Rhesus monkey gastric mucins: oligomeric structure, glycoforms and *Helicobacter pylori* binding[1]

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Mucins isolated from the stomach of Rhesus monkey are oligomeric glycoproteins with a similar mass, density, glycoform profile and tissue localization as human MUC5AC and MUC6. Antibodies raised against the human mucins recognize those from monkey, which thus appear to be orthologous to those from human beings. Rhesus monkey muc5ac and muc6 are produced by the gastric-surface epithelium and glands respectively, and occur as three distinct glycoforms. The mucins are substituted with the histo blood-group antigens B, Le^a (Lewis a), Le^b, Le^x, Le^y, H-type-2, the Tn-antigen, the T-antigen, the sialyl-Le^x and sialyl-Le^a structures, and the expression of these determinants varies between individuals. At neutral pH, *Helicobacter pylori* strains expressing BabA (blood-group antigen-binding adhesin) bind Rhesus monkey gastric mucins via the Le^b or H-type-1 structures, apparently on muc5ac, as well as on a smaller putative mucin, and binding is inhibited by Le^b or H-type-1 conjugates. A SabA (sialic acid-binding adhesin)-positive *H. pylori* mutant binds to sialyl-Le^x-positive mucins to a smaller extent compared with the BabA-positive strains. At acidic pH, the microbe binds to mucins substituted by sialylated structures such as sialyl-Le^x and sialylated type-2 core, and this binding is inhibited by DNA and dextran sulphate. Thus mucin–*H. pylori* binding occurs via at least three different mechanisms: (1) BabA-dependent binding to Le^b and related structures, (2) SabA-dependent binding to sialyl-Le^x and (3) binding through a charge-mediated mechanism to sialylated structures at low pH values.

Key words: gastric mucosa, glycoform, *Helicobacter pylori*, Lewis antigen, mucin, Rhesus monkey.

INTRODUCTION

Histologically, the stomach is divided into three regions: cardia, corpus/fundus and antrum. The surface epithelium is connected via the gastric pits (foveolae) and the neck region is connected to the gastric glands located in the submucosa. The surface/foveolar epithelium is formed by a single layer of mucin-producing cells and mucins are also produced by the glands, in particular in antrum. The glands in corpus/fundus secrete acid (parietal cells) as well as pepsinogen (chief cells), and the gastric surface is covered by a mucus layer that maintains a pH gradient varying from acidic in the lumen to neutral at the cell surface to protect against e.g. proteolytic enzymes and acid. The polymer matrix of mucus is formed by high-molecular-mass oligomeric glycoproteins referred to as mucins. In the human stomach, MUC5AC and MUC6 are the major secreted mucins, the former being produced by the surface/foveolar epithelium and the latter by the glands [1]. However, in conditions such as intestinal metaplasia and cancer of the stomach, the intestinal MUC2 mucin may also appear in this location. The Le^a (Lewis a) and Le^b blood-group antigens mainly appear in the surface epithelium, whereas the Le^x and Le^y antigens are expressed in the mucous, chief and parietal cells of the glands [1,2]; however, their distribution is not always that distinct [2,3].

Helicobacter pylori infection is the primary cause of gastric and duodenal ulcers and is a pivotal risk factor for gastric cancer [4,5]. During natural infection, *H. pylori* is mainly found in the gastric mucus layer [6]; however, it is also attached to the epithelial surface, within epithelial cells and in the lamina propria [7]. In a healthy stomach, the Le^b blood-group antigen has been shown to mediate the attachment of *H. pylori* to the human gastric mucosa [8] and the MUC5AC mucin [9], whereas sialylated Lewis antigens contribute to binding in inflamed tissue [10]. We have shown previously that, at neutral pH, *H. pylori* binding to human gastric mucins is strain- and blood-group-dependent [9]. In contrast, binding to human gastric mucins at acidic pH seems to be a common feature of all *H. pylori* strains, which is independent of the expression of blood-group structures on host mucins (S. Lindén, J. Mahdavi, J. Hedenbro, T. Borén and I. Carlstedt, unpublished work). *H. heilmannii* can also colonize the stomach and, although less pathogenic than *H. pylori*, this bacterium may cause milder forms of gastritis [11].

Rhesus monkeys naturally have persistent *H. pylori* infection [12], leading to loss of mucus [13,14] and gastritis [15]. Gastric ulcers and gastric carcinoma have also been reported in this species [16]. These animals can be experimentally infected with *H. pylori*, and individuals differ in their susceptibility to particular bacterial strains [13,14]. In addition, the gastroduodenal anatomy and physiology of the Rhesus monkey is very similar to that of humans, and the monkeys are known to produce human-type blood-group structures such as the A-B-O and Lewis antigens [17].

The aim of the present study was to investigate the structure, tissue localization, blood-group expression and *H. pylori* binding properties of gastric mucins from the Rhesus monkey. We observed that in the stomach of a healthy monkey, the surface

Abbreviations used: BabA, blood-group antigen-binding adhesin; DBA, *Dolichos biflorus*; HSA, human serum albumin; GdmCl, guanidinium chloride; Le^a, Lewis a; MAL, *Maakia amurensis* II; PNA, peanut agglutinin; SabA, sialic acid-binding adhesin.

[1] The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defence or Uniformed Services University of the Health Sciences.

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epithelium and glands express muc5ac and muc6 respectively, but do not express muc2. Receptors for *H. pylori* adhesins such as Le^b, sialyl-Le^x and sialyl-Le^a are present, and the amounts vary between individuals. Binding of BabA (blood-group antigen-binding adhesin)-positive *H. pylori* to Rhesus monkey gastric mucins is inhibited by Le^b or H-type-1 conjugates at neutral pH, and by DNA and dextran sulphate at acidic pH. Binding of BabA-negative, SabA (sialic acid-binding adhesin)-positive *H. pylori* is proportional to the level of sialyl-Le^x. We conclude that the Rhesus monkey is an excellent model for investigating the role of gastric mucins in *H. pylori* colonization and persistence, and consequently results from studies on this species may be highly relevant to human diseases.

EXPERIMENTAL

Materials

Our research was conducted according to the principles enunciated in the Guide for the Care and Use of Laboratory Animals [18] in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All procedures involving animals were reviewed and approved by the Animal Care and Use Committee of our institution. The stomachs of two monkeys (C2H and DHO) were frozen on solid CO_2 immediately after death. Archived paraffin-embedded tissue blocks from antrum and corpus of these two monkeys and 16 additional animals (aged 2–13 years; mean 5.8 years) as well as from cardia and duodenum from four (9EU, 81D52, A6W and D6G) of the latter were studied by histochemistry. All animals were *H. pylori*- and CagA-negative at the time of biopsy, 12 were *H. heilmannii*-negative (F754, F436, 86D02, 86D06, T4C, 8V5, 82A49, 8PZ, EOE, 9A5, 85D08 and E6C) and six were *H. heilmannii*-positive (C2H, DHO, 9EU, 81D52, A6W and D6G). All the sections investigated had gastritis scores of $\leqslant 1$ according to the Sydney system [19].

