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N-Acetylneuraminyllactose-Binding Fibrillar Hemagglutinin of *Campylobacter pylori*: a Putative Colonization Factor Antigen

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***Campylobacter pylori* is the causative agent of gastritis and possibly of peptic and duodenal ulcers in adults. Histological observations show *C. pylori* attached to gastric epithelium as well as in the mucus layer of the stomach. We found that clinical isolates of *C. pylori* possess a cell-bound hemagglutinin detectable with human erythrocytes (all phenotypes tested) and those of a variety of animal species. The *C. pylori* hemagglutinin is antigenic, heat sensitive, and destroyed by pronase and papain but resistant to pepsin and trypsin. The hemagglutinin has fibrillar morphology; *C. pylori*-erythrocyte interaction displays very intimate contact, which is typical of fibrillae-mediated attachment. Fibrillae were removed from *C. pylori* by solubilization with *N*-octylglucose. After partial purification and removal of *N*-octylglucose by dialysis, the protein reaggregated, with the assembly of fibrillar structures. Hemagglutination inhibition was observed with the sialoproteins fetuin, α_2-macroglobulin, and glycophorin A but not with asialofetuin or asialoglycophorin A. The erythrocyte receptor was more sensitive to destruction by a neuraminidase specific for the *N*-acetylneuraminyl-α(2-3)-galactopyranosyl [NeuAc(2-3)Gal] sequence than one specific for NeuAc(2-6)Gal. Hemagglutination-inhibition assays with *N*-acetylneuraminyl-α(2-3)-lactose [NeuAc(2-3)-lactose] and NeuAc(2-6)-lactose confirmed that the *C. pylori* hemagglutinin preferentially binds to the NeuAc(2-3)Gal isomer of NeuAc-lactose. Based upon the above-described properties of the *C. pylori* fibrillar hemagglutinin, we conclude that this antigen should be designated as a putative colonization factor antigen.**

The relationship between colonization of the stomach by *Campylobacter pylori*, formerly *Campylobacter pyloridis*, and occurrence of chronic gastritis has been well established since the first successful isolation of this organism was reported in 1983 (12, 14, 28, 30, 36, 40, 42). Currently, the role of *C. pylori* in peptic and duodenal ulcer disease is the subject of intense investigation (3, 6, 12, 18, 28, 36, 40). Although this spiral-shaped motile bacterium is well adapted to the mucus environment (14), *C. pylori* also adheres to the epithelial cell surface of the gastric mucosa. Only gastric-type epithelium is colonized, and the organism preferentially attaches at or near intercellular junctions (14, 28, 36). Postattachment changes in epithelial cell morphology, including pedestal formation of the type seen with enteropathogenic *Escherichia coli* on intestinal epithelium (24, 39), are also observed (14, 36).

Tissue- and cell-specific attachment of bacterial pathogens is a key aspect of the host-parasite relationship, and thus the above-cited observations indicate that the basic mechanism of colonization by *C. pylori* is not unique although its target site, gastric-type epithelium, is unusual. Specific target-cell attachment by bacterial pathogens is mediated by specific structures which radiate from the bacterial cell surface. Morphologically, three different types of adhesive structures have been observed. These include, first, fimbriae, or pili, which are long and usually rigid hairlike structures. There are many examples of these, including the colonization factor antigens (CFAs) of enterotoxigenic *E. coli* (2, 8, 15, 23), the digalactoside-binding fimbriae of uropathogenic *E. coli* (19), the attachment pili of *Neisseria gonorrhoeae* (4, 35), the fimbrial hemagglutinin of *Bordetella pertussis* (1), *Pseudomonas aeruginosa* pili (45), and fimbriae of *Vibrio cholerae* O1 (7) and non-O1 (16) serotypes.

A second structural variety of bacterial adhesin, the fibrillae, are like fimbriae but smaller in diameter, much less rigid or curly, and usually very numerous, giving the impression of a fine network or capsule; examples of these are the fibrillar component of CFA/II of the enterotoxigenic *E. coli* (23) and other fibrillar *E. coli* adhesins (15, 17, 38), the lipoteichoic acid-M protein fibrillae of *Streptococcus pyogenes* (2), and the virulence-associated fibrillae of *Yersinia enterocolitica* (20). A third variety of bacterial adhesin is beyond resolution of the electron microscope and is therefore termed afimbrial or nonfimbrial (11, 26, 30, 33, 37).

Besides structural similarities, bacterial adhesins, or CFAs, also share the properties of (i) moderate to strong hydrophobicity (9, 10, 27, 41) and (ii) the ability of recognize and attach to specific receptors (2, 19, 37). Collectively, these properties explain the fact that most bacterial CFAs are hemagglutinins (2, 9). Hemagglutination (HA) may appear to be of broad specificity (i.e., HA with erythrocytes from numerous different animal species), as in the case of the mannose-binding enterobacterial common fimbriae (9, 32), owing to the widespread occurrence of the receptor structure. Other bacterial adhesins display HA activity with only one or a few species of erythrocyte, owing to more limited occurrence of their receptors (9).

We postulated that the interaction of *C. pylori* with gastric-type epithelium is mediated by a CFA detectable as a hemagglutinin. We report here that *C. pylori* isolates possess a fibrillar surface antigen which is a hemagglutinin and that an essential feature of the erythrocyte receptor recognized by this adhesin is the NeuAc(2-3)Gal component of NeuAc-lactose (for abbreviations, see below).

MATERIALS AND METHODS

Abbreviations. The following abbreviations are used in this paper: NeuAc-lactose, *N*-acetylneuraminyl-lactose; NeuAc

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(2-3)Gal(1-4)Glu, *N*-acetylneuraminyl-α(2-3)-galactopyranosyl-β(1-4)-glycopyranose isomer of NeuAc-lactose; NeuAc(2-6)Gal(1-4)Glu, *N*-acetylneuraminyl-α(2-6)-galactopyranosyl-β(1-4)-glycopyranose isomer of NeuAc-lactose; NeuAc(2-3)Gal, *N*-acetylneuraminyl-α(2-3)-galactopyranosyl sequence; NeuAc(2-6)Gal, *N*-acetylneuraminyl-α(2-6)-galactopyranosyl sequence.

