Identification of erythrocyte-binding antigens in *Helicobacter pylori*

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The surface antigens of *Helicobacter pylori* conferring erythrocyte-binding activity were obtained by adsorption onto formaldehyde-treated dog and goat erythrocytes from supernatant fractions of sonicated bacteria and elution using a high concentration of NaCl. The desorbed material was analysed by SDS-PAGE and immunoblotting with anti-whole-cell serum to agar-grown bacteria which had been absorbed with broth-grown, non-haemagglutinating cells (haemagglutination-associated antiserum). Two polypeptides with molecular masses of 25 and 59 kDa were revealed as erythrocyte-binding antigens. Strains which agglutinated both dog and goat erythrocytes possessed both these erythrocyte-binding antigens, whereas an antigenically cross-reactive 24 kDa polypeptide was present in a strain which only agglutinated goat erythrocytes. Haemagglutinin material was extracted from *H. pylori* using *n*-octylglucopyranoside and purified by Sepharose chromatography and sucrose density gradient ultracentrifugation. The purified extract directly agglutinated erythrocytes in a neuraminyl-lactose-sensitive and neuraminidase-sensitive manner. The 59 kDa polypeptide was not present in the purified haemagglutinin preparation. The haemagglutination-associated antiserum reacted strongly with the 25 kDa polypeptide band which was the most prominent polypeptide band on analysis of the purified haemagglutinin preparation by SDS-PAGE and silver staining. Thus, *H. pylori* possesses at least two adhesins, one of which recognises a *N*-acetylneuraminic acid (α 2–3) moiety of receptors, the other being of unknown receptor specificity. Differences in the antigenicity and molecular masses of these adhesins in individual strains may underlie differences in receptor-binding specificities and haemagglutination profiles.

Introduction

In vitro haemagglutinating activity of *Helicobacter pylori* has been demonstrated with a variety of erythrocyte species (Huang *et al.*, 1988; Emödy *et al.*, 1988; Evans *et al.*, 1988; Nakazawa *et al.*, 1989; Robinson *et al.*, 1990). Different haemagglutination phenotypes and patterns have been suggested (Huang *et al.*, 1988; Emödy *et al.*, 1988; Robinson *et al.*, 1990). Evans *et al.* (1988) reported a fibrillar haemagglutinin that bound specifically to *N*-acetylneuraminyl-lactose (NeuAc-lactose) and proposed that this moiety was the adhesin of *H. pylori* primarily involved in gastric mucosal colonization. Unfortunately,

a molecular analysis of this fibrillar haemagglutinin was not done.

Reports of glycolipid receptors for *H. pylori* have appeared (Lingwood *et al.*, 1989; Slomiany *et al.*, 1989; Saitoh *et al.*, 1991). Lingwood *et al.* (1989) reported that lipid extracts of human red blood cells and of human and pig stomach tissue were specifically recognized by isolates of *H. pylori*. On the basis of chemical analysis, they proposed that the active component was a novel glycerolipid and that it was the specific gastric epithelial receptor for *H. pylori* adherence. The reports of Slomiany *et al.* (1989) and Saitoh *et al.* (1991) using haemagglutination (HA) inhibition tests and a solid-phase TLC-immunostaining technique, respectively, identified ganglioside GM₃ as a receptor molecule for *H. pylori*.

Several *in vitro* models utilizing mammalian cell lines have been developed for evaluation of *H. pylori* adherence. It was reported that *H. pylori* strains adhered to HEp-2, INT-407, Y-1 adrenal, and HeLa cell lines (Neman-Simha & Megrand, 1988; Fauchère *et al.*, 1989; Neman-Simha *et al.*, 1989; Evans *et al.*, 1989). Evans *et*

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate; HA, haemagglutination; HAA, haemagglutination-associated antiserum; HSA, haemagglutinin-specific antibody; NeuAc, *N*-acetylneuraminic acid; NeuAc-lactose, *N*-acetylneuraminyl-lactose; NOG, *n*-octyl- β -D-glucopyranoside; SAM, superficial adhering material; SDOC, sodium deoxycholate.

al. (1989) also reported that *H. pylori* adherence to Y-1 mouse adrenal cells was rapid, neuraminidase-sensitive, and blocked by a sialoglycoprotein rich in NeuAc-lactose. However, neuraminidase sensitivity was not demonstrated in other studies using HeLa cells (Fau-chère *et al.*, 1989) and human gastric carcinoma (KATO III) cells (Dunn *et al.*, 1991) as model systems for *H. pylori* adherence.

Despite some discrepancies, the findings from the various studies on haemagglutination caused by *H. pylori* all indicate that molecular characterization of the haemagglutinins is necessary for a better understanding of this property and the nature of the ligand-receptor interaction(s). Huang *et al.* (1988) described *H. pylori* strains which agglutinated human red blood cells (HA phenotype 1) and others which did not (HA phenotype 2). Accordingly, attempts were made (i) to purify haemagglutinin from *H. pylori* strain 136292, which showed HA phenotype 1 activity and relatively higher HA titres compared with the other strains tested previously; (ii) to analyse, using Western blotting, the erythrocyte-binding antigens of three strains (one HA phenotype 1 and two HA phenotype 2), which exhibit different abilities and specificities in HA reactions observed previously (Huang *et al.*, 1988); and (iii) to characterize the erythrocyte-binding antigens by analysis of purified haemagglutinin.