The bacterial strains, culture conditions and biotinylation procedure, as well as most of the reagents, have been described previously [9]. Strains CCUG17875 and P466 express the Le^b/H-type-1-binding adhesin BabA and the 17875*babA1A2* mutant expresses SabA (binds to sialyl-Le^x) [9]. The biotin blocking system and alkaline phosphatase-conjugated pig anti-rabbit serum were obtained from Dako (Glostrup, Denmark). Biotinylated *Ulex europeus* I, PNA (peanut agglutinin), MAL (*Maakia amurensis* II), DBA (*Dolichos biflorus*), *Helix pomatia*, Elderberry bark, *Vicia villosa* B_4, *Lotus tetraglobus*, *Griffonica simplicifolia* II and *Galantus nivalis* lectins as well as the Strept AB complex/horseradish peroxidase were obtained from Vector Laboratories (Peterborough, U.K.). Polyclonal antibodies against MUC5AC (LUM5-1 [20] and LUM5AC-2), MUC6 (LUM6-3 [21]) and MUC2 (LUM2-3 [22]) were raised in rabbits using peptides conjugated with keyhole-limpet haemocyanin. The peptide sequence used for LUM5AC-2 was CPPEAPIFDEDKMQ. A monoclonal antibody raised against MUC5AC (clone 45M1) and neuraminidase from *Vibrio cholerae* were obtained from Sigma. Anti-sialyl-Le^x (clone KM93) and anti-sialyl-Le^a (clone IH4) were obtained from Seikagaku America (Ijamsville, MD, U.S.A.), anti-Le^a (clone 7LE) and anti-Le^b (clone 2-25LE) were gifts from Dr Jaques Bara (INSERM, Paris Cedex, France), and anti-Le^a (clone BG-5), Le^b (BG-6), Le^x (BG-7) and Le^y (BG-8) were obtained from Signet Laboratories (Dedham, MA, U.S.A.). Anti-(blood-group A) (clone A003), anti-(blood-group B) (clone B005) and an antibody recognizing Le^b, BLe^b, ALe^b and H-type-1 structures (clone 96FR2.10) [23] were obtained from Biotest (Breieich, Germany). Anti-sialyl-di-Le^x (FH6) and anti-sulpho-Le^a (IRMA) were provided by Dr H. Clausen (University of Copenhagen, Copenhagen, Denmark) and Dr E. C. I. Veerman (Vrije Universiteit, Amsterdam, The Netherlands) respectively. Hybond-P PVDF membranes were from Amersham Biosciences (Uppsala, Sweden).

Methods

Histochemistry

Genta-stained [24] sections were used to determine the *H. pylori* and *H. heilmannii* status, the gastritis score according to the Sydney system (0 = none, 1 = mild, 2 = moderate and 3 = marked) [19] and the location of sialomucins. Sulphomucins were detected by the method of Bravo and Correa [25].

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections (4 μm) were dewaxed, rehydrated and subjected to antigen retrieval by ebullition in a pressure cooker in 10 mM sodium citrate buffer (pH 5.5) for 9 min. In addition, sections to be treated with the LUM5AC-2, LUM6-3 and LUM2-3 antibodies were incubated with 10 mM 1,4-dithiothreitol in 0.1 M Tris/HCl buffer (pH 8.0) at room temperature (20 °C) for 30 min, followed by 25 mM iodoacetamide at room temperature for 30 min to expose the epitopes; other antibodies were not analysed by this treatment. Sections were then treated with 3 % (v/v) hydrogen peroxide for 30 min at room temperature, washed with 0.15 M NaCl, 0.1 M Tris/HCl buffer (pH 7.4), and placed in Shandon coverplate-disposable immunostaining chambers. Non-specific binding was blocked by normal goat serum (1:5) in 0.15 M NaCl, 0.1 M Tris/HCl buffer (pH 7.4) containing 0.05 % Tween 20 for 1 h and the sections were then treated with the biotin blocking system. The sections were incubated with a primary antibody diluted in 0.15 M NaCl, 0.1 M Tris/HCl buffer (pH 7.4) containing 0.05 % Tween 20 (45M1 and LUM6-3, 1:2000; LUM2-3 and LUM5AC-2, 1:500; clones 7LE, BG-5, 2-25LE, BG-6, BG-7, BG-8, KM93 and IH4, 1:100; FH6, 1:50; and IRMA, 1:10), a secondary antibody (biotinylated goat anti-mouse and goat anti-rabbit antisera, 1:500) and Strept AB complex/horseradish peroxidase; the bound antibodies were visualized using diaminobenzidine for 15 min. The sections were counterstained with Harris haematoxylin. Proportions of the surface/foveolar epithelium and the glands that were stained with the antibodies against various blood-group antigens were estimated either by direct observation of the sections (unsialylated structures) or by using a locally developed image analysis program (sialylated Lewis antigens). With the latter procedure, stained and unstained areas were outlined and the percentage of stained areas was calculated as the average of three randomly selected fields/view. In both cases, the results were grouped into four categories: high level of staining (approx. 90–100 %; indicated by +++ in Table 1), medium level of staining (25–90 %; indicated by ++), low level of staining (1–25 %; indicated by +) and virtually no staining (< 1 %; indicated by −).

Isolation of mucins

The frozen stomachs of the DHO and C2H monkeys were thawed. The mucosal surface of the C2H stomach (but not the DHO one) was rinsed to remove some debris causing partial loss of surface mucin. The entire mucosa of C2H was dissected from the underlying muscle layer, whereas that of DHO was divided into a surface and a gland preparation by gently scraping off the surface epithelium with a microscope slide before dissecting the residual gland mucosa from the underlying muscle layer as performed

previously on human samples [21]. The material was extracted and subjected to density-gradient centrifugation as described in [21]. The residual 'insoluble' mucin remaining after extraction was brought into solution by reduction and, after alkylation, was subjected to density-gradient centrifugation as above.

Analytical methods

Density measurements were performed using a Carlsberg pipette as a pycnometer. Carbohydrate was measured as periodate-oxidizable structures using a microtitre-based assay [26] and sialic acid was detected using an automated method [27]. In addition, sialic acid (carboxylate groups) and sulphate were assayed by staining aliquots of samples blotted on to PVDF membranes with 1 % Alcian Blue 8GX. Both sialic acid and sulphate were stained in 3 % acetic acid at pH 2.5, whereas mainly sulphate residues were stained in 0.1 M HCl (pH 1). The difference in reactivity at pH 2.5 and 1 is ascribed to sialic acid. Staining was quantified with a Hoefer densitometer. ELISA was performed using antibodies and lectins as described previously [9]. For analyses using LUM6-3, LUM5AC-2 and LUM2-3 antibodies, mucins were treated with 2 mM 1,4-dithiothreitol in 6 M GdmCl (guanidinium chloride), 5 mM sodium EDTA, 0.1 M Tris/HCl buffer (pH 8.0) at 37 °C for 1 h, followed by 5 mM iodoacetamide in the same buffer at room temperature for 1 h to expose the epitopes. Other antibodies were not analysed by this treatment. The following dilutions were used for the primary antibody/lectins: 1:1000 for LUM5-1, LUM6-3 and LUM2-3; 1:500 for LUM5AC-2; 1:100 for clones A003, B005, 7LE, 2-25LE, KM93 and IH4; 1:500 for clone 96Fr2.10; and 2 μg/ml for biotinylated *U. europeus*, PNA, MAL, *G. nivalis*, DBA, *H. pomatia*, Elderberry bark, *V. villosa*, *L. tetraglobus* and *G. simplicifolia*. $CaCl_2$ was added to the lectin solutions to a final concentration of 0.1 mM before performing the incubations.

Microtitre-based assays and inhibition experiments with *H. pylori*

These analyses were performed as described previously [9] except that the pH value was adjusted to 3 or 7.4 in the Boehringer blocking reagent for ELISA containing 0.05 % Tween and 10 mM citric acid. The inhibitors used were H-type-1–HSA (human serum albumin) and Le^b–HSA conjugates (1 μg/ml) or dextran sulphate (1 μg/ml) and salmon sperm DNA (0.1 μg/ml). Neuraminidase digestion was performed with 0.2 unit/ml in 1 mM $CaCl_2$, 50 mM sodium acetate buffer (pH 5.5) at 37 °C for 100 min after the mucins were coated on to the microtitre plates.

Reduction of mucins

Mucin subunits were obtained by reduction using 10 mM 1,4-dithiothreitol in 6 M GdmCl, 5 mM Na-EDTA, 0.1 M Tris/HCl buffer (pH 8.0) at 37 °C for 5 h, followed by alkylation in 25 mM iodoacetamide at room temperature for 5 h.

Chromatography

For gel chromatography, samples were dialysed against 4 M GdmCl, 10 mM sodium phosphate buffer (pH 7) and chromatographed on a CL2B column (16 cm × 51.5 cm) eluted with the same buffer at a flow rate of 0.10 ml/min. Fractions (1 ml) were collected. Anion-exchange chromatography was performed as described previously [9].