Reagents and chemicals. Bovine serum albumin (BSA), *N*-octylglucose (NOG), *N*-acetylneuraminic acid, NeuAc-lactose from bovine colostrum [mixture of NeuAc(2-3)Gal(1-4)Glu, 85%, and NeuAc(2-6)Gal(1-4)Glu, 15%], NeuAc-lactose from human milk [mixture of NeuAc(2-3)Gal(1-4)Glu, 15%, and NeuAc(2-6)Gal(1-4)Glu, 85%], *N*-acetyl-D-galactosamine, thiodigalactoside, fetuin, asialofetuin, human glycophorin A, asialoglycophorin A, human α_2-macroglobulin, trypsin, papain, pepsin A, pronase, and *Clostridium perfringens* neuraminidase were obtained from Sigma Chemical Co., St. Louis, Mo. Neuraminidase from *Arthrobacter ureafaciens* was obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Bovine, horse, and sheep erythrocytes were obtained from Bethyl Laboratories, Montgomery, Tex. African green monkey erythrocytes were obtained from Charles River Primates, Port Washington, N.Y.; adult chicken erythrocytes were purchased from Texas Animal Specialties, Humble, Tex.

Human erythrocytes of defined antigenicity were obtained from Gamma Biologicals, Houston, Tex. These included null erythrocytes representative of the major high-frequency antigens, A, B, H, D, C, E, c, e, Le^a, Le^b, K, k, Fy^a, Fy^b, Jk^a, Jk^b, Do^a, Do^b, Lu^b, M, N, S, s, P1, Xg^a, Vel, Lan, Kn^a, Er^a, Rg^a, Ch^a, McM^a, JMH−, Jo^a, Hy, In^b, Gil, Di^b, and RAF, and also the following null phenotypes representing low-frequency antigens: O^h, I−, En^{a-}, U−, Tj(a−), Rh^{null}, Le(a−b−), K^o, Fy(a−b−), Jk(a−b−), Ge(−2−3), Lu(a−b−), Co(a−b−), and IFC−. Cord erythrocytes were also tested, as well as erythrocytes positive for the following low-frequency antigens: Go(a), Rd, Wr(a), Mit, Rd, Sw(a), and Miltenberger I and II.

Bacterial cultures and culture conditions. Strains of *C. pylori* were isolated from antral stomach biopsies as previously described (13). Stock cultures were prepared, from blood-agar plates, in Campylobacter Albimi Cysteine medium (Remel, Lenexa, Kans.) containing 20% (vol/vol) glycerol and maintained at −70°C. Blood-agar medium consisted of brain heart infusion (Difco Laboratories, Detroit, Mich.), 1.5% agar, and 7% whole horse blood no older than 10 days. For large-scale production *C. pylori* was grown on blood-agar plates at 37°C for 48 to 72 h in an atmosphere of 12% CO_2 and 98% humidity. Broth cultures were grown, without shaking, under similar atmospheric conditions for 24 to 48 h in a medium consisting of brain heart infusion broth (Difco) plus 10% horse serum, 0.05% yeast extract (Difco), and 0.04% rabbit hemoglobin (crystalline, from Sigma Chemical Co.). Cells were harvested by centrifugation at 17,000 × *g* for 10 min, washed twice with phosphate-buffered saline (PBS) (pH 7.3), and kept at 4°C until used.

HA assays. HA tests were performed on microscope slides at room temperature (RT), mixing equal volumes (usually 20 μl) of erythrocytes (2.0% in PBS) and *C. pylori* cells suspended at 5×10^7 cells per ml in PBS. HA-inhibition (HAI) tests were performed by mixing 10 μl of the bacterial cells with 10 μl of the test substance, followed 1 min later by 20 μl of either bovine or human erythrocytes. HA reactions were recorded as 4+, 3+, 2+, 1+, or negative 1 min after addition of erythrocytes.

ELISA for quantitating fetuin-binding activity. The fetuin-binding hemagglutinin of *C. pylori* was determined by an enzyme-linked immunosorbent assay (ELISA) as follows. Wells of 96-well microtiter plates (Linbro Scientific Co., Hamden, Conn.) were coated with 100 μl per well of a 1.0-μg/ml solution of fetuin prepared in 0.05 M Tris chloride buffer (pH 8.0; Tris-8 buffer) containing 0.025% sodium azide as preservative. Plates were kept at 37°C in a humid chamber for 18 to 24 h. Excess plastic binding sites were blocked with 1.0% BSA in PBS after removal of fetuin and one wash with PBS. PBS plus 0.02% Tween-20 was used for subsequent washing steps. All reagents were applied at 100 μl per well. Samples were tested at 1:500 dilution in PBS unless otherwise specified. After 60 min at RT the wells were washed and an optimum dilution, usually 1:300, of rabbit anti-*C. pylori* was added. After 60 min at RT, wells were washed and an optimal dilution of alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (Southern Biotechnology, Birmingham, Ala.) was added. After 60 min at RT, wells were washed and alkaline phosphate substrate (Sigma Chemical Co.) was added. Optical density readings were obtained after 120 min at 37°C, employing a Flow Titertek Multiskan MC ELISA Reader. Rabbit serum and conjugate reagent were diluted in PBS–0.02% Tween-20 containing 0.1% BSA.

Treatment of *C. pylori* cells with proteinase enzymes. Blood-agar-grown *C. pylori* was harvested after 48 h of incubation and washed once with PBS by centrifugation at 7,500 × *g* for 10 min. The bacterial pellet was suspended in 0.1 M acetate buffer (pH 3.0) to approximately 10^9 bacteria per ml. A mixture of 0.5 ml of pepsin A (110 U/ml in the acetate buffer) and 0.5 ml of cell suspension was incubated for 1 h at 37°C. The bacteria were then washed once with PBS to adjust the pH to 7.3 and then assayed for HA activity.

Similar procedures were used to test the effect of trypsin (123 U/ml in 0.1 M phosphate buffer, pH 7.4), protease (27 U/ml in 0.1 M phosphate buffer, pH 7.6), and papain (26 U/ml in 0.1 M phosphate buffer, pH 6.2). Controls consisted of bacterial cells treated with the appropriate buffer, minus enzyme, and erythrocytes treated with the indicated enzymes.

