Fig. 1E

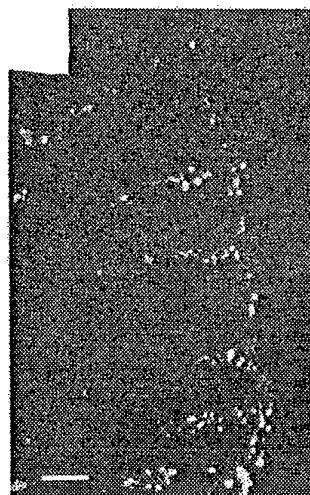


Fig. 1F

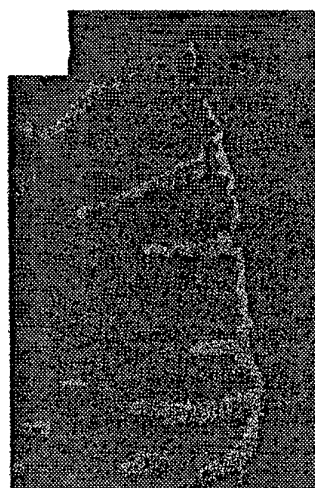


Fig. 1G

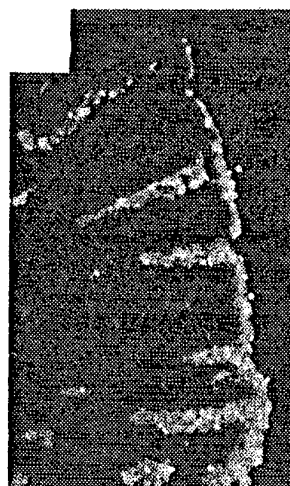


Fig. 1H

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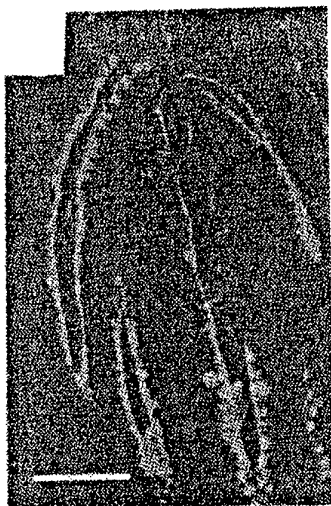


Fig. 1I



Fig. 1J



Fig. 1K

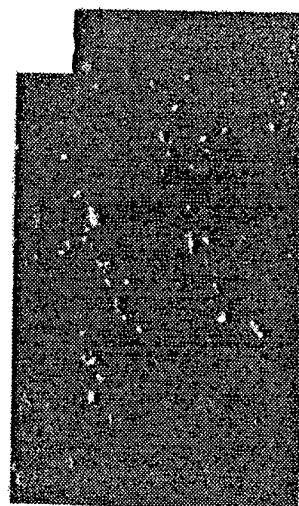


Fig. 1L

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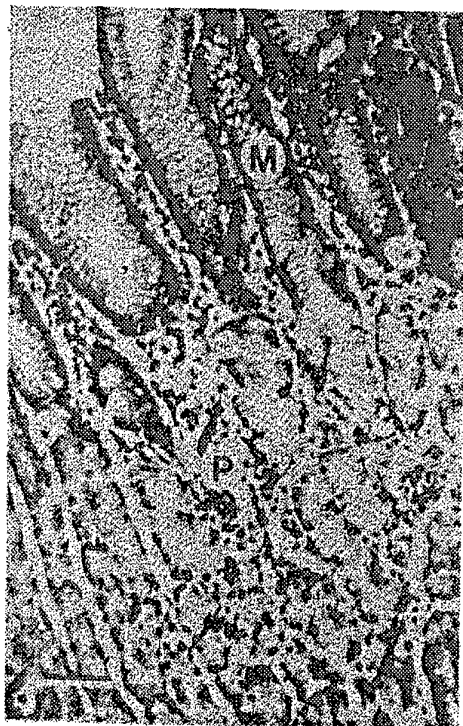


Fig. 2A



Fig. 2B

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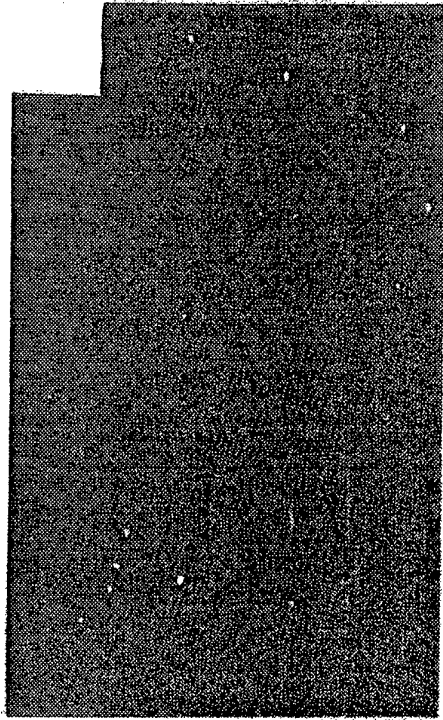