Statistics

Binding assays using antibodies, lectins and *H. pylori* were performed on the individual fractions (80/chromatogram) from anion-exchange chromatography and the absorbance values obtained were subjected to statistical analysis to investigate which carbohydrate structures are most probably receptors for the *H. pylori* strains. Data are reported as correlations either for all three samples ($n = 240$) or (in some cases) for an individual sample ($n = 80$). Pearson product–moment correlations were used to describe the relationships between pairs of variables and a bootstrap re-sampling procedure was used to assess significance of differences between correlation coefficients. The intercooled Stata 7.0 software was used for the calculations.

RESULTS

Tissue localization of mucin gene products

The antibodies used in the present study were designed to recognize human mucins. However, 45M1, LUM5AC-2, LUM6-3 and LUM2-3 worked well in the Rhesus monkey, whereas the reactivity with LUM5-1 was weak. In antrum, muc5ac was detected in the surface/foveolar cells using the antibodies 45M1 (Figure 1a), LUM5AC-2 (Figure 1b), LUM5-1 (results not shown) and muc6 in the glands (Figure 1c). A similar distribution was also found in cardia (results not shown). In corpus, a small amount of muc6 was found in the glands, but some was present in mucous cells and lumen in the neck region (results not shown). In duodenum, the Brunner's glands were also positive for muc6 (Figure 1d). The goblet cells in the surface epithelium in duodenum were stained with the antibody raised against human MUC2 and muc2 was thus produced (Figure 1e). No muc2 was detected in the stomach of any of the animals studied as expected for any healthy gastric mucosa. The tissue localizations of the mucins were similar in all monkeys and it is concluded that the distribution of muc2, muc5ac and muc6 in the gastric/duodenal mucosa of Rhesus monkey is the same as that of MUC2, MUC5AC and MUC6 in humans.

Density-gradient centrifugation of soluble gastric mucins

Mucins were isolated from two monkeys. Tissue from one (C2H) was not divided into surface and gland material, whereas in the other (DHO), the mucins were isolated separately from these two locations and designated DHO surface and DHO glands respectively. UV-absorbing material was found as a peak at 1.47 g/ml, suggesting that DNA was present at this density [28]; UV-absorbing material was also found at the top of the gradients where non-glycosylated proteins are expected to appear (Figures 2a, 2c and 2e). Carbohydrate (periodate-oxidizable material) was mainly found as a peak at 1.4 g/ml (Figures 2a, 2c and 2e), coinciding with reactivity against muc5ac, as detected using the 45M1 antibody (Figures 2b, 2d and 2f) as well as the LUM5-1 and LUM5AC-2 antisera (results not shown) and muc6 (LUM6-3 antibody; Figures 2b, 2d and 2f). In DHO, most of the muc5ac was found in the preparation from the surface mucosa and was of slightly higher density compared with muc6, whereas most of the muc6 was found in the preparation of the gland tissue. Sialic acid was mainly found at 1.4 g/ml, but a small amount was also present at the top of the gradients (Figures 2a, 2c and 2e). The level of sialic acid in relation to carbohydrate differed between the two monkeys, and sialic acid analysis performed with and without prior alkali treatment showed identical results, suggesting that the gastric mucins are not O-acetylated [29]. Both samples reacted with an antibody raised against the B structure and with the *U. europeus* lectin recognizing the H-type-2 structure, but not with an antibody raised against the A structure (results not shown). Approx. 10 % or less of the mucins as determined with

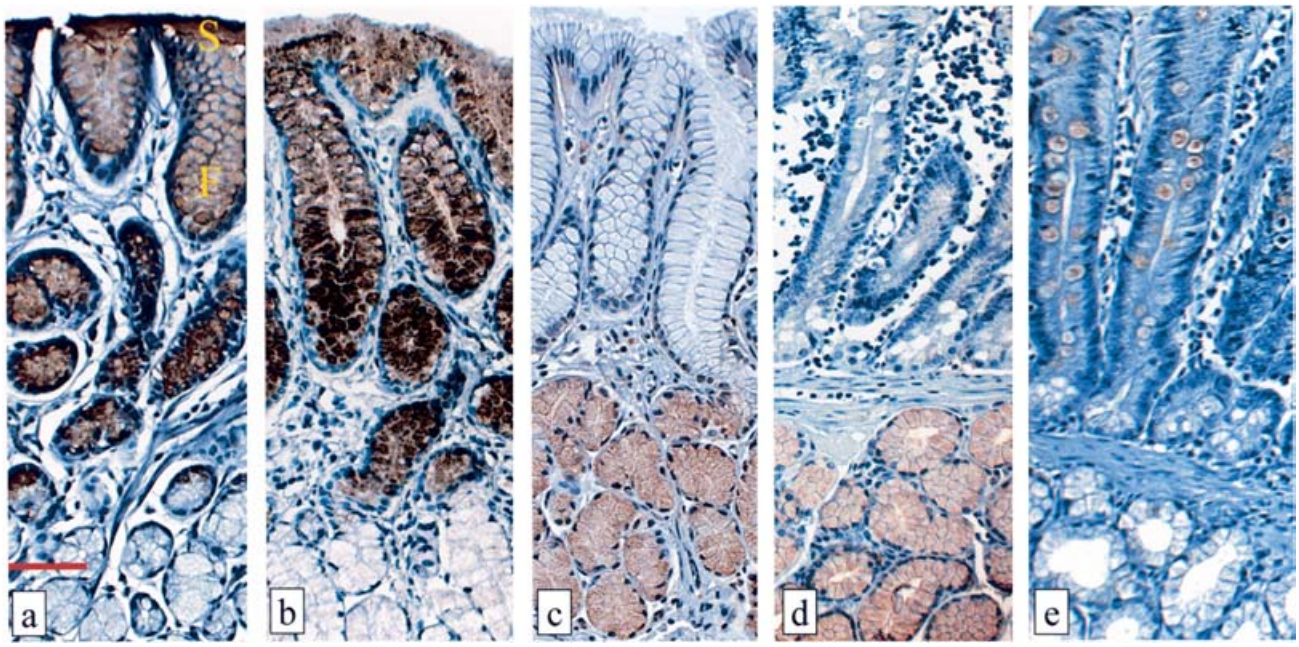

Figure 1 Tissue localization of mucins

Histologically, the stomach is divided into cardia, corpus/fundus and antrum. The surface/foveolar epithelium (indicated by S and F respectively in **a**) is formed by a single layer of mucin-producing cells. The stomach empties into the duodenum where the mucosa is composed of a columnar epithelium with absorptive cells interspersed with goblet cells, and with Brunner's glands in the submucosa. Sections of Rhesus monkey antrum were stained brown for muc5ac with the 45M1 antibody (**a**) and LUM5AC-2 antiserum (**b**), and for muc6 with the LUM6-3 antiserum (**c**). Sections from duodenum were stained brown for muc6 and muc2 with the LUM6-3 (**d**) and LUM2-3 antisera (**e**) respectively. Scale bar: in (**a**), 50 μm.