Treatment of erythrocytes with neuraminidase. Neuraminidase sensitivity of the erythrocyte receptor for the *C. pylori* hemagglutinin was tested by pretreating (at 37°C for 10 min) a 2% suspension of human or bovine erythrocytes mixed with equal volumes of twofold dilutions of neuraminidase (starting at 4.0 U/ml in PBS) of either *C. perfringens* or *A. ureafaciens*. Erythrocytes were then twice washed in PBS and tested for HA with blood-agar-grown *C. pylori*.

Preparation of rabbit immune sera. In early experiments, the anti-*C. pylori* rabbit serum used for the ELISA was obtained from animals hyperimmunized with whole formalinized bacteria. Later experiments employed monospecific antihemagglutinin serum, which gave the same results. Monospecific rabbit anti-fetuin-binding hemagglutinin was prepared by hyperimmunizing rabbits with NOG-extracted, column-purified, and dialysis-precipitated protein. First the antigen was administered subcutaneously in Freund complete adjuvant; six booster injections were given at 4-day intervals by the intramuscular route. Rabbits were bled 15 days after the last booster dose. Antibody titers were determined by the fetuin ELISA described above. Monospecific polyclonal serum was prepared by adsorbing the hymperimmune serum with packed cell pellets of *C. pylori* 8826 (a representative HA-positive strain of *C. pylori*) which had been grown in broth; these cells were HA negative. One milliliter of packed cells was suspended in 4 ml of the serum,

and the mixture was incubated for 1 h at 37°C. After centrifugation at 17,000 × *g* for 15 min, the serum was transferred to a tube containing a similar cell pellet, and the procedure was repeated until the adsorbed serum agglutinated plate-grown strain 8826 cells but not broth-grown 8826 cells. Rabbit serum prepared in this manner inhibited HA when mixed with *C. pylori* cells before addition of erythrocytes.

Immunolabeling of *C. pylori* cells for examination by electron microscopy. *C. pylori* cells obtained from 48-h blood-agar plate cultures were harvested and washed once with PBS. Cells were fixed with 1% glutaraldehyde (Sigma Chemical Co.) in PBS for 5 min at RT, recovered by centrifugation at 1,000 × *g* for 10 min, and suspended in PBS. A 15-μl portion of the cell suspension was placed on a carbon-stabilized nitrocellulose film copper specimen grid, and after 3 min the excess volume was removed with a filter paper. Approximately 15 μl each of the following reagents were added, in this order: PBS containing 2% BSA; monospecific polyclonal (adsorbed) antihemagglutinin rabbit serum; PBS containing 2% BSA; gold-labeled goat anti-rabbit immunoglobulin G (10 nm size; Sigma Chemical Co.) diluted as recommended by the manufacturer; and finally glass-distilled water. Grids were then air dried. Selected grids were negatively stained with 2% phosphotungstic acid (pH 4.5) and others were observed without staining, using a CM10 PW6020 Philips transmission electron microscope at 60 kV. Controls consisted of cells treated by the same procedure with preimmune rabbit serum and broth-grown (HA-negative) cells of strain 8826.

Electron microscopy of human erythrocytes agglutinated with *C. pylori*. Freshly prepared bacterial cells, obtained from blood-agar plate cultures as described above, were adjusted to 10^8/ml. Equal volumes of bacteria and freshly washed human erythrocytes (2% in PBS) were mixed on a clean microscope slide. The agglutinated erythrocytes were placed in a conical centrifuge tube, which was darkened with aluminum foil, and fixed overnight at 4°C with 3% glutaraldehyde buffered with 0.1 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] and 0.05% ruthenium red (pH 6.5). The pellet was then washed three times with 0.1 M PIPES buffer, and the pellet was suspended and kept for 40 min in a mixture consisting of equal parts of 4% osmium tetroxide, PIPES buffer, and 150 mg of ruthenium red per dl. The sample was embedded in Spear resin after dehydration with a graded series of ethanol. Sections (1 μm) were cut from the polymerized blocks with glass knives. Suitable sections were used to prepare 100-Å (10-nm) sections with a diamond knife. The sections were stained with uranyl acetate and lead citrate and then examined with a Jeol KEO 100C transmission electron microscope at 80 kV.

Electron microscopic examination of purified *C. pylori* fetuin-binding hemagglutinin. Protein obtained from the agarose A-1.5m column, eluted with Tris-8 buffer containing 0.5% NOG, was dialyzed to reaggregate the hemagglutinin. The precipitate was collected by centrifugation at 27,000 × *g* for 15 min and suspended in PBS to the original volume. Carbon-stabilized, nitrocellulose-film, copper specimen support grids were coated with a 1:10 dilution of the specimen, drained with filter paper after 2 min, air dried, and negatively stained with 2% phosphotungstic acid (pH 4.5).

Preparation of *C. pylori* homogenate. A thick suspension, approximately 6 ml of packed cells in 12 ml of PBS, was homogenized in a Sorvall Omni-Mixer at 4°C at maximum speed for 15 1-min intervals, alternated with 15 1-min intervals at rest. The cells were then removed by centrifugation at 27,000 × *g* for 15 min, and the clear supernatant was passed through an agarose A-0.5m column. Elution was with the Tris-8 buffer. Fractions containing the void-volume peak were pooled and examined by electron microscopy as follows. A sample was mixed with an equal volume of a 1:200 dilution of monospecific rabbit antihemagglutinin serum and incubated for 30 min at 37°C. The immunoprecipitate was then examined by transmission electron microscopy (TEM) using carbon-stabilized nitrocellulose grids and the negative staining procedure as indicated above.

Agglutination of erythrocytes with latex beads coated with *C. pylori* fetuin-binding hemagglutinin. Polystyrene latex beads (Difco Laboratories) measuring 0.81 to 1.77 μm were used. Two milliliters of the latex was suspended in 20 ml of distilled water, mixed, and filtered through a Whatman no. 40 filter paper. The filtrate was adjusted to an optical density at 640 nm of 2.0 in PBS. A mixture consisting of 0.1 ml of latex suspension, 5.0 ml of PBS, and 0.5 ml of *C. pylori* protein (1.5 mg/ml) was incubated at 37°C for 30 min. The beads were then washed twice with 10 volumes of PBS, and the suspension was adjusted to an optical density of 0.3 in PBS containing 0.1% BSA. Agglutination tests were conducted by mixing equal volumes of coated latex beads and freshly washed bovine erythrocytes (2.0% in PBS).