Methods

***H. pylori* strains and growth conditions.** Three *H. pylori* strains were principally used in this study, namely 136292, 172460 and 184858. Strain 136292 is an HA phenotype 1 strain which is strongly haemagglutinating (human, guinea-pig, sheep, dog, goat and rat erythrocytes), whereas strain 172460 strongly agglutinates only goat erythrocytes but also weakly agglutinates rat erythrocytes. Strain 184858 only agglutinates rat erythrocytes weakly. Both the latter strains are HA phenotype 2 strains (Huang *et al.*, 1988). Two additional HA phenotype 1 strains (185706 and 133538) and one further HA phenotype 2 strain (216983) were used in certain experiments.

H. pylori were grown on 7% (v/v) lysed horse blood *Campylobacter* (LBC) agar plates (without selective supplement) in an anaerobic jar (Oxoid) using a Gas Generating Kit for campylobacters (Oxoid) at 37 °C for 48–72 h (Huang *et al.*, 1988). To grow *H. pylori* in broth medium, bacteria were inoculated into 200 ml serum broth medium containing 2.5% (w/v) nutrient broth No. 2 (Oxoid), 0.5% (w/v) yeast extract (Oxoid) and 2% (v/v) newborn calf serum (Gibco) in a one litre flask. The flask was placed in an anaerobic jar with a Gas Generating Kit for campylobacters and incubated at 37 °C for 48 h with constant shaking at 150 r.p.m. (New Brunswick Orbital Incubator).

HA assay. Citrated dog, goat, sheep and rat blood were obtained fresh from the BioResources Unit, Trinity College, Dublin, Republic of Ireland. Citrated ox and horse blood were obtained from Abbotstown Veterinary Institute, Dublin, Republic of Ireland. Human A and O blood were outdated samples from the local blood bank. Washing of red blood cells, preparation of bacterial suspensions and the microtitration

method for haemagglutination have been detailed previously (Huang *et al.*, 1988).

HA inhibition test. The following solutions were prepared in PBS (0.145 M-NaCl, 10 mM-sodium phosphate buffer, pH 7.2) for use in HA inhibition tests: NeuAc-lactose from bovine colostrum [mixture of NeuAc(α 2-3)Gal(β 1-4)Glc, 85% and NeuAc(α 2-6)Gal(β 1-4)Glc, 15%] and NeuAc-lactose from human milk [mixture of NeuAc(α 2-3)Gal(β 1-4)Glc, 15% and NeuAc(α 2-6)Gal(β 1-4)Glc, 85%] (2 mg ml⁻¹); N-acetylneuraminic acid (NeuAc) from egg yolk and synthetic NeuAc (2 mg ml⁻¹). These sugars were obtained from Sigma.

Each well in the inhibition assays contained 25 μ l of a twofold dilution of bacterial suspension or haemagglutinin sample, 25 μ l test inhibitor, and 25 μ l erythrocyte suspension (both 3 and 1.5% packed cell volume suspensions were used). Controls contained 25 μ l PBS instead of test inhibitor. Titrations were incubated and read as for the microtitration HA assay (Huang *et al.*, 1988). Tests were performed in parallel with dog, goat, horse, ox, sheep and human A erythrocyte suspensions with each of the *H. pylori* strains tested.

Neuraminidase treatment of erythrocytes. Erythrocytes were sedimented, washed twice and resuspended to 3% (v/v) packed cell volume in either PBS (pH 7.2) or acetate buffered saline (0.145 M-NaCl, 10 mM-sodium acetate/acetic acid buffer, pH 5.2). Dog, goat, horse, ox, sheep and human A erythrocyte suspensions (3% packed cell volume in buffered saline) were treated with *Clostridium perfringens* type X neuraminidase (Sigma) at 0.1 U ml⁻¹ for 1 h at 37 °C. Neuraminidases from *Vibrio cholerae* (Sigma) and from *Arthrobacter ureafaciens* (Boehringer Mannheim) were also used (0.1 U ml⁻¹ at 37 °C for 1 h) in the treatment tests. Two batches of each type of neuraminidase were tested. The neuraminidase-treated erythrocytes were sedimented, washed twice with PBS and resuspended to the original volume in PBS. Haemagglutinin samples as well as *H. pylori* bacteria were tested with the treated and control suspensions of erythrocytes. Lectins from *Arachis hypogaea* [Sigma; β -D-Gal(1-3)-D-GalNAc-specific] and *Limulus polyphemus* (Sigma; NeuAc-specific) were used to check the efficiency of the neuraminidase treatment. Peanut agglutinin does not agglutinate normal human erythrocytes, but strongly agglutinates neuraminidase-treated erythrocytes. Neuraminidase-treated human erythrocytes are not agglutinated by *Limulus polyphemus* agglutinin.

Protease and heat treatments of purified haemagglutinin. These were performed as previously described for *H. pylori* (Huang *et al.*, 1988).

Production of antisera to *H. pylori* whole cells. *H. pylori* strains 136292, 172460 and 184858 were used individually. Bacteria lawned onto LBC agar plates and grown as above (37 °C, 48 h) were harvested and washed twice with PBS (pH 7.2), once with PBS containing 0.5% (w/v) formaldehyde, and finally resuspended in PBS containing 0.5% formaldehyde [1 g (wet wt) bacteria per 100 ml suspension]. Adult New Zealand white rabbits were immunized intravenously at 7-d intervals for 6 weeks with a constant dose of 0.5 ml formaldehyde-treated bacterial suspension for each injection. The rabbits were bled 12 d after the last injection. The sera were separated and stored at -20 °C.

Preparation of HA-associated antiserum (HAA). Antiserum raised against agar-grown bacteria of strain 136292 was absorbed with broth-grown bacteria of the same strain which had been serially passaged in the broth medium until harvested bacteria were non-haemagglutinating as tested by the microtitration method. Absorption was performed by incubating the antiserum with washed broth-grown bacterial cells [about 2 g (wet wt) per 100 ml antiserum] at 4 °C for 6–12 h followed by removal of the bacteria by centrifugation at 8000 g for 10 min at 4 °C. This procedure was repeated three times.