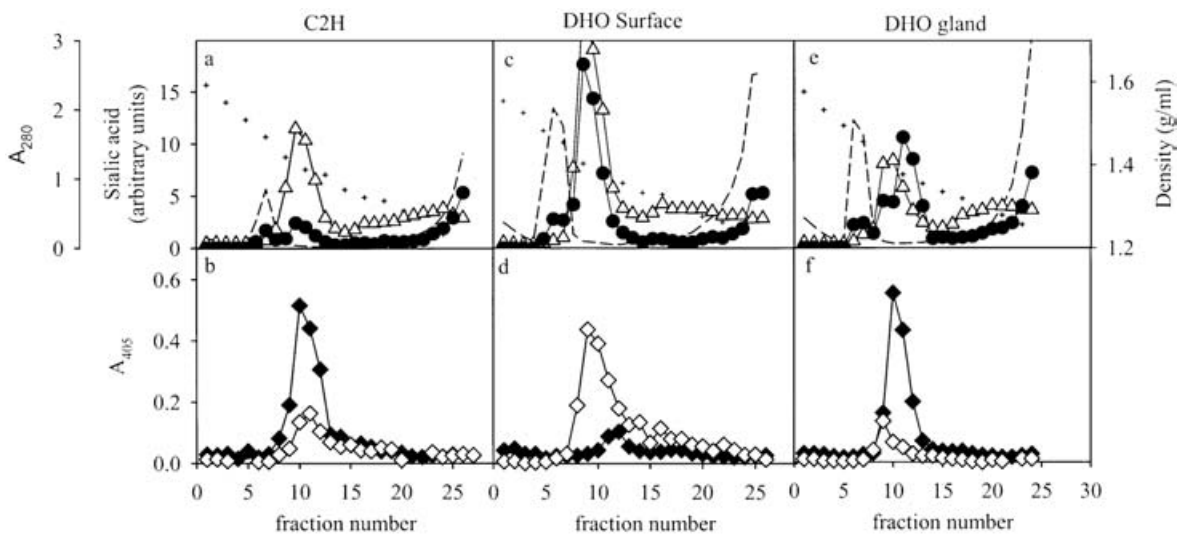

Figure 2 Density-gradient centrifugation of soluble gastric mucins

Mucosal extracts from C2H (**a**, **b**), DHO surface (**c**, **d**) and DHO gland (**e**, **f**) were subjected to CsCl density-gradient centrifugation. The gradients were emptied from the bottom of the tubes, and fractions were analysed for (**a**, **c**, **e**) density (+), A_{280} (- - -), carbohydrate (△), sialic acid (●) and (**b**, **d**, **f**) muc5ac (◇) and muc6 (◆). The apparent differences in the relative amounts of muc5ac and muc6 is most probably due to the fact that the mucosal surface of the C2H stomach (but not the DHO one) was rinsed to remove some debris causing partial loss of surface mucin.

carbohydrate, sialic acid and muc5ac analysis was left in the 'insoluble' residue (results not shown). No muc2 or O-acetylated sialic acids were found, in agreement with the absence of intestinal metaplasia from the tissue sections studied.

H. pylori binding to mucins and Lewis antigens

Fractions from the density gradients were analysed by a microtitre-based assay for *H. pylori* binding. At pH 7.4, the Le^b-binding BabA-positive strains CCUG17875 (Figures 3a, 3d and 3g) and p466 (results not shown) bound not only to the mucins at 1.4 g/ml, but also to material of lower density. Mucins from DHO reacted with antibodies raised against the Le^b (Figures 3d and 3g) and Le^a antigens (results not shown), whereas mucins from C2H did not (Figure 3a), although they reacted with an antibody recognizing both the H-type-1 structure and Le^b (clone 96FR2.10; results not shown). In DHO, where muc5ac and muc6 were enriched in the surface and gland material respectively,

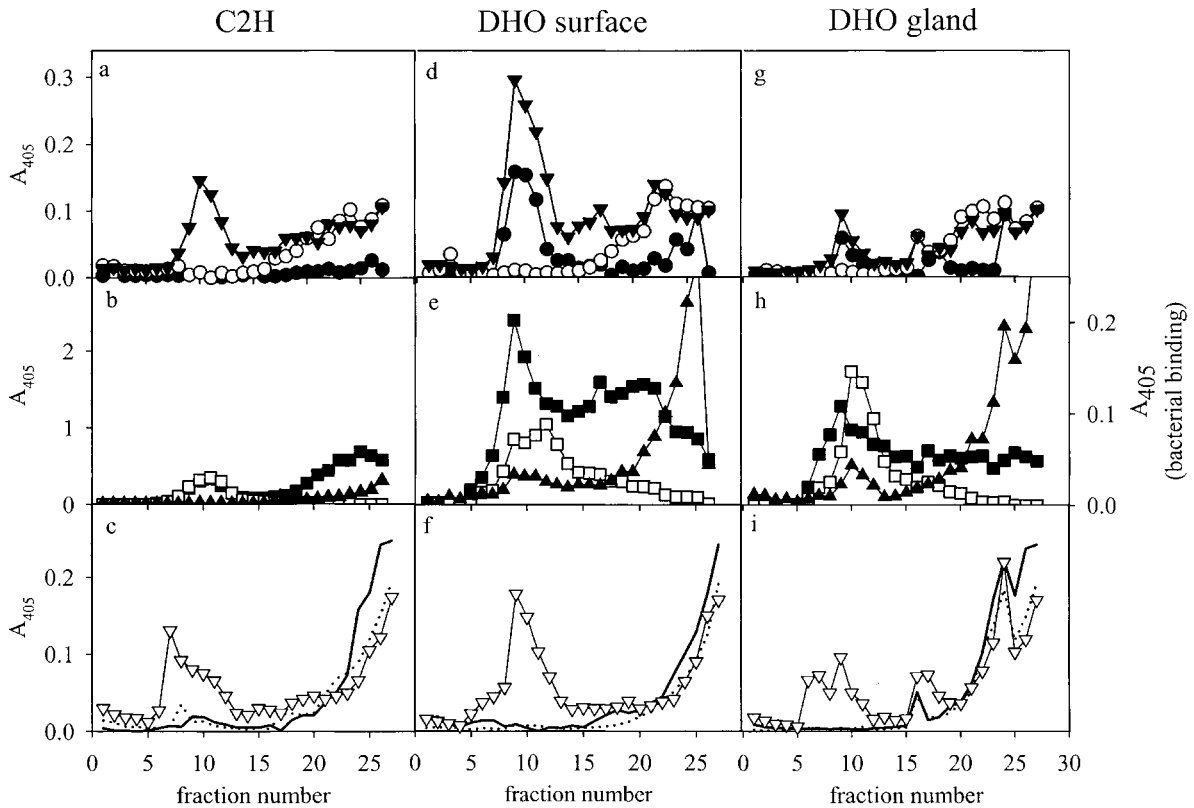

Figure 3 Lewis antigens and *H. pylori* binding to mucins

Mucosal extracts from C2H (**a**–**c**), DHO surface (**d**–**f**) and DHO gland tissue (**g**–**i**) were subjected to CsCl density-gradient centrifugation. The fractions were analysed for (**a**, **d**, **g**) Le^b (2-25LE) (●), binding with the BabA-positive *H. pylori* strain CCUG17875 at pH 7.4 (▼) and binding with CCUG17875 inhibited by the Le^b–HSA conjugate at pH 7.4 (○), (**b**, **e**, **h**) sialyl-Le^a (■), sialyl-Le^x (□) and binding with the *babA1A2* mutant at pH 7.4 (▲) and (**c**, **f**, **i**) binding with the strain CCUG17875 at pH 3 (▽), binding with the strain CCUG17875 inhibited by dextran sulphate at pH 3 (—) and binding with strain CCUG17875 inhibited by DNA at pH 3 (·····).

the BabA-dependent binding coincided with muc5ac and Le^b reactivity rather than with muc6. Binding to mucins was inhibited by Le^b–HSA (Figures 3a, 3d and 3g) and H-type-1–HSA (results not shown) conjugates, whereas binding to material of low density was not affected. The SabA-positive sialyl-Le^x-binding *babA1A2* mutant bound to the mucins at pH 7.4, and binding was abolished by neuraminidase treatment (results not shown). The apparent binding was less than that of CCUG17875 (cf. Figures 3a, 3d and 3g with Figures 3b, 3e and 3h), and a similar degree of binding was observed both when detecting the biotinylated *H. pylori* with streptavidin and with a rabbit anti-*H. pylori* antiserum (results not shown). The reactivity with the antibody against sialyl-Le^x coincided with the SabA-binding mucin peak at 1.4 g/ml, whereas the anti-sialyl-Le^a antibody also reacted with material of lower density (Figures 3b, 3e and 3h). At pH 3, *H. pylori* bound not only to material present at 1.47 g/ml coinciding with DNA and to the mucin band at 1.4 g/ml, but also to low-density material (cf. Figures 2a, 2c and 2e with Figures 3c, 3f and 3i). Binding at 1.47 g/ml, as well as to the mucins, was inhibited by dextran sulphate and DNA, whereas binding to the low-density material was not affected (Figures 3c, 3f and 3i). In the gland sample, binding to the mucins was completely abolished by neuraminidase treatment, whereas in the surface sample some residual binding was observed after this treatment (results not shown). The *H. pylori* binding reactivity left in the 'insoluble' residue was approx. 10% or less and was found at the same density as for the soluble material and no differences in *H. pylori*-binding properties were observed (results not shown).