RESULTS

HA of erythrocytes by *C. pylori*. *C. pylori* isolates from antral stomach biopsies of 10 different individuals were grown on blood-agar plates and tested for HA using erythrocytes from the following animal species: rabbit, guinea pig, adult chicken, horse, cow, sheep, and African green monkey. Erythrocytes of all of the animal species tested strongly hemagglutinated (4+ reactions) at RT with all of the *C. pylori* isolates. Whole human blood was negative for HA; however, 4+ reactions were seen after the erythrocytes were washed twice with PBS. All of the *C. pylori* strains grown in broth were HA negative.

Lack of correlation between human erythrocyte antigenicity and HA with C. pylori. Attempts were made to find a correlation between a particular human erythrocyte antigen and HA with *C. pylori*. First, HA tests were performed with fresh, washed erythrocytes obtained from approximately 50 different blood donors; all were positive. Second, a large collection of human erythrocytes was screened. These were chosen to include (i) erythrocytes lacking the known high-frequency antigens such as those in the ABO, Rh, Kel, Duffy, and Lewis blood groups; (ii) null phenotypes representing low-frequency antigens such as Gerbich, Vel, Rodgers, and Holly-Gregory; and (iii) erythrocytes positive for representative low-frequency antigens. (See Materials and Methods for a complete listing.) All of these different erythrocyte specimens were HA positive with the clinical isolates of *C. pylori*.

Preliminary characterization of the *C. pylori* hemagglutinin. It was found by titration that 0.6×10^7 to 1.0×10^7 *C. pylori* cells per ml was required to produce a 4+ HA reaction with either human or bovine erythrocytes. Suspensions of 5×10^7 bacterial cells per ml, which had been pretreated with trypsin, pepsin A, pronase, or papain separately were tested for HA with bovine and human erythrocytes. The *C. pylori* hemagglutinin was not destroyed by either trypsin or pepsin, but it was sensitive to both pronase and papain. Destruction of the HA activity by pronase and papain indicates the protein nature of the hemagglutinin. Similar proteinase treatments of the human and bovine erythrocytes had no effect on HA with untreated *C. pylori*.

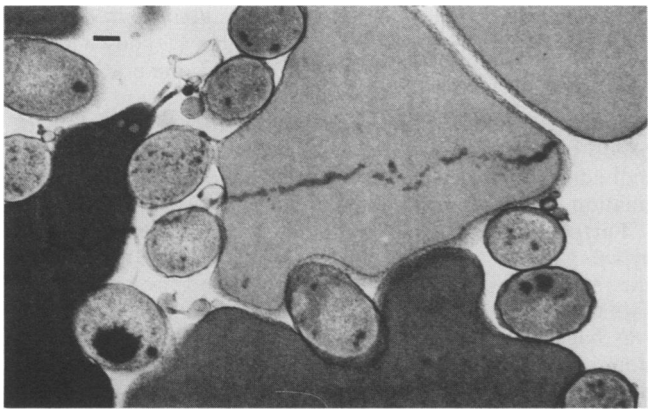

FIG. 1. Thin-section view of *C. pylori* cells interacting with human erythrocytes. Note intimate contact with indentation of the erythrocytes in contact with the bacteria. Bar, 0.1 μm.

Heat sensitivity of the *C. pylori* hemagglutinin was tested by incubating different samples of a *C. pylori* cell suspension at temperatures from 40 to 56°C, at 2°C increments, for 10 min. The cells produced 4+ HA after treatment at 48°C and 2+ HA after heating at 50°C and were HA negative after exposure to 52°C. Supernatant fluids obtained by centrifugation of the heat-treated bacteria (50, 52, and 54°C) did not produce HA. Supernatants of heat-treated (53°C, 10 min) *C. pylori* cells did not bind to fetuin, as shown by the ELISA method described below.

Bovine erythrocytes agglutinated with *C. pylori* cells were examined by electron microscopy in an attempt to visualize a bacterial structure responsible for HA. The bacterial cell-erythrocyte interaction was very close and not characteristic of an interaction mediated by fimbriae (Fig. 1 and 2). Interestingly, the indentation of the erythrocyte surface in contact with the *C. pylori* cell (Fig. 1) was quite similar to that seen in photomicrographs of this organism interacting with stomach mucosal cells (14, 36).

Evidence for the role of sialic acid(s) in HA of erythrocytes by *C. pylori*. Pretreatment of human or bovine erythrocytes with *C. perfringens* neuraminidase (0.0156 U/ml), which is specific for NeuAc(2-3)Gal(1-4)Glu, destroyed the agglutinability of the erythrocytes by *C. pylori*. Also a neuraminidase from *A. ureafaciens*, which preferentially cleaves NeuAc(2-6)Gal(1-4)Glu, destroyed the agglutinability of the erythrocytes, but at a higher concentration, 0.188 U/ml. These results suggested to us that the receptor on the erythrocytes is a sialoprotein containing the NeuAc(2-3)-Gal isomeric form of NeuAc-lactose.

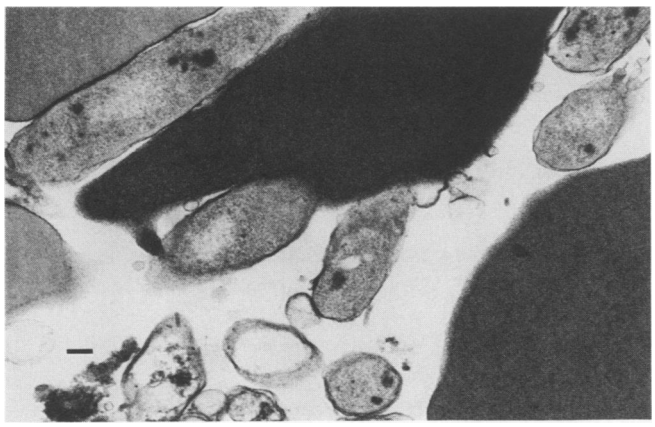

FIG. 2. Thin-section view of *C. pylori* cells interacting with human erythrocytes. Note contact along length of the bacteria. Bar, 0.1 μm.