Preparation of haemagglutinin-specific antibody (HSA). Of the haemagglutinin sample (see Results), 3 ml (approximately 1 mg

Fig. 1. Flow scheme of purification of *H. pylori* haemagglutinin.

protein) was centrifuged (Eppendorf centrifuge, 7000 *g*, 5 min) and the pellet resuspended in 1 ml PBS containing 1.5% (w/v) bovine serum albumin (BSA, Sigma) and incubated for 15 min at 18–20 °C. Antiserum raised against whole cells of agar-grown *H. pylori* strain 136292 (500 µl) was added to the suspension. After incubation for 30 min at 18–20 °C, the mixture was centrifuged to sediment the complex of antibody–haemagglutinin material. The pellet was washed three times with PBS, and then resuspended in 1 ml glycine HCl buffer (0.2 M, pH 2.2) for 10 min. After centrifugation (7000 *g*, 5 min) the supernatant fraction containing desorbed antibodies was neutralized with 1 M-NaOH. This constituted the HSA.

Tests on the effects of detergents on HA activity. Graded quantities of washed cells of *H. pylori* strain 136292 were each suspended in 1 ml PBS containing various concentrations (from 0.2 to 1%, w/v, of detergents and 2 mM-PMSF) and 2 mM-benzamidine to yield various final bacterial cell concentrations (2, 4 and 6%, wet w/v). The bacterial suspensions were incubated for 15 min at 18–20 °C. The bacteria were recovered by centrifugation (6000 *g*, 10 min), washed once with PBS, resuspended in PBS (about 5×10^{10} cells ml⁻¹) and tested for their HA titres against dog erythrocytes using the microtitration method. The following detergents were tested: *n*-octyl- β -D-glucopyranoside (NOG), 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulphonate (CHAPS), and sodium deoxycholate (SDOC) (all from Sigma).

Preparation of haemagglutinin sample. An overview of the final standard purification scheme is presented in Fig. 1. Washed cells of *H. pylori* strain 136292 (about 0.5 g wet wt) from 30 LBC-agar plates were suspended in 12 ml PBS containing 0.4% NOG, 2 mM-PMSF and 2 mM-benzamidine for 15 min at 18–20 °C. The bacteria were recovered by centrifugation (6000 *g*, 10 min), the supernatant fraction discarded and the bacteria resuspended in 12 ml PBS with protease

inhibitors and 1% (w/v) NOG, for a further 15 min. After centrifugation the supernatant fraction was dialysed against PBS for 24 h at 4 °C with three changes of PBS. The precipitate which formed after dialysis was harvested by centrifugation (8000 *g*, 10 min) and washed once in PBS, once in PBS containing 1% CHAPS, twice more in PBS and then dispersed or dissociated in 2 ml PBS containing 6 M-guanidine HCl (Sigma). After centrifugation at 10000 *g* for 15 min the supernatant fraction was applied onto a Sepharose CL-6B gel bed (1.5 × 40 cm) using PBS containing 6 M-guanidine HCl as elution buffer. Fractions containing the void-volume material were pooled (about 5 ml in total). Of the pooled sample, 2.5 ml aliquots were directly applied onto linear sucrose density gradients [12 ml, 20–50% (w/w) sucrose in distilled water containing 6 M-guanidine HCl]. After ultracentrifugation (100000 *g*, 20 h, 18 °C) the gradients were fractionated and fractions (about 1.5 ml each) monitored for A_{280} . Selected fractions were dialysed against PBS and tested for their haemagglutinating activities.

Erythrocyte-binding tests. Citrated fresh dog and goat erythrocytes were washed three times with PBS and prepared as 5% (v/v) suspensions of packed cell volumes in PBS. Formaldehyde (40%, w/v) was added dropwise, with stirring, to the erythrocyte suspensions to a final concentration of 10% (w/v). Gentle stirring continued over 24 h at 4 °C. The formaldehyde-treated cells were sedimented (3500 *g*, 5 min) and washed six times with 0.15 M-NaCl and finally prepared as 2.5% (v/v) suspensions of packed cell volume in PBS.

Agar-grown *H. pylori* cells (strains 136292, 172460 and 184858 separately) were washed three times with PBS and resuspended as 2% (v/v) packed cell volume suspensions in PBS. The tube containing each bacterial suspension was placed in a beaker with iced salt water and sonicated (MSE ultrasonicator) at full power (10 × 10 s bursts with 20 s pauses in between). Debris was removed by centrifugation (10000 *g*, 15 min, 4 °C).

Each supernatant fraction was assayed in an erythrocyte-binding test as follows. To 200 µl formaldehyde-treated erythrocyte suspension in a Eppendorf tube 200 µl 5% (w/v) BSA in PBS was added and the mixture incubated at 18–22 °C for 15 min. To this 600 µl supernatant fraction of sonicated bacteria was added and the mixture incubated for a further 30 min. The Eppendorf tube was briefly centrifuged (about 7000 *g*, 10 s) to pellet the erythrocytes. The pellet was washed three times with PBS. After the last washing the pellet was suspended in 80 µl 1.5 M-NaCl for 15 min at room temperature. After centrifugation (7000 *g*, 10 s) the supernatant fraction was removed and dialysed against distilled water overnight at 4 °C. Of each dialysed sample 40 µl was analysed by SDS-PAGE and Western blotting.