Size fractionation of mucins and mucin subunits

Mucins were pooled from the CsCl density gradients (fractions 8–14; Figure 2a) and subjected to gel chromatography (Figure 4 shows the results from C2H). Carbohydrate (Figure 4a), muc5ac (results not shown) and muc6 (results not shown) eluted in the void volume of the column. Binding with the *babA1A2* mutant at pH 3 coincided with muc5ac, muc6 and carbohydrate reactivity (Figure 4a). Binding with the BabA-positive *H. pylori* strain (CCUG17875) at neutral pH also coincided with muc5ac, muc6 and carbohydrate reactivity, but also occurred with molecules of smaller size (Figure 4a). After reduction, carbohydrate (Figure 4b), muc5ac (results not shown) and muc6 (results not shown) eluted as a broad included peak starting in the void volume, showing that both muc5ac and muc6 from Rhesus monkey are oligomeric structures held together by disulphide bonds. Again, binding with the *babA1A2* mutant at pH 3 coincided only with the muc5ac and muc6 subunits (Figure 4b), whereas binding with a BabA-positive *H. pylori* strain (CCUG17875) at neutral pH coincided with both muc5ac and muc6 reactivity and the smaller-size material. The latter component appears to be insensitive to reduction, since the size did not change after reduction

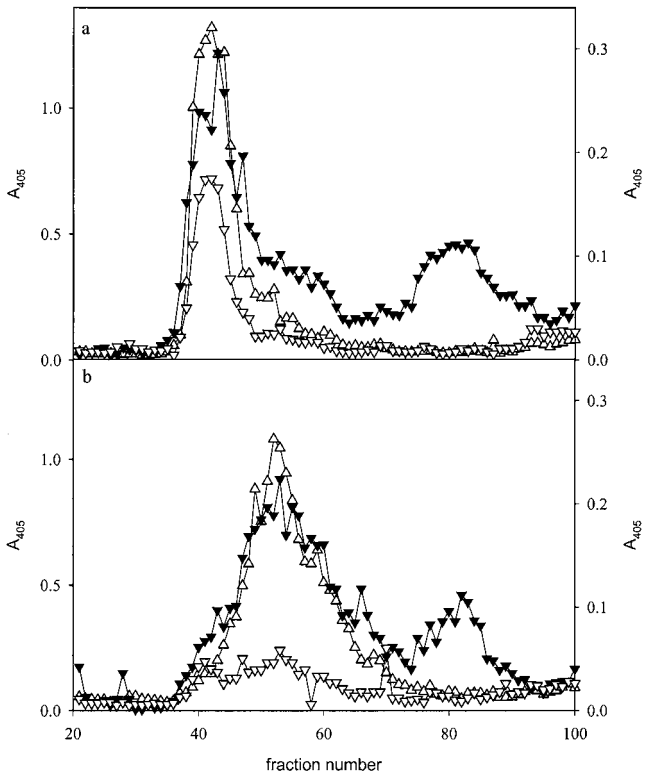

Figure 4 Gel chromatography of whole mucins (a) and reduced mucin subunits (b)

Mucins from the C2H monkey were pooled from the CsCl density gradients (fractions 8–14; Figure 2a) and subjected to gel chromatography on a CL2B column. Fractions were analysed for carbohydrate (△), binding with the BabA-positive *H. pylori* strain CCUG17875 at pH 7.4 (▼) and binding with the SabA-positive *babA1A2* mutant at pH 3 (▽).

(cf. Figures 4a and 4b), and did not react with antibodies against MUC5AC, MUC6, Le^b or the PNA (recognizing Galβ1,3GalNAc) and *G. nivalis* (recognizing Manα1,3Man) lectins (results not shown).

H. pylori binding to mucin glycoforms

The mucin band (pooled from the CsCl density gradients, fractions 8–14; Figure 2) was separated by anion-exchange chromatography into three glycoforms, which were either not retained by the column (glycoform 1), eluted at the beginning of the gradients (glycoform 2) or retained more (glycoform 3) as indicated in Figure 5(a). The glycoforms were revealed using carbohydrate analysis as well as muc5ac and muc6 reactivity (Figures 5a, 5d and 5g). Sulphate (as detected by Alcian Blue at pH 1) was found only in glycoform 3, and sialic acid (as the difference between Alcian Blue stainings at pH 2.5 and 1) was found mainly in glycoforms 2 and 3 (results not shown). Consequently, the glycoforms can be described as neutral mucins (glycoform 1), sialomucins (glycoform 2) and a mixture of sialo- and sulphomucins (glycoform 3). The sulphated species within glycoform 3 have a slightly higher charge density compared with the sialylated species, and the mucin preparations containing gland material (C2H and DHO gland) contained more sulphated material than the DHO surface preparation (results not shown). Reactivity with the *V. villosa* lectin was found only in the two preparations containing the glands, indicating that the Tn-antigen (GalNAcα1-O-serine) is only expressed there (results not shown). When present, *V. villosa* reactivity occurred in all three glycoforms, together with the PNA (recognizing Galβ1,3GalNAc), *H. pomatia* (GalNAc) and *G. simplicifolia* (terminal GlcNAc) lectin reactivities (results not shown). Reactivity with the MAL lectin (NeuAcα2,3Galβ1,4GlcNAc) was associated with glycoforms 2 and 3 as well as with material in between glycoforms 2 and 3 (results not shown). No reactivity was detected with the Elderberry bark (Neu5Acα2,6Gal/GalNAc) or DBA (GalNAcα1,3GalNAc) lectins (results not shown). At pH 7.4, the BabA-positive *H. pylori* strain (CCUG17875) bound to all three glycoforms, but mainly to glycoforms 2 and 3 (Figures 5b, 5e and 5h). In the DHO samples, this corresponds to the reactivity with the Le^b antibody (Figures 5b, 5e and 5h). In the Le^b-negative C2H sample, reactivity was detected with the antibody recognizing Le^b, BLe^b, ALe^b and H-type-1 structures (clone 96Fr2.10; Figures 5b, 5e and 5h), which may explain why the strain CCUG17875 binds to mucins from this animal (Figure 5b).

To assess which mucins and carbohydrate structure are relevant for *H. pylori* mucin binding, statistical correlations between antibody/lectin and *H. pylori* reactivity (as A_{405} values from the microtitre-based assays) were determined for the fractions obtained from anion-exchange chromatography. At pH 7.4, the correlation between the binding of the strain CCUG17875 ($P = 0.001$) and the elution pattern of muc5ac ($R = 0.4840$) was significantly higher compared with that of muc6 ($R = 0.1682$), and the correlation between microbe binding and Le^b ($R = 0.7186$) was in the same order of magnitude as the correlation between the two assays performed on the same chromatogram using a single antibody (clone FR2.10) at two different concentrations ($R = 0.7462$). The correlation between the strain CCUG17875 and the antibody recognizing Le^b, BLe^b, ALe^b and H-type-1 structures (clone FR2.10) ($R = 0.5869$) was higher compared with sialyl-Le^x ($R = 0.3479$), PNA ($R = 0.2234$) and MAL ($R = 0.0184$; $P = 0.7773$). Unless otherwise stated, $P < 0.05$. The results are consistent with the notion that the strain CCUG17875 at pH 7.4 preferably binds to Le^b or other fucosylated structures on muc5ac. Binding with the SabA-positive mutant (*babA1A2*) at pH 7.4 was mainly observed in glycoforms 2 and 3, corresponding to the reactivity with the sialyl-Le^x antibody (Figures 5c, 5f and 5i) ($R = 0.7851$). The correlation of *babA1A2* binding to sialyl-Le^x was significantly higher ($P < 0.001$) compared with those of the other structures (clone FR2.10, $R = 0.4962$; MAL, $R = 0.3647$; PNA, $R = 0.2819$; carbohydrate, $R = 0.2460$; 2-25LE, $R = 0.1763$).