TABLE 1. HA of bovine erythrocytes by *C. pylori* and inhibition of HA by NeuAc-lactose and by sialoproteins

Test substance	Concn (mg/ml) producing 100% HAI
NeuAc-lactose, bovine	0.078
NeuAc-lactose, human	>1.000
N-Acetylneuraminic acid	>1.000[a]
N-Acetyl-D-galactosamine	>1.000
Thiodigalactoside	>1.000
Mannose	>1.000
Fetuin	0.500
Asialofetuin	>1.000
Glycophorin A	0.250
Asialoglycophorin A	>1.000
α_2-Macroglobulin	1.000

[a] Agglutination of bacteria, but no HAI.

This contention was further supported by data obtained when several different sialic acids and sialoproteins were tested for HAI. Complete (100%) HAI was shown with 0.078 mg of bovine NeuAc-lactose, consisting primarily of the NeuAc(2-3)Gal isomer, per ml, whereas no inhibition was seen, even at 1.0 mg/ml, with NeuAc-lactose from human milk, which consists primarily of the NeuAc(2-6)Gal isomer (Table 1). Note also that sialoproteins which contain the NeuAc(2-3)Gal isomer of NeuAc-lactose, i.e., human erythrocyte glycophorin A, fetuin, and human α_2-macroglobulin, also inhibited HA of erythrocytes by *C. pylori*. HA in the presence of fetuin at 0.25 mg/ml was minimal (2+). The corresponding asialoproteins did not produce HAI. These results strongly indicate that the receptor on the erythrocytes is a sialoprotein containing NeuAc(2-3)Gal.

Solubilization of the *C. pylori* hemagglutinin by NOG. In preliminary experiments it was found that *C. pylori* cells exposed to 1% NOG became HA negative. The following experiment was carried out to determine a concentration of NOG for obtaining the fetuin-binding hemagglutinin in soluble form. *C. pylori* cells from 21 blood-agar plates were washed with PBS, and each of five identical pellets was suspended in PBS containing 0.0, 0.1, 0.2, 0.4, or 0.8% NOG, respectively. Cells were recovered by centrifugation at 17,000 × *g* for 12 min after 20 min at RT, washed twice in PBS, and then suspended in PBS to the original volume (3.0 ml). The NOG-containing supernatants (extracts) were dialyzed for 18 h against PBS at 4°C and then centrifuged at 27,000 × *g* for 15 min. The resultant pellets were suspended in 3.0 ml of PBS.

The pretreated bacterial cells, the dialyzed supernatants, and the recovered precipitates were assayed by the fetuin ELISA technique (Table 2). Note that the bacteria exposed to 0.4 and 0.8% NOG were HA negative and also that, after extraction with 0.4% NOG, most of the fetuin-binding activity remained in the supernatant fraction after dialysis. Essentially the same results were obtained with 0.5% NOG, and this concentration was used in subsequent experiments.

Partial purification of fetuin-binding protein of *C. pylori* and comparison of product from plate-grown and broth-grown cells. *C. pylori* cells harvested from 16 blood-agar plates were extracted with 0.5% NOG, and the extract was brought to 90% saturation with ammonium sulfate. After centrifuga-

TABLE 2. Effect of various NOG concentrations on HA activity of *C. pylori* and on solubilization of fetuin-binding antigen(s) as determined by an ELISA

% NOG used for extraction	HA of bovine erythrocytes after treatment	Antigen titer[a]		
		Cells	Supernatant	Precipitate
0.00	+	1:1,600		
0.10	+	1:1,600	<1:200	<1:200
0.20	+	1:800	1:200	<1:200
0.40	−	1:400	1:3,200	1:200
0.80	−	1:200	1:1,600	1:800

[a] NOG-extracted cells were washed and suspended in 3.0 ml of PBS; NOG extracts (3.0 ml each) were dialyzed against PBS, and the resultant precipitate, if any, was collected by centrifugation and suspended in 3.0 ml of PBS. Titer was determined by ELISA and was defined as the highest dilution giving an optical density of ≥0.100 at a wavelength of 510 nm.

tion, the precipitate was dissolved in a minimum volume of Tris-8 buffer containing 0.025% sodium azide. After dialysis against this buffer, 0.5% NOG was added and the sample (2.5 ml) was applied to an agarose A-1.5m column. Elution was carried out with the same Tris-8 buffer containing 0.5% NOG (Fig. 3). The fetuin-binding activity eluted as a single broad peak. For comparison of molecular weight, *C. pylori* urease (600,000) eluted at fraction 34, yeast alcohol dehydrogenase (150,000) eluted at fraction 42, and purified BSA (66,000) eluted at fraction 45.

Since broth-grown *C. pylori* cells did not hemagglutinate erythrocytes, the following experiment was designed to examine such cells for the fetuin-binding protein. Broth-grown cells, approximately equal in packed cell volume to that used in the above experiment, were extracted with 0.5% NOG and processed as described above, and the final product was chromatographed as described above using the same agarose A-1.5m column (Fig. 4). Note that none of the proteins which eluted from this column had fetuin-binding activity. This indicates that the protein peak seen in Fig. 3, detected by its fetuin-binding activity, is the *C. pylori* hemagglutinin.

Agglutination of bovine erythrocytes by latex beads coated with cell-free *C. pylori* hemagglutinin. Latex beads were coated with the fetuin-binding protein eluted from the agarose A-1.5m column (pool of fractions 39 to 47) shown in Fig. 3. Coated beads readily attached to the bovine erythrocytes and also caused the erythrocytes to agglutinate (Fig. 5). When the coated latex beads were preexposed to fetuin, the beads had a tendency to aggregate, with a relatively few individual beads attaching to the erythrocytes, but no agglutination of the erythrocytes was seen (Fig. 6).

Further purification of the fetuin-binding protein of *C. pylori*. Agarose column fractions (39 to 47, Fig. 3) containing the fetuin-binding activity were pooled and brought to 90% saturation with ammonium sulfate. The resultant precipitate was back-extracted by suspension in a 45% saturated solution of ammonium sulfate, and the resultant pellet was discarded. The supernatant contained approximately one-third of the original protein; this was reprecipitated by 90% saturation with ammonium sulfate and suspended in a minimum volume of PBS. Upon dialysis against PBS a light, flocculant precipitate formed. This precipitate was recovered by high-speed centrifugation, and the remaining supernatant was applied to an agarose A-1.5m column after addition of 0.5% NOG, eluting with the Tris-8 buffer containing 0.5% NOG (Fig. 7). Note the two peaks of fetuin-binding activity, one centered at fractions 50 to 51 and the other at fraction 45 (Fig. 7). Note that the protein eluting at fraction 45 had greater fetuin-binding activity than the smaller species eluting at fractions 50 to 51. Dialysis of the protein in either of these peaks (Fig. 7) in PBS to eliminate the NOG resulted in formation of a flocculant precipitate consisting of reaggregated hemagglutinin.