SDS-PAGE and gel staining. SDS-PAGE was performed essentially as described by Laemmli (1970) using 12% (w/v) acrylamide separating gels. Bacteria (200 µg protein) were boiled in 100 µl sample buffer for 5 min and the lysate centrifuged (7000 *g*, 2 min) to obtain a supernatant fraction. For visualizing protein profiles, gels were stained with Coomassie brilliant blue R-250. In some experiments gels were silver-stained by the method of Wray *et al.* (1981). Carbohydrate was detected by the highly sensitive periodic acid–silver stain (Dubray & Bezard, 1982).

Immunoblotting technique. The Western blotting technique of Burnette (1982) was used for immunoblotting. After electrophoresis, the polypeptides separated by SDS-PAGE were transferred electrophoretically to a nitrocellulose filter (BA85, pore size 0.45 µm, Schleicher & Schuell). This was then soaked in Tris/HCl-buffered saline (TBS; 10 mM-Tris/HCl, 0.15 M-NaCl, pH 7.5) containing 2% BSA for 60 min at 18–20 °C and then transferred for 90 min to antiserum, which had been diluted 1:100 in TBS containing 1% BSA. After being washed with TBS the filter was incubated with a 1:1000 dilution of horseradish-peroxidase-conjugated Protein A (Sigma) in TBS containing 1% (w/v) BSA for 60 min. After being washed again,

Table 1. HA-inhibition by NeuAc-lactose from bovine colostrum and from human milk when tested with bacterial cells of *H. pylori* strain 136292 and erythrocytes of six species

Results shown are representative of duplicate experiments with strain 136292; similar patterns of HA inhibition were also obtained in duplicate experiments with strains 185706, 133538, 216983, and 172460 (strain 172460 was tested only with goat red blood cells), although absolute titres differed from those below; final concentrations of NeuAc-lactose and erythrocytes in wells were 667 µg ml⁻¹ and 0.5% (v/v), respectively.

Haemagglutination conditions	HA titres for indicated erythrocyte species					
	Human A	Sheep	Dog	Goat	Bovine	Horse
<i>H. pylori</i> cells + PBS	32	32	128	64	32	32
<i>H. pylori</i> cells + bovine NeuAc-lactose in PBS	4	4	32-64	64	16-32	16-32
<i>H. pylori</i> cells + human NeuAc-lactose in PBS	8	32	128	64	32	32

the blots were developed in TBS containing 0.06% (w/v) 4-chloro-1-naphthol and 0.01% (v/v) H₂O₂. The reaction was stopped by washing in distilled water.

Protein determination. Protein concentration was determined by the method of Markwell *et al.* (1978). BSA was used as standard. This method is a modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples.

Electron microscopy. (a) Negative staining method. Samples to be examined were placed on copper grids bearing a carbon-coated formvar film. The grids were drained with filter paper after 2 min, negatively stained with 1% (w/v) silicotungstate for 1 min, and finally air-dried. Grids were examined with a Hitachi H-700 transmission electron microscope at 100 kV.

(b) Thin section method. Bacterial suspensions or haemagglutinin samples were mixed with equal volumes of 3% (v/v) fresh erythrocyte suspensions in wells of a white tile and rocked. After haemagglutination the supernatant fractions were removed with a Pasteur pipette and the agglutinated erythrocytes set in 2% (w/v) SeaPlaque low-gelling-temperature (28–30 °C) agarose (FMC BioProducts). Small (about 1 mm³) blocks of the gelled agarose were cut and fixed for 2 h at room temperature with 3% (v/v) glutaraldehyde in 0.1 M-sodium cacodylate buffer (pH 7.2). After being washed six times with 0.05 M-potassium phosphate buffer (pH 7.2), the blocks were further fixed with 2% (w/v) OsO₄ in 0.1 M-sodium cacodylate buffer (pH 7.2) for 2 h at room temperature (18–20 °C). After dehydration with a graded series of ethanol solutions and treatment with transitional fluid (propylene oxide), the specimens were embedded in resin (Emix resin kit). Thin sections (70 nm) were cut with a glass knife (Ultramicrotome) and stained with uranyl acetate and lead citrate.

Results

HA inhibition

In a broad survey (Huang *et al.*, 1988) NeuAc-lactose (at a final concentration of 1.67 mg ml⁻¹) was reported not to inhibit haemagglutination by bacterial cells of each of three HA phenotype 1 *H. pylori* strains including strain 136292. In addition, NeuAc and galactose as a mixture of monosaccharides did not inhibit. Shortly after publica-

tion of these findings, the report of Evans *et al.* (1988) appeared describing a NeuAc-lactose-binding fibrillar haemagglutinin on *H. pylori*. They noted that NeuAc-lactose from bovine colostrum [85% NeuAc(α2-3)Gal] but not NeuAc-lactose of human origin [85% NeuAc(α2-6)Gal] inhibited haemagglutination of bovine and human erythrocytes. Although not stated in Huang *et al.* (1988), the NeuAc-lactose used in those studies was of human origin. Accordingly, in light of the findings of Evans *et al.* (1988), it became necessary for us to reassess our original observations on NeuAc-lactose as a potential inhibitor of *H. pylori* haemagglutination.

NeuAc-lactose from bovine colostrum and from human milk, NeuAc from egg yolk and synthetic NeuAc were tested in the microtitration assay for their abilities to inhibit haemagglutination. Replicate bacterial titrations were performed. Also, in order to improve the sensitivity of HA inhibition, 1.5% (v/v packed cells) erythrocyte suspensions were used instead of the standard 3% erythrocyte suspensions that had previously been used (Huang *et al.*, 1988).