At pH 3, *babA1A2* (Figures 5c, 5f and 5i) bound to glycoform 2, to a component reacting with MAL eluting between glycoforms 2 and 3 (results not shown) and to glycoform 3. The overall correlation was similar for sialyl-Le^x ($R = 0.6235$) and MAL ($R = 0.6164$), which is in agreement with the observation that the correlation of *babA1A2* binding to MAL and sialyl-Le^x at pH 3 does not differ significantly ($P = 0.938$). In the individual samples, the correlation between C2H and DHO gland was higher for MAL, whereas in the DHO surface the correlation was higher for sialyl-Le^x. Binding did not occur for the sulphated subpopulation of glycoform 3, indicating that charge is not the only requirement for adhesion.

The correlations between binding with the BabA-positive strain (CCUG17875) at pH 7.4, the SabA-positive mutant (*babA1A2*) at pH 7.4 and the SabA-positive mutant (*babA1A2*) at pH 3 are significantly different ($P = 0.001$). Together, the results suggest that there are three different modes of binding, most probably corresponding to (i) BabA-dependent binding to Le^b and related structures (e.g. H-type-1), (ii) SabA-dependent binding to sialyl-Le^x and (iii) binding through an as-yet unknown mechanism to charged structures like sialyl-Le^x and sialyl-core-type-2

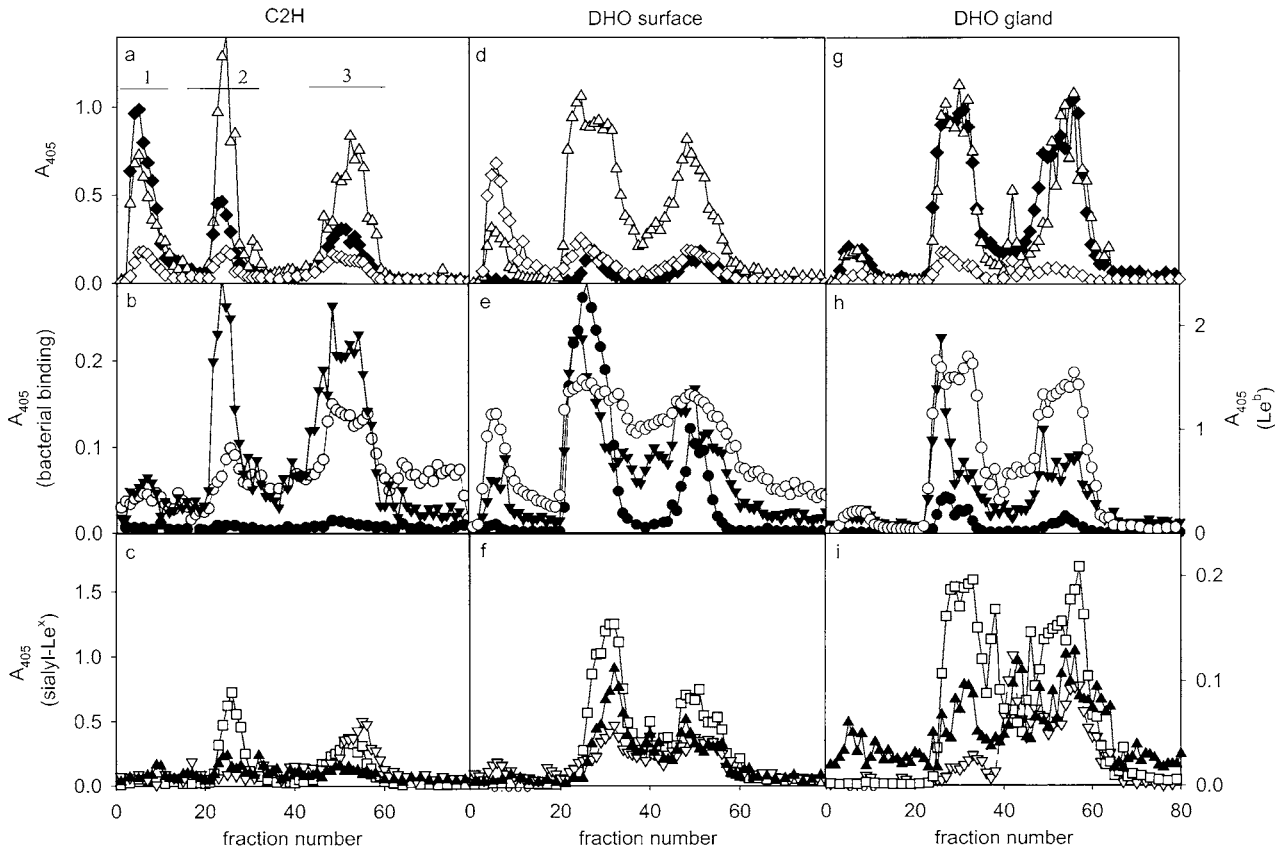

Figure 5 ***H. pylori*** **binding to mucin glycoforms**

Mucins were pooled from the CsCl density gradients (fractions 8–14; Figure 2), reduced, alkylated and subjected to anion-exchange chromatography on a Mono Q column; (**a**–**c**) mucins from C2H, (**d**–**f**) mucins from DHO surface and (**g**–**i**) mucins from DHO gland. Fractions were analysed for (**a**, **d**, **g**) carbohydrate (△), muc5ac (LUM5AC-2) (◇), muc6 (LUM6-3) (◆), (**b**, **e**, **h**) Le^b (2-25LE) (●), reactivity with the antibody recognizing Le^b, BLe^b, ALe^b and H-type-1 structures (clone 96FR2.10) (○), binding with the BabA-positive *H. pylori* strain CCUG17875 at pH 7.4 (▼), (**c**, **f**, **i**) sialyl-Le^x (□), binding with the SabA-positive *babA1A2* mutant at pH 7.4 (▲), and binding with the SabA-positive *babA1A2* mutant at pH 3 (▽). Glycoforms 1–3 are indicated by short horizontal lines (**a**).

(NeuAcα2,3Galβ1,4GlcNAc, as reacting with MAL), but with less discrimination compared with the sialyl-Le^x-binding SabA adhesin at neutral pH.

Tissue localization of carbohydrate structures

To assess further the tissue distribution of mucin glycoforms and expression of carbohydrate structures relevant for *H. pylori* binding, a large number of tissue specimens were investigated using histochemistry and immunohistochemistry. In the majority of the animals studied, the neck region of the gastric-surface and foveolar epithelium stained faintly blue with Alcian Blue (Figure 6a), suggesting that sialomucins co-localize with muc5ac (see Figures 1a and 1b). No distinct stain for sulphomucins was observed in the stomach (results not shown). In duodenum, the goblet cells were stained intensely blue and some of the Brunner's glands faintly blue by Alcian Blue (Figure 6b). The Bravo and Correa method [25] stained both the goblet cells and the Brunner's glands with a mixture of black and green (Figure 6c), indicating that both sialomucins and sulphomucins are present in these locations.