Electron microscopy of reaggregated *C. pylori* protein possessing fetuin-binding activity. As noted above, the *C. pylori* protein possessing fetuin-binding activity readily precipitated upon dialysis against PBS. An experiment was performed in which a sample of the flocculant precipitate was removed after 4 h of dialysis and prepared for observation by TEM; another sample was similarly examined after 24 h of dialysis (Fig. 8 and 9). After 4 h of dialysis, the protein appeared as an aggregate of long, parallel, flexible, intertwining structures of 2 to 4 nm in diameter. After 24 h of dialysis, the aggregated protein had the structure of closely packed,

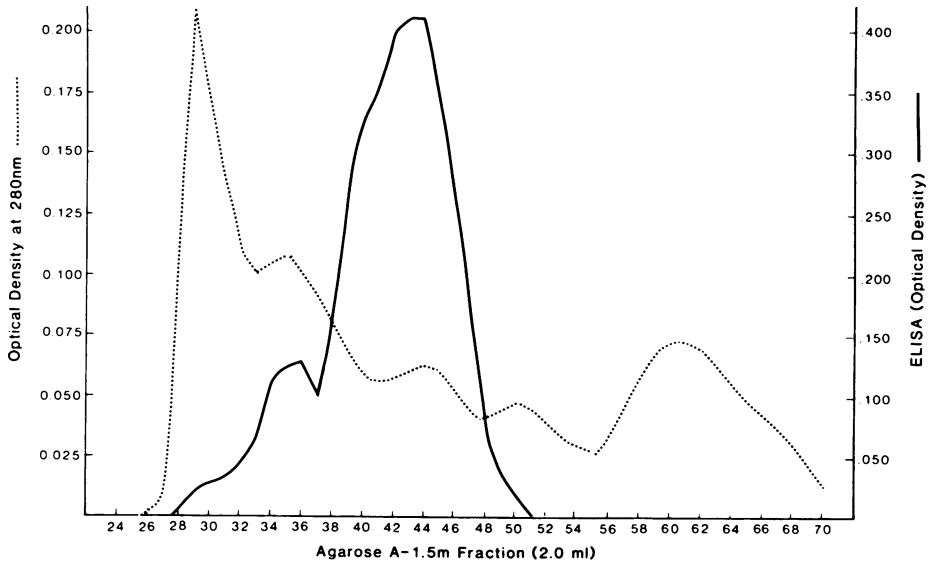

FIG. 3. Agarose chromatography of solubilized *C. pylori* hemagglutinin, detected as fetuin-binding antigen in an ELISA. The applied sample was a 0.5% NOG extract of cells grown on blood-agar plates; elution buffer contained 0.5% NOG.

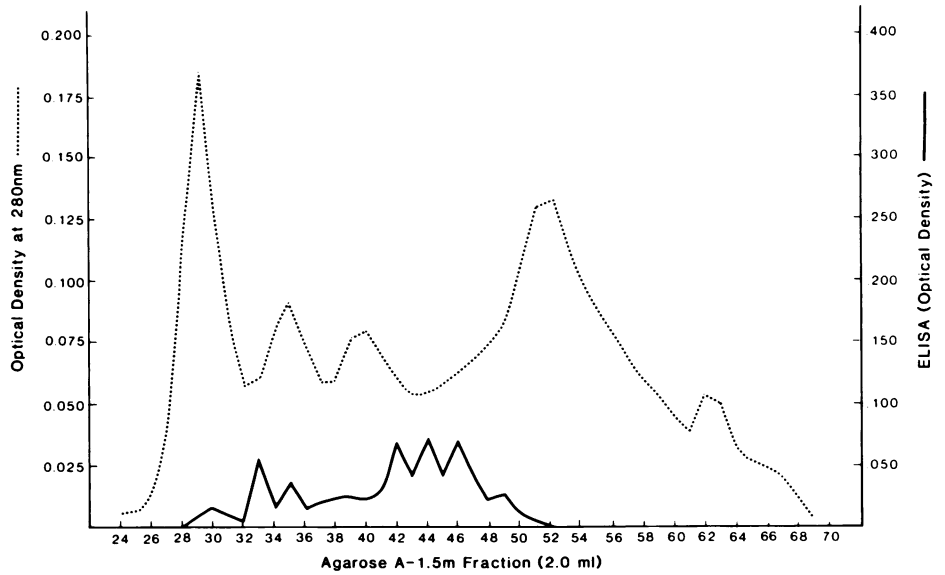

FIG. 4. Chromatography of NOG extract of *C. pylori* cells, as in Fig. 3 except that the bacteria were grown in broth. Note absence of large peak of fetuin-binding activity.

parallel, flexible, 2-nm-diameter structures resembling fibrillae. These results indicate that dilution or removal of NOG by dialysis produces various stages of reassembly of the fibrillar hemagglutinin. The small aggregates which chromatographed on agarose A-1.5m in the presence of 0.5% NOG are detectable as fetuin-binding antigen.

Electron microscopy of *C. pylori* cells and cell-free homogenate. The following experiment was performed to ascertain whether the fetuin-binding protein extracted with 0.5% NOG represents the native hemagglutinin molecule. Cell-free homogenates were obtained by blending thick suspensions of *C. pylori* cells. A void volume fraction obtained by agarose A-0.5m column chromatography, containing the fetuin-binding activity, was visualized by TEM after treatment with the specific rabbit antihemagglutinin serum. Flexible fibrillar structures of approximately 2 nm in diameter were seen, and these structures were similar to those seen when the hemagglutinin was reaggregated after dissociation by 0.5% NOG. This crude preparation also contained flagella, but these structures were not aggregated by the antihemagglutinin antibody.