In no experiment with any of the six test erythrocyte species (human A, sheep, dog, goat, bovine and horse) was inhibition by egg yolk NeuAc or synthetic NeuAc observed. NeuAc-lactose, from both bovine and human milk, caused some inhibition when strain 136292 was tested using the microtitration system (Table 1). Essentially identical findings were obtained with another four strains tested including two HA phenotype 2 strains, *viz.* 172460 and 216983. The ability of NeuAc-lactose to inhibit haemagglutination varied with the erythrocyte species rather than the bacterial strain tested. With NeuAc-lactose from bovine colostrum, no inhibition was observed with goat red blood cells, slight inhibition (*i.e.* drop in end-point reading from 3+ or 2+ to 1+ or ±) with bovine and horse erythrocytes, weak inhibition (two–fourfold reduction in HA titre) with dog red blood

cells, and marked inhibition with human and sheep erythrocytes. For NeuAc-lactose from human milk, however, a fourfold inhibition effect was found only with human red blood cells. However, even when using almost ten times the minimum inhibitory concentration of bovine NeuAc-lactose as reported by Evans *et al.* (1988), complete inhibition of *H. pylori* haemagglutination was not achieved.

Treatment of erythrocytes with neuraminidase

Neuraminidase (ex *Clostridium perfringens*) treatment of dog, goat, horse, bovine, sheep and human A erythrocytes at a concentration of 0.1 U ml^{-1} at 37°C for 1 h did not affect the HA titres of the three HA phenotype 1 strains (133538, 136292 and 185706) and two HA phenotype 2 strains (172460 and 216983) of *H. pylori*. Lectin agglutinability (*Arachis hypogaea* agglutinin and *Limulus polyphemus* agglutinin) was used to control the efficiency of the neuraminidase treatment of the erythrocytes. Neuraminidase treatment of erythrocytes rendered them inagglutinable by horseshoe lectin (NeuAc-specific) but agglutinable by peanut agglutinin indicating removal of the NeuAc. In addition, erythrocytes treated with neuraminidases from *Vibrio cholerae* (Sigma) and from *Arthrobacter ureafaciens* (Boehringer Mannheim) (0.1 U ml^{-1} at 37°C for 1 h) were also agglutinated to the same titres as control erythrocytes.

The failure of neuraminidase treatment to inhibit haemagglutination, the variation in HA inhibition effects observed with bovine NeuAc-lactose with different erythrocyte species and the different HA phenotypes already observed (Huang *et al.*, 1988) strongly suggested that multiple haemagglutinins might be present on *H. pylori*.

Whole bacterial cell protein profiles of H. pylori strains examined by SDS-PAGE and by Western blotting using anti-whole-cell sera

H. pylori strains grown on LBC agar plates showed very similar protein profiles by SDS-PAGE (Fig. 2a). The molecular masses of the major polypeptide bands in the gels appeared to be 68, 63, 59, 54–55 (doublet), 45, 30 and 22 kDa. There were, however, some differences between strains in minor bands seen. To test the reactivities of anti-whole-cell sera, Western blotting of whole-cell samples of strains 136292, 184858, and 172460 was performed (Fig. 2b, c, d). The 63, 45 and 22 kDa polypeptide bands, reacted weakly or not at all with homologous antiserum, whereas some minor bands, e.g. the 25 kDa polypeptide band, reacted strongly with anti-whole-cell sera. The 59 and 25 kDa polypeptide bands of

all three strains displayed antigenic cross-reactivities between strains. With respect to the 25 kDa polypeptide, strain 172460 showed differences from the other two strains not only in antigenic reactivity, but also in its molecular mass value which was slightly smaller (24 kDa). In the case of the 17 and 16 kDa polypeptides, the three antisera manifested totally different reactivities. The 17 and 16 kDa antigens of the three strains were immunostained only by the anti-strain 184858 serum and by the anti-strain 136292 serum, respectively.

Identification of the erythrocyte-binding antigens of H. pylori

Formaldehyde-treated dog and goat erythrocytes yielded the same HA titres as freshly washed untreated erythrocytes did when tested with three strains of *H. pylori* (136292, 172460 and 184858), suggesting that the receptors recognized by the haemagglutinin(s) of *H. pylori* had not been affected by the formaldehyde treatment. After incubation of the formaldehyde-treated erythrocytes with the supernatant fractions from sonicated bacterial cells of strains 136292, 184858 and 172460 followed by elution of absorbed material with a high concentration of NaCl, the desorbed dialysed fractions were analysed by SDS-PAGE, electrotransfer, and immunoblotting with a pooled anti-whole-cell antiserum (a mixture of equal volumes of the anti-whole-cell sera against these three strains).

Two antigenic polypeptides with molecular masses of 25 and 59 kDa, respectively, were detected in both lanes on to which had been applied, respectively, material desorbed from formaldehyde-treated dog and goat erythrocytes incubated with supernatant fractions of sonicated cells of strain 136292 (Fig. 3, lanes 4 and 5). For strain 172460 (Fig. 3, lanes 6 and 7) only material eluted from goat erythrocytes showed a well immunostained band, which had a molecular mass slightly lower (24 kDa) than that of the 25 kDa antigen of strain 136292. A 59 kDa band was also visible but faint. However, corresponding 25 kDa and 59 kDa bands in strain 184858 were not detected (Fig. 3, lanes 2 and 3). It is improbable that the failure to detect antigens corresponding to these polypeptides was due to failure to elute them from the erythrocytes but rather that they did not bind to the erythrocytes, as strain 184858 does not agglutinate either of these erythrocyte species.