Fig. 2C

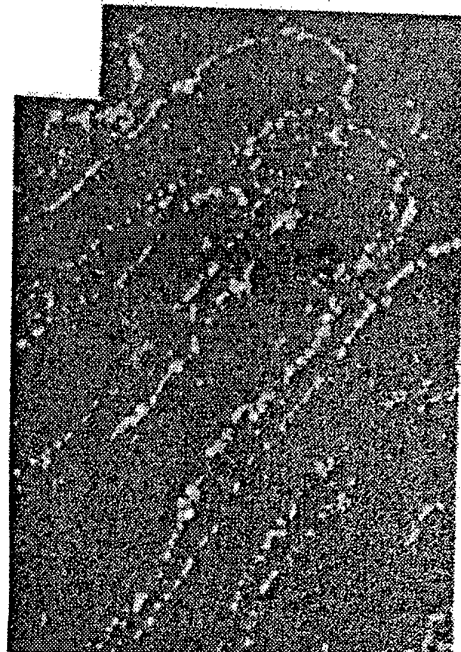


Fig. 2D

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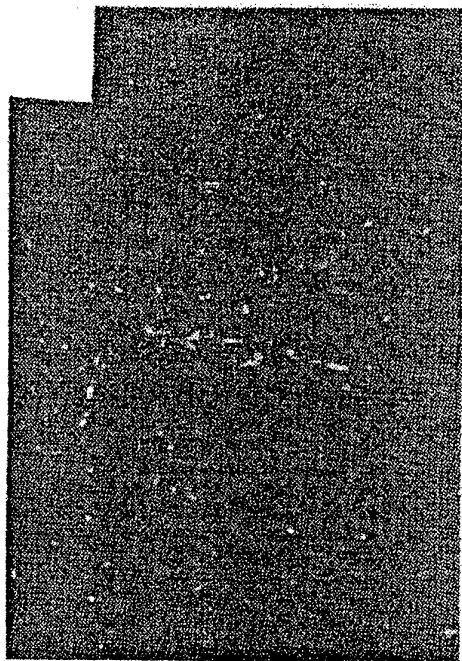


Fig. 2E

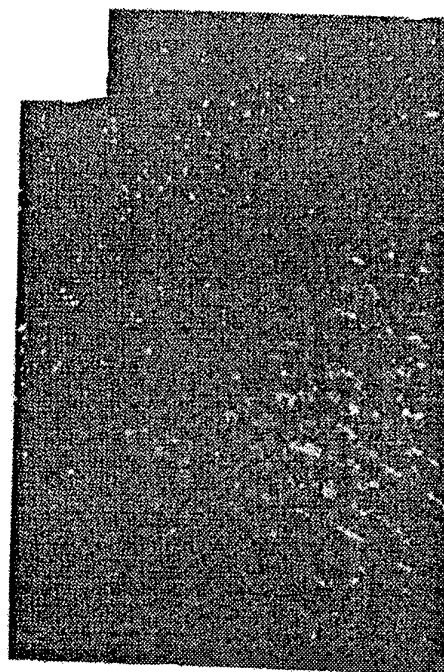


Fig. 2F

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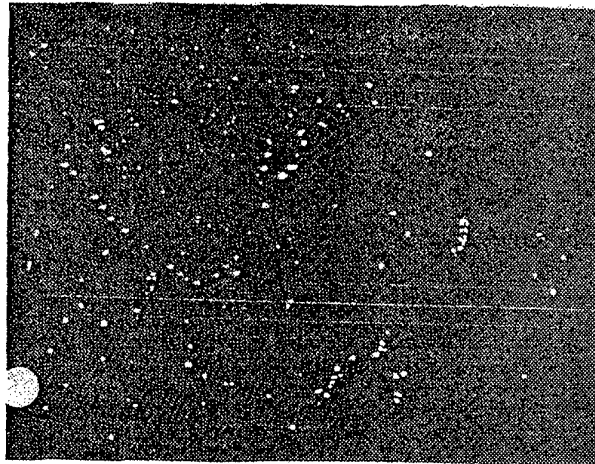


Fig. 3A



Fig. 3B

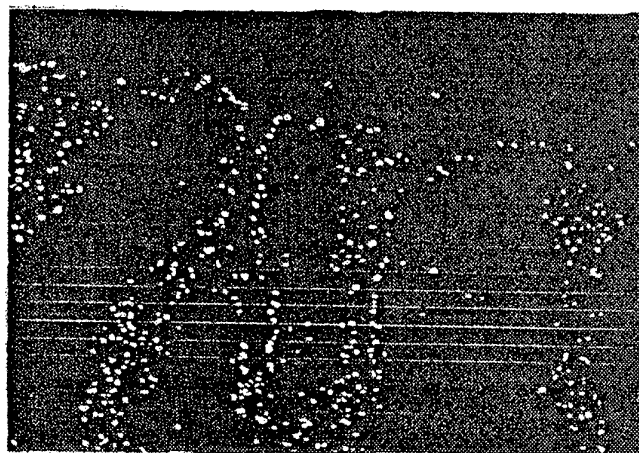


Fig. 3C

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB 94/00023

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5: A61K 31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5: A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WOI, MEDLINE, CA, US PATENTS-FULLTEXT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	WO, A1, 9403184 (NEOSE PHARMACEUTICALS, INC.), 17 February 1994 (17.02.94) --	1-16
A	US, A, 4935406 (JAMES C. COLEMAN ET AL), 19 June 1990 (19.06.90) --	1-16
A	US, A, 5116821 (JARED L. RANDALL ET AL), 26 May 1992 (26.05.92) -- -----	1-16

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

27 May 1994

Date of mailing of the international search report

01-06-1994

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB 94/00023

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 17-32
because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐

The additional search fees were accompanied by the applicant's protest.

☐

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

07/05/94

International application No.

PCT/IB 94/00023

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO-A1-	9403184	17/02/94	NONE	
US-A-	4935406	19/06/90	AU-B- 609999	09/05/91
			AU-A- 3663489	04/01/90
			EP-A- 0348143	27/12/89
US-A-	5116821	26/05/92	NONE	