The immunogold method was employed, using the specific antihemagglutinin serum, to locate the hemagglutinin on the surface of agar-grown *C. pylori* cells (Fig. 10 and 11). Note

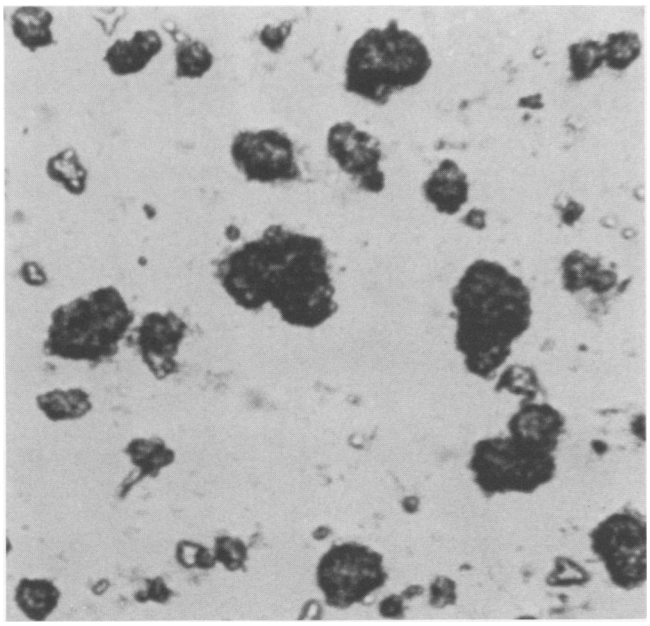

FIG. 5. Mixture of erythrocytes and latex beads (size, 0.81 to 1.77 μm) coated with a protein preparation possessing fetuin-binding activity. Note agglutination of the erythrocytes.

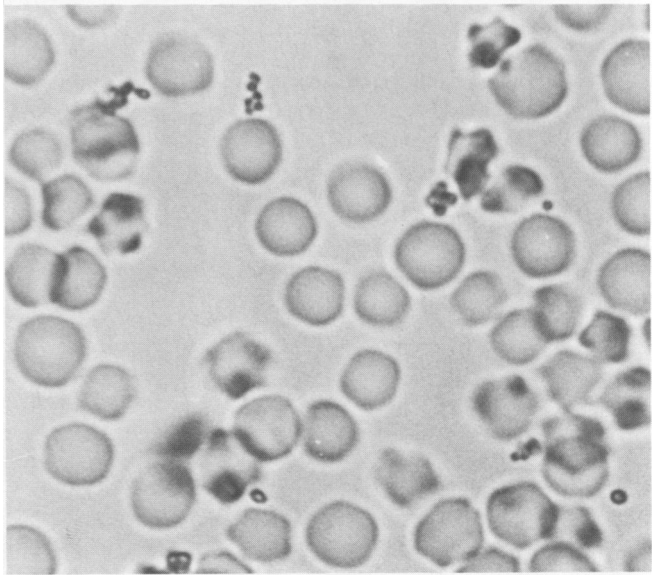

FIG. 6. Mixture of erythrocytes and latex beads coated with protein possessing fetuin-binding activity, as in Fig. 5, except that the coated beads were preexposed to fetuin. Note aggregation of beads with few beads bound to erythrocytes; also note that the erythrocytes are not agglutinated.

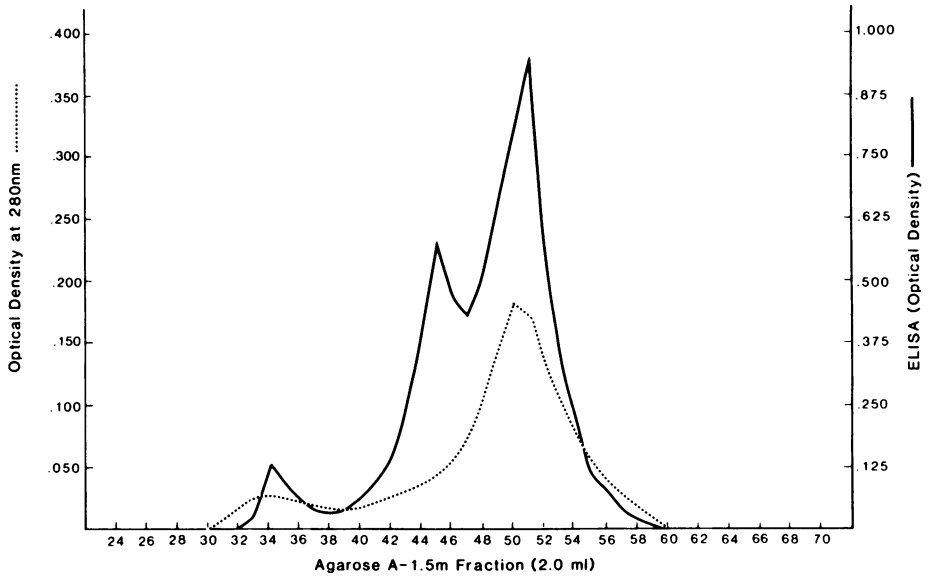

FIG. 7. Agarose chromatography of fetuin-binding protein (*C. pylori* hemagglutinin) after further purification by ammonium sulfate back-extraction. The applied sample was a supernatant, cleared by centrifugation, after dialysis against PBS. Elution buffer contained 0.5% NOG. Note that this preparation contains a peak of fetuin-binding activity (fraction 50 to 51) not seen with the crude preparation.

(Fig. 10) that the antibody reacted with a layer of material surrounding the bacterial cells; the fibrillar structure was not visible at this magnification. Also, immunogold-labeled antibody may be seen reacting with clumps of the antigen separated from the cells in this preparation, but not with the flagella. Control cells, i.e., cells pretreated with pronase to destroy the hemagglutinin, did not bind the immuno-label (not shown here). Figure 11 shows an isolated spiral-shaped cell completely surrounded by a relatively thick layer of the antigen.