Extraction and purification of haemagglutinin-containing material

Different concentrations of NOG were tested for their effects on the haemagglutinating activity of strain

Fig. 2. SDS-PAGE analysis of whole-cell protein profiles of three *H. pylori* strains and antigenic analysis by Western immunoblotting. (a) Agar-grown bacteria, stained with Coomassie brilliant blue, 50 μ g protein per lane. Lanes: 1–3, strains 136292, 184858 and 172460, respectively. (b, c, d) Electrotransferred protein profiles of the three strains reacted with anti-whole-cell sera (1:100 dilutions) against strains 136292, 184858 and 172460, respectively; 25 μ g protein per lane. Lanes as for (a).

136292. A concentration of 0.4% (w/v) NOG was found to have little effect on the HA titres, whereas 0.8% NOG almost completely removed the haemagglutinating ability of the bacteria. The other detergents tested, namely CHAPS and SDOC, had no significant effects even at final concentrations of 1% (w/v). Attempts to extract active haemagglutinin from strain 136292 directly with 1% (w/v) NOG failed. Pretreatment of the bacteria with 0.4% NOG, followed by centrifugation and extraction with 1% NOG yielded material which precipitated on dialysis. The harvested precipitate agglutinated erythrocytes. Interestingly, haemagglutinating extracts were also obtained by using 1% (w/v) CHAPS or 1% (w/v) SDOC in place of 0.4% NOG for the initial extraction. The haemagglutinating precipitate could be dispersed or dissociated in 1.0% NOG or in guanidine HCl (from 5 to 8.6 M) but not in 1% CHAPS. The 'solubilized' material in either NOG or guanidine HCl could be recovered by

dialysis against PBS. The resulting precipitate showed no significant loss of haemagglutinating activity.

Based on these findings a scheme was developed to purify the haemagglutinin as described in Methods (Fig. 1). After gel chromatography of the haemagglutinin material 'solubilized' in 6 M-guanidine HCl on a Sepharose CL-6B column in the presence of 6 M-guanidine HCl, more than 95% of the applied material was eluted in the void volume of the column as detected by measuring A_{280} , indicating that the extract was not truly solubilized by the guanidine HCl. Following sucrose density gradient ultracentrifugation of the pooled void-volume fractions from Sepharose chromatography in the presence of 6 M-guanidine HCl, a sharp greyish-white band was present in the gradient. The fractions containing this material had the maximum absorbances at 280 nm. After dialysis the peak fraction (about 2 ml) had a protein content of about 0.3 mg ml⁻¹.

Fig. 3. Erythrocyte-binding antigens of *H. pylori* identified by immunoblotting. Lanes: 1, whole-cell lysate of strain 136292; 2 and 3, eluates from formaldehyde-treated dog (lane 2) and goat (lane 3) erythrocytes pre-incubated with the supernatant fraction of a sonicated bacterial suspension of strain 184858; 4 and 5, eluates from formaldehyde-treated dog (lane 4) and goat (lane 5) erythrocytes pre-incubated with the supernatant fraction of a sonicated bacterial suspension of strain 136292; 6 and 7, eluates from formaldehyde-treated dog (lane 6) and goat (lane 7) erythrocytes pre-incubated with the supernatant fraction of a sonicated bacterial suspension of strain 172460. Pooled serum comprising equal volumes of anti-whole-cell sera to the three corresponding strains was used as the first antibody.

Analysis of the purified haemagglutinin of *H. pylori* strain 136292

By serial twofold dilution assay, the haemagglutinin sample from strain 136292 purified by NOG extraction, Sepharose CL-6B column chromatography and density gradient ultracentrifugation, agglutinated dog, goat and human erythrocytes at a minimum concentration of about 10, 75 and 75 $\mu\text{g ml}^{-1}$, respectively, for a 1.5% erythrocyte suspension (final concentration).

The previous findings (Huang *et al.*, 1988) that heat and protease treatments of bacteria resulted in the abolition of the ability of *H. pylori* to agglutinate erythrocytes were reproduced with the haemagglutinin sample. After heating at 56 °C for 5 min or treatment

with α -chymotrypsin (100 $\mu\text{g ml}^{-1}$, final concentration), β -chymotrypsin (200 $\mu\text{g ml}^{-1}$), trypsin (200 $\mu\text{g ml}^{-1}$) or pronase (100 $\mu\text{g ml}^{-1}$) at 37 °C for 20 min and then washing with PBS, the haemagglutinin sample (150 $\mu\text{g protein ml}^{-1}$) showed no haemagglutinating activity against dog, goat, or human erythrocytes.

However, a difference between the behaviour of bacterial cells of strain 136292 and the haemagglutinin sample derived from this strain was found in relation to the use of neuraminidase-treated erythrocytes in the HA test. Whereas neuraminidase treatment of erythrocytes did not affect the HA titres with bacterial cells, the same treatment of dog, goat and human red blood cells caused abolition of haemagglutination by the purified haemagglutinin sample.

Also, in contrast to the previous findings that NeuAc-lactose from bovine colostrum was inhibitory to different degrees on haemagglutination of erythrocytes by *H. pylori* strain 136292, when similar HA inhibition assays (NeuAc-lactose, final concentration 667 $\mu\text{g ml}^{-1}$; erythrocytes, final concentration 1%, v/v) were carried out with the purified haemagglutinin sample (final concentration 100 $\mu\text{g ml}^{-1}$), complete inhibition occurred with all the test erythrocyte species (human A, sheep, dog, goat, bovine and horse).

In Coomassie-brilliant-blue-stained SDS-PAGE gels the haemagglutinin sample (Fig. 4a, lane 2) revealed three major polypeptides on the basis of staining intensity with molecular masses of 75, 68 and 54–55 (doublet) kDa, respectively. Less intensely stained bands had molecular masses of 30, 25 and 16 (doublet) kDa. In contrast, the 25 kDa polypeptide band was the most prominent in silver-stained SDS-PAGE gels (Fig. 4b, lane 2).