The results for the Lewis-type structures are summarized in Table 1. In antrum, all surface mucus cells and 10–70% of the glands were stained in 11 monkeys [both with 2-25LE (Figure 6d), BG-6 and 96Fr2.10 antibodies; results not shown]. In corpus, staining was usually more intense than in antrum, but with negative patches interspersed among positive ones. In cardia, localization of Le^b was similar to that in antrum, and in duodenum, Le^b was found in the goblet cells (results not shown). In the duodenum of the animals that were Le^b-negative in the stomach, Le^b was still found in a few Brunner's glands and at the bottom of the crypts (results not shown). All monkeys that were Le^b-positive were also Le^a-positive, and those with a high proportion of Le^b-positive surface/foveolar epithelium also had a high proportion of Le^a in the same location. Furthermore, staining of serial sections indicated that cells produce both structures and also that they could occur independently. In the Le^a-positive animals, Le^a was found in the surface/foveolar and neck region of antrum, corpus and cardia, whereas no Le^a was detected in the glands (Figure 6e). In duodenum, Le^a was located in the goblet cells, although in one monkey some cells in the bottom of the crypts and the Brunner's glands were also positive (results not shown). When present, sialyl-Le^a was located in the surface/foveolar epithelium in all regions of the stomach (results not shown). In duodenum, goblet cells were stained in the monkeys that were sialyl-Le^a-positive in the stomach, and also in one of the monkeys that was sialyl-Le^a-negative in the stomach (A6W; results not shown). No sulpho-Le^a was detected in any of the gastric specimens, but in two (A6W and 9EU) of the five monkeys where tissue was available from the duodenum, a few cells in the Brunner's glands

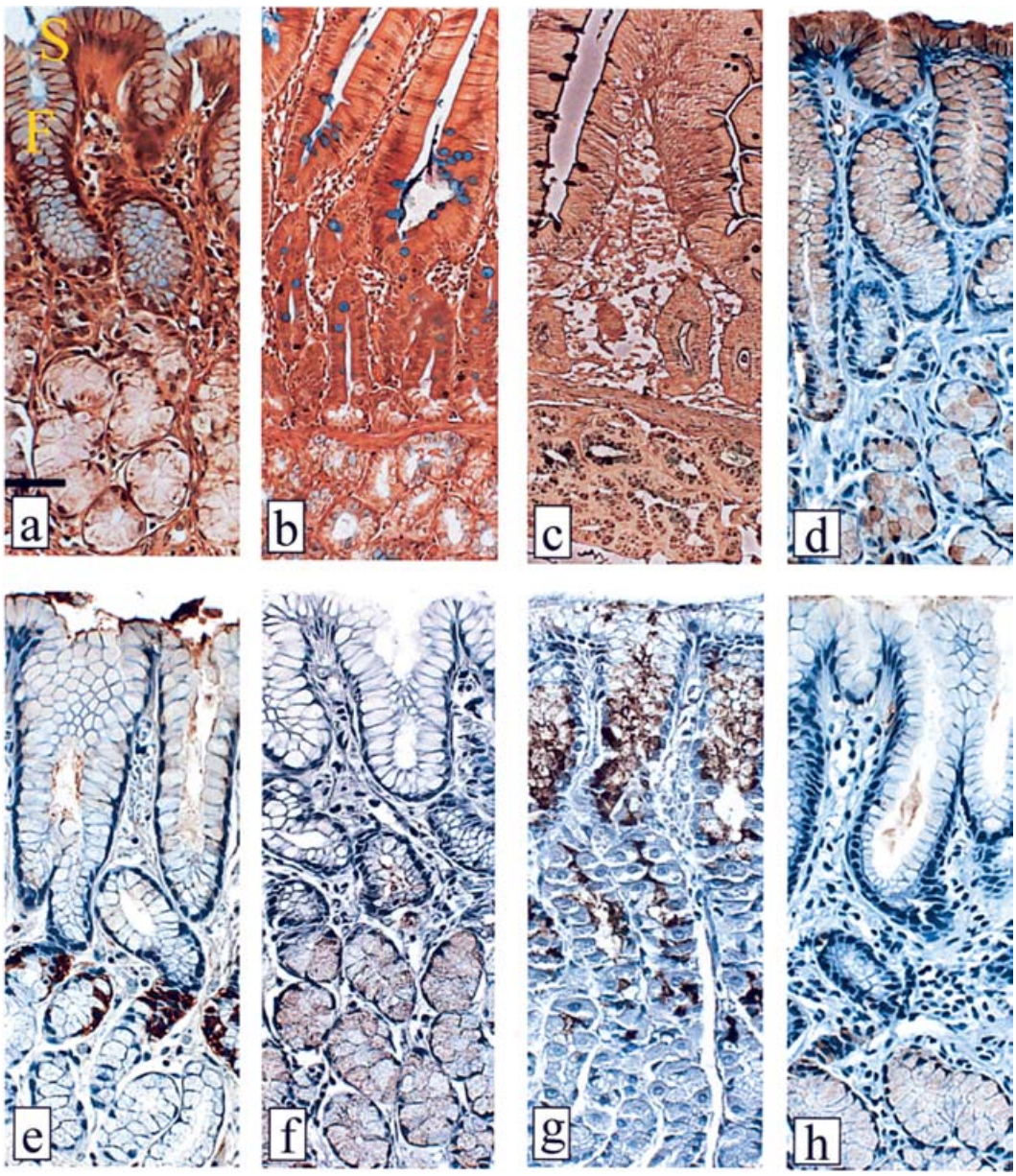

Figure 6 Tissue localization of carbohydrate structures

Sections from antrum (**a**) and duodenum (**b**) were stained by the method of Genta et al. [24], where sialo- and sulphomucins were visualized as blue colour. Duodenum was also stained by the method of Bravo and Correa [25] (**c**), which stains sialic acids blue and stains sulphate black. Sections from antrum were stained brown for Le^b (2-25LE) (**d**), Le^a (BG-5) (**e**) and Le^y (BG-8) (**f**), from corpus for Le^x (BG-7) (**g**), and from antrum for sialyl-Le^x (KM93) (**h**). In (**a**), S and F indicate surface and foveolar epithelium respectively. Scale bar, 50 μm.

were positive (results not shown). Since no goblet cells were stained, although they stained for sulphomucin, the goblet cells must contain sulphated structures other than sulpho-Le^a. Le^y was found in the glands of all monkeys both in antrum and corpus, but rarely in the surface/foveolar epithelium (Figure 3f). In contrast, Le^x was detected more frequently in the foveolar epithelium and in the mucin-producing cells of the neck region than in the glands (Figure 3g). However, the sialyl-Le^x antibody stained the antral glands in 13 of the 17 monkeys, indicating that Le^x is also mainly a glandular product, but sialylation masks the epitope so that it is not recognized by the Le^x antibody. In duodenum, sialyl-Le^x was present in goblet cells and Brunner's glands (results not shown). Sialyl-di-Le^x was negative in gastric specimens of all monkeys except for three (85D08, A6W and 82A49), in which a few cells in the antral and corpus glands were stained (results not shown). In duodenum, sialyl-di-Le^x was occasionally detected in goblet cells (results not shown).

DISCUSSION

Mucin coding sequences are conserved in a number of species, and divergence increases in the order human, monkey, rabbit/rat/cow and mouse [30]. In the present study, we used antibodies raised against the human MUC5AC, MUC6 and MUC2 mucins to detect and study the corresponding glycoproteins from Rhesus monkey. In the stomach, muc5ac was localized to the surface/foveolar

Table 1 Lewis antigens in antrum and corpus

Lewis antigens were analysed using immunohistochemistry. Areas of the surface/foveolar epithelium and the glands that were stained for the various carbohydrate structures were estimated and the results were grouped into four categories: high level of staining (approx. 90–100 %; indicated by +++), medium level of staining (25–90 %; indicated by ++), low level of staining (1–25 %; indicated by +) and virtually no staining (<1 %; indicated by −). n.d., not determined.