DISCUSSION

Colonization of the stomach by *C. pylori* is an important factor in chronic gastritis, and there is evidence that this organism also contributes to the development of peptic and

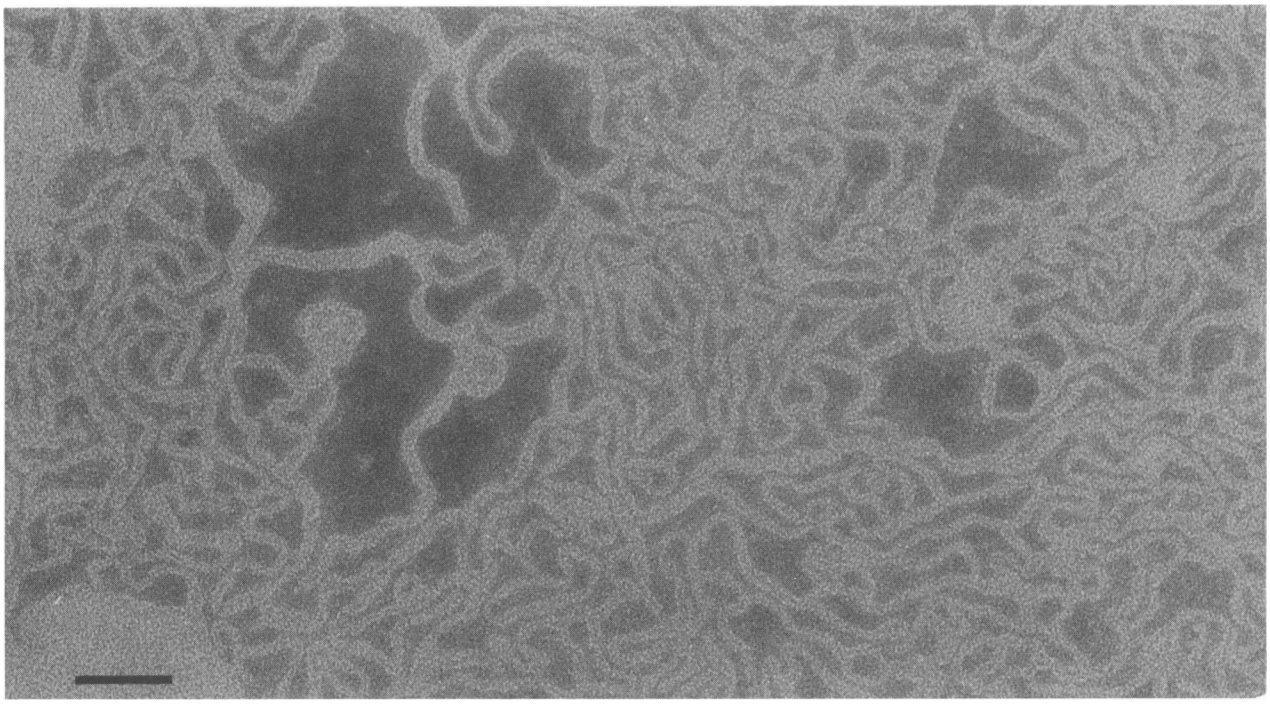

FIG. 8. Electron micrograph of precipitated fetuin-binding protein obtained after 4 h of dialysis against PBS. This protein is equivalent to that eluting at fraction 45 in Fig. 7. The width of the flexible structures is from two to four times that of the structures seen after 24 h of dialysis (shown in Fig. 9). Bar, 50 nm.

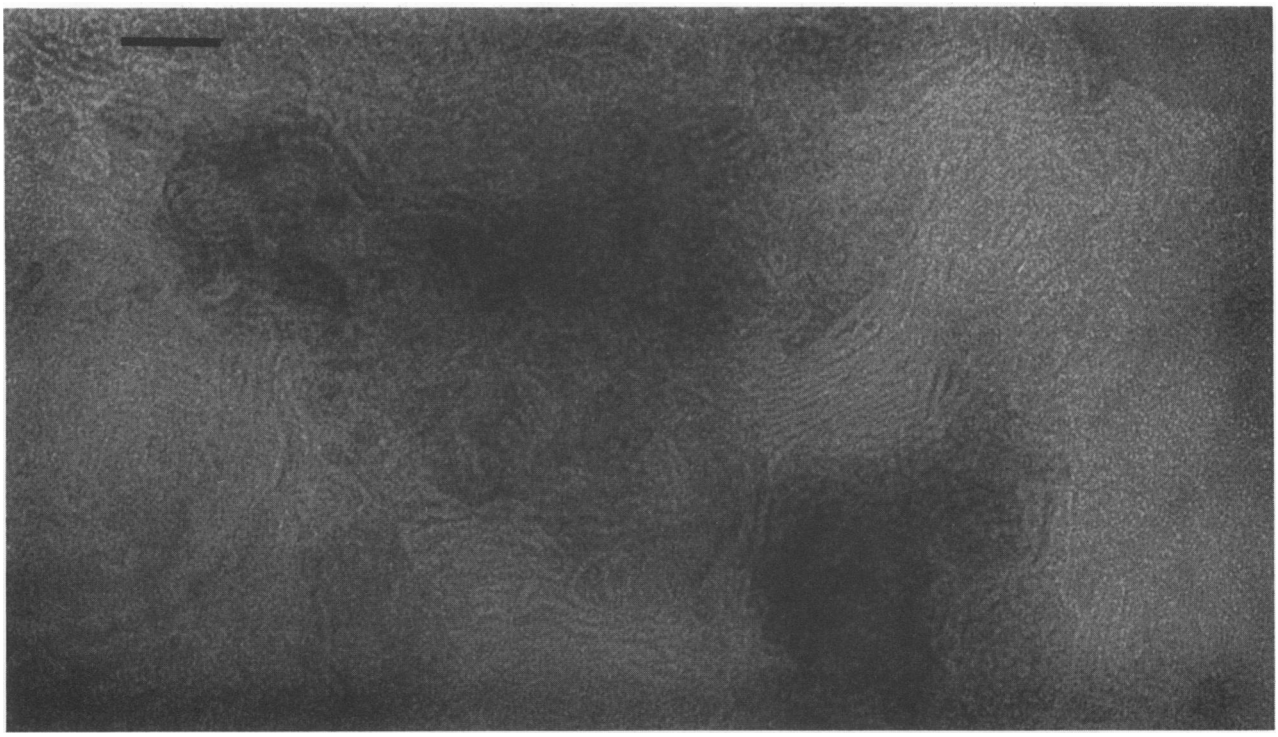

FIG. 9. Electron micrograph of precipitated fetuin-binding protein; sample is the same as that shown in Fig. 8 except for the duration of dialysis, 24 h. Note the linear, flexible structures of approximately 2-nm width. These structures are interpreted to be reassembled fibrillae. Bar, 50 nm.

duodenal ulcers