Whole cells of strain 136292 and the haemagglutinin sample were solubilized in the sample buffer and run as paired samples in triplicate, immunoblotted and probed with antisera with different specificities. Antiserum to whole cells of strain 136292 reacted strongly with the 25 kDa polypeptide band and less intensely with the 68, 54 and 16 kDa polypeptide bands of the haemagglutinin sample (Fig. 4c, lane 2). The HAA, prepared from the antiserum to agar-grown whole cells of strain 136292 by absorption with broth-grown, non-haemagglutinating bacteria, reacted with the 25 kDa polypeptide band and less intensely with the 16 kDa polypeptide band in both the whole cell and haemagglutinin samples (Fig. 4d, lanes 1 and 2). In contrast, a 59 kDa polypeptide band of the whole cell sample, which was absent in the purified haemagglutinin sample, reacted strongly with this absorbed antiserum (Fig. 4d, lane 1). With this exception, a similarity was found in the reactivities of the HAA and the HSA against the haemagglutinin material. However, the 25 kDa polypeptide bands of both the

Fig. 4. Analysis of the haemagglutinin sample by SDS-PAGE and immunoblotting. (a, b) SDS-PAGE gels stained with Coomassie brilliant blue and by the silver stain method, respectively. (c, d, e) Western blotting with anti-whole-cell serum, HAA and HSA, respectively. Lanes: 1, whole cells (50 µg protein per lane) of strain 136292; 2, haemagglutinin sample prepared from strain 136292 [15 µg protein per lane except lane 2 of (a) in which 45 µg protein was applied].

whole cell and haemagglutinin samples reacted more strongly with the HSA than with the HAA (Fig. 4e). The HSA also reacted with the 16 kDa polypeptide.

When the haemagglutinin sample (15 µg protein per lane) was run in an SDS-PAGE gel, no significant carbohydrate was detected by the sensitive silver stain method of Dubray & Bezard (1982) (data not shown).

Examination of H. pylori haemagglutinin by electron microscopy

The negatively stained haemagglutinin sample revealed vesicle-like structures of various sizes (Fig. 5a). Thin sectioning of erythrocytes agglutinated by the haemagglutinin sample showed close apposition of the vesicle surface to the surface of erythrocytes and bridging between erythrocytes by the vesicles (Fig. 5b, c). The attachment of the vesicles to the erythrocytes was visually similar to that seen between *H. pylori* bacteria and the erythrocytes (Fig. 5d, e). However, despite numerous attempts the detailed morphological and structural nature of the haemagglutinin on the surface of the vesicles was not resolved.

Discussion

Fresh erythrocytes have previously been used for receptor-binding assays similar to that employed in this study (e.g., Haynes *et al.*, 1988). However, a major problem arising when fresh erythrocytes were used herein was the haemolysis which occurred at the elution step where either an acidic buffer (usually pH 2.2 or thereabouts) or a high salt concentration had to be used. To overcome this problem formaldehyde-fixed erythrocytes were utilized successfully instead of fresh ones. Formaldehyde-treated erythrocytes have long been used for passive HA assays, but the exact surface changes caused by the treatment with formaldehyde remain unclear. No matter what the changes might be, identical HA titres and patterns were shown by untreated erythrocytes and by erythrocytes treated with formaldehyde, indicating that the receptors involved in haemagglutination with *H. pylori*, at least for the strains used in this study, were not significantly affected by this chemical treatment.

Both the 25 and 59 kDa polypeptide antigens of the haemagglutinating strain 136292 were found to absorb to

Fig. 5. Electron microscopy of the purified haemagglutinin of *H. pylori* strain 136292 and of the interactions of strain 136292 and its haemagglutinin with erythrocytes. (a) Negatively stained haemagglutinin sample of *H. pylori* strain 136292. Sample stained with silicotungstate. Bar, 100 nm. (b, c) Thin section view of interaction between haemagglutinin sample of *H. pylori* strain 136292 and dog erythrocytes. (c) is a higher magnification view. Samples stained with uranyl acetate and lead citrate. Bars, 1 µm. (d, e) Thin section view of interaction between agar-grown bacterial cells of *H. pylori* strain 136292 and dog erythrocytes. (e) is a higher magnification view. Bars, 1 µm (d) and 100 nm (e).

formaldehyde-treated erythrocytes as revealed by the direct binding, elution and immunoblotting assay. However, of these two bands only the 25 kDa polypeptide antigen was present in the haemagglutinin sample which showed clear NeuAc-lactose (ex bovine colostrum) sensitive and neuraminidase-sensitive (by treatment of erythrocytes) HA activity. These findings strongly indicated that the 25 kDa polypeptide antigen of strain 136292 was a NeuAc-lactose-specific erythrocyte-binding antigen. This antigen most probably recognizes the NeuAc(α 2-3) moiety of the receptor as suggested by several studies (Evans *et al.*, 1988; Slomiany *et al.*, 1989; Robinson *et al.*, 1990; Saitoh *et al.*, 1991).