	Le^b		Le^a		Sialyl-Le^a		Le^x		Sialyl-Le^x		Le^y	
Monkey	Surface	Gland	Surface	Gland	Surface	Gland	Surface	Gland	Surface	Gland	Surface	Gland
Antrum												
F754	+++	++	+++	−	+	−	−	−	+	−	−	+++
86D02	+++	++	+++	−	++	−	−	−	−	−	−	++
T4C	+++	++	+++	−	+	−	−	−	+	+	−	+++
8V5	+++	++	+++	−	+	−	−	−	−	+	−	+
F436	+++	++	+++	−	+	−	+	−	−	++	−	+++
82A49	+++	++	+++	−	++	−	−	−	−	−	−	++
8PZ	+++	++	+++	−	+	−	−	−	+	++	−	+
EOE	+++	+	+++	−	+	−	−	−	−	+++	−	++
9A5	+++	++	+++	−	++	−	−	−	−	−	−	++
9EU	+++	+	+++	−	+	−	−	−	−	+++	−	+++
81D52	+++	++	+++	−	++	−	n.d.	n.d.	−	+	n.d.	++
86D06	−	−	−	−	−	−	+	−	−	++	+	++
E6C	−	−	−	−	+	−	+	−	−	++	++	+++
85D08	−	−	−	−	−	−	−	−	−	++	−	++
A6W	−	−	−	−	−	−	−	−	−	+++	−	++
C2H	−	−	−	−	−	−	−	−	−	+++	−	+++
D6G	−	−	−	−	−	−	n.d.	n.d.	−	+++	n.d.	n.d.
Corpus												
F754	++	−	++	−	++	−	+	++	+	−	−	+++
86D02	++	−	++	−	++	−	−	+	+	−	−	+++
T4C	++	−	++	−	++	−	+	−	+	−	−	+++
8V5	++	−	++	−	++	−	+	++	−	−	−	+++
F436	++	−	+++	−	++	−	++	++	++	−	−	++
82A49	+++	+	+++	−	++	−	+	−	+	−	−	+++
8PZ	+++	+	++	−	++	−	++	+	+	−	−	+++
EOE	++	−	++	−	++	−	++	+	−	−	−	+++
9A5	+++	−	++	−	++	−	+	+	−	−	−	+++
9EU	++	−	++	−	++	−	+	+	−	−	−	+++
81D52	+++	−	++	−	++	−	−	−	−	−	−	+++
DH0	++	−	++	−	++	−	+	−	++	−	++	+++
E6C	−	−	−	−	++	−	+	++	−	−	−	+++
85D08	−	−	−	−	−	−	+	−	−	−	−	+++
A6W	−	−	−	−	−	−	−	−	−	−	n.d.	n.d.
C2H	−	−	−	−	−	−	+	+	−	−	−	+++
D6G	−	−	−	−	−	−	+	+	−	−	n.d.	n.d.

epithelium and muc6 to the glands as in humans. In addition, biochemical properties such as mass, density, oligomeric nature and charge of the three glycoforms are very similar to those of human MUC5AC and MUC6, indicating that the Rhesus monkey produces muc5ac and muc6 orthologues to the human counterparts. The presence of mucin orthologues to the MUC2 [31] and MUC7 [32] mucins in monkeys has been shown previously.

The mucins from both monkeys studied using biochemical methods reacted with antibodies raised against Le^a, Le^b and B structures. As in blood-group B- and H-positive humans [9], these antigens were also present in material with lower density than muc5ac and muc6, indicating that these structures also occur in other molecules. Although Le^x and Le^y structures that are formed on the H-type-2 structures were present in the monkey mucins, little reactivity with the *U. europeus* lectin was found for the muc5ac and muc6 mucins, indicating that in these two animals, most mucin-bound H-type-2 structures are masked. In contrast, on human gastric mucins from Scandinavians, the H-type-2 structure is exposed in individuals producing this structure [9]. Furthermore, all monkeys that produced Le^b also produced Le^a, indicating that the *Lewis* gene product successfully competes for the substrate (core type-1 chains) with that of the *Secretor* gene. Consequently, H-type-1 and -2 structures seem to be less prevalent in the monkey compared with humans of Scandinavian origin [9]. Interestingly, there is considerable variation among human ethnic groups, e.g. Polynesians express the secretor phenotype weakly [33]. Le^b was detected in the surface/foveolar epithelium and, in most Le^b-positive monkeys, also to some extent in the antral glands with all three Le^b antibodies. The Lewis type-1 structures (Le^a and Le^b) were, in general, evenly distributed in the surface/foveolar epithelium of the antrum, whereas the corpus had the simultaneous presence of strong positive and negative zones in the same tissue sample. The presence of Le^b-positive and -negative areas in the same tissue sample has been demonstrated previously in humans [34] and, as in humans [1,2,35], sialyl-Le^a expression was generally restricted to muc5ac-producing cells of Le^a-positive individuals. Lewis type-2 structures (Le^x and Le^y) were mainly located in the glands and, to some extent, also in the surface/foveolar epithelium. As in humans, mucin substitution of carbohydrate structures such as blood-group antigens and sialic acid vary between individuals.

At neutral pH, the BabA-positive *H. pylori* strain (CCUG17875) seems to bind to Le^b or similar structures present

on the muc5ac mucin and to a putative monomeric mucin. The highest correlation between binding of the BabA-positive *H. pylori* strain to carbohydrate was found in Le^b/fucosylated structures, and a higher correlation was found in muc5ac compared with that in muc6. No Le^b was detected on mucins from the C2H monkey, but reactivity was found with the antibody recognizing Le^b, BLe^b, ALe^b and H-type-1 (clone 96Fr2.10) structures, and binding was inhibited by Le^b–HSA and H-type-1–HSA conjugates, indicating that binding occurs in H-type-1 through the Le^b-binding BabA adhesin. Adhesion also occurred on a smaller, apparently monomeric, component of mucin density that may represent a cell-associated mucin. This component is present in very small amounts relative to muc5ac and muc6, in keeping with the fact that the secreted oligomeric mucins are present both as a thick mucus layer attached to the mucosa and in a highly condensed storage form in the secretory granules, whereas cell-associated mucins only occur as a single molecular 'layer' at the luminal plasma membrane. In mucin preparations from human tissue, we have also observed binding to a putative membrane-associated MUC1 of similar size (S. Lindén, J. Mahdavi, J. Hedenbro, T. Borén and I. Carlstedt, unpublished work). Other investigators have shown that the role of cell-associated mucins such as MUC1 could be dynamic in *H. pylori* infection, e.g. the MUC1 exodomain may be cleaved off or subjected to glycosylation changes [36].

Binding to mucins with the SabA-positive *babA1A2* mutant was found to correlate with the presence of sialyl-Le^x in the sample but not to sialyl-Le^a, in accordance with previously published results, although 50% of the strains that bind the sialyl-Le^x antigen also bind the sialyl-Le^a antigen [10]. In a previous study on healthy human mucins, no such binding was detected; however, no sialyl-Le^x was detected either. The difference in sialyl-Le^x expression could possibly be explained by the fact that monkeys have a low-grade gastritis and/or that humans have less acidic mucins.

At acidic pH, *H. pylori* bound to material of similar density as DNA in addition to mucins, and this interaction was inhibited by dextran sulphate and DNA, suggesting an electrostatic binding. Mucin binding at acidic pH occurred mainly with the charged glycoforms and appeared to correlate with sialyl-Le^x and MAL reactivity. However, there was no binding to the sulphated sub-population of glycoform 3, indicating that charge is a necessary but not sufficient structural feature for adhesion.

H. heilmannii has previously been shown to be less pathogenic and to cause less gastritis compared with *H. pylori* [11]. *H. heilmannii* was present in six of the monkeys. However, no differences were observed except that there was a tendency towards higher levels of sialyl-Le^x in the antral glands of *H. heilmannii*-positive animals. Gastric mucins from pig [37] and rat [38] have also been shown to carry negative charges, suggesting that the proportion of acidic mucins is lower in the human stomach compared with other species. Mucins from other regions of the human alimentary tract (e.g. oral cavity [39] and intestine [22]), where a higher bacterial density is normally present, also have a higher charge density; possibly, production of acidic mucins is induced by bacterial challenge as a defence mechanism. A decrease in acidic mucins in the gastric juice after eradication of *H. pylori* has been described previously [40].

In summary, the present study suggests that *H. pylori* can bind to gastric mucins through three different mechanisms: (1) BabA-dependent binding to Le^b and other fucosylated structures, (2) SabA-dependent binding to sialyl-Le^x and (3) charge-dependent binding to sialyl-Le^x and sialyl-type-2 at acidic pH. Most of the animals used for investigating *H. pylori* infection are different from humans regarding gastric anatomy, physiology and inflammatory response, as well as mucin and carbohydrate expression. In contrast, Rhesus monkey gastric mucins are very similar to human gastric mucins, suggesting that host–microbe interactions in the two species are similar and Rhesus monkey is an excellent model for such studies.

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