The 59 kDa polypeptide antigen proved to be another erythrocyte-binding antigen but of unknown receptor specificity. This was suggested both by the erythrocyte-binding test and by the Western blotting analysis of whole-cell protein and the purified haemagglutinin using the HAA, which reacted with both the 59 and 25 kDa antigens of the whole-cell sample but only with the 25 kDa antigen of haemagglutinin sample. This HAA was prepared by absorption of anti-whole-cell serum against agar-grown bacteria of strain 136292 with non-haemagglutinating broth-grown bacteria of the same strain. Thus, the HAA was supposed to recognize antigens that were associated with haemagglutination. It seems unlikely that the 25 kDa and the 59 kDa polypeptide antigens were associated as polypeptides of the same protein, not only because the 59 kDa polypeptide antigen was completely removed from the haemagglutinin sample of strain 136292, but also because the 59 kDa polypeptide antigen did not bind as well as the 25 kDa polypeptide antigen to goat erythrocytes in the case of strain 172460 (Fig. 3, lane 7). Failure to detect the 59 kDa polypeptide in the partially purified haemagglutinin may have been due to complete solubilization by NOG extraction, since any truly soluble material would not be obtained by the protocol used (Fig. 1).

These findings might explain why neuraminidase treatment and NeuAc-lactose addition did not strongly inhibit haemagglutination with whole bacterial cells but did with the haemagglutinin sample. Robinson *et al.* (1990) suggested that different haemagglutinins might be present on *H. pylori* cells and, thus, that more than one type of receptor might be involved in the ligand-receptor interaction. Indeed, the different affinities of intact and sonicated *H. pylori* cells for the glycolipid receptors GM₃ and galactosylceramide sulphate reinforces this view (Saitoh *et al.*, 1991). The present study agrees with this concept and provides evidence for the presence of more than one adhesin at a molecular level. It seems reasonable to speculate that the 25 kDa polypeptide recognises NeuAc(α 2-3) of ganglioside GM₃ and that

the 59 kDa polypeptide may be the ligand for galactosylceramide sulphate.

Strain 172460 only agglutinated goat erythrocytes. Its erythrocyte-binding antigen was found to cross-react with the 25 kDa polypeptide antigen of strain 136292, but had a slightly lower molecular mass than that of strain 136292. However, the specificity of the receptor on goat erythrocytes for the 24 kDa polypeptide antigen of strain 172460 is not known. Agglutination of goat erythrocytes by strain 172460 was not inhibited by NeuAc-lactose from human milk and bovine colostrum. Moreover, its 24 kDa polypeptide antigen did not absorb to dog erythrocytes as did the 25 kDa polypeptide antigen of strain 136292 (Fig. 3), suggesting that its receptor specificity is different. Interestingly, strain 184858 which did not agglutinate human, dog or goat erythrocytes also contained both 25 and 59 kDa polypeptide antigens, but neither was found to bind to the erythrocytes tested. These results indicate that the antigenic cross-reactivities belie HA activities.

Another possibility is that the 25 and 59 kDa polypeptide antigens may be two universal types of receptor-binding proteins for all or most of the strains of *H. pylori*. The 25 and 59 kDa polypeptides, respectively, from different strains may be serologically related, closely or distantly. In receptor-binding specificity, they may be different, at least *in vitro* as suggested by this study. Uropathogenic *Escherichia coli* can express serologically identical fimbriae with different receptor-binding specificities (Lund *et al.*, 1988). By analogy, it is possible that the 25 kDa polypeptides of different *H. pylori* strains may differ in structure, reflected by different molecular masses and serological reactivity, and thus in the extent to which their epitope recognition sites can bind to NeuAc(α 2-3), thereby imparting different properties in binding and HA assays.

In the studies of Fauchère and coworkers (Fauchère *et al.*, 1989; Fauchère & Blaser, 1990), some easily extractable adherent material [designated superficial adhering material (SAM)] of *H. pylori* bound to HeLa cell membranes. This binding was not inhibited by addition of NeuAc or by pretreatment of HeLa cell membranes with *C. perfringens* neuraminidase, and a major antigen of approximately 60 kDa was present in the chromatography fraction exhibiting the maximum adherence activity. From the property of the binding and the molecular mass of the antigen reported, this SAM antigen seems similar to the 59 kDa antigen described in this study. However, Fauchère and coworkers seemed unsure whether the 60 kDa antigen was the receptor-binding antigen or was copurified with urease, as the SAM-derived antigen was recognized by an anti-urease serum tested by them.

The haemagglutinin was extracted from *H. pylori* in such a way as to retain the ability to agglutinate erythrocytes directly. Electron microscopy demonstrated vesicular material, the surfaces of which attached to the surfaces of erythrocytes. A similar attachment was seen between the bacteria and the erythrocytes. However, the morphological nature of the haemagglutinin itself was not revealed. Evans *et al.* (1988) reported that the *H. pylori* haemagglutinin was fibrillar but no molecular analysis was given, making comparisons with the present material difficult. However, on the basis of the electron micrographs presented by Evans *et al.* (1988), their fibrillar haemagglutinin may have been contaminated by LPS or the haemagglutinin may have been absorbed onto LPS vesicles which are morphologically similar by electron microscopy to the material described by these authors (Kuno *et al.*, 1986).

The relationship, if any, between the 25 kDa polypeptide antigen and other polypeptide bands, particularly the 16 kDa polypeptide, seen in SDS-PAGE gels is not certain. Although both the 16 and 25 kDa polypeptides reacted with both the HAA and the HSA, it is unclear whether the 16 kDa polypeptide antigen in the purified haemagglutinin sample was a breakdown product of the 25 kDa polypeptide. Monoclonal or polyclonal antibodies specific to the 25 kDa polypeptide or the 16 kDa polypeptide would be required to make this distinction. However, given that the purified haemagglutinin was prepared in the presence of protease inhibitors, it seems unlikely that the 16 kDa polypeptide is related to the 25 kDa polypeptide as a breakdown product. The role, if any, of the haemagglutinin antigens described herein in adherence of *H. pylori* to antral or duodenal epithelium remains to be established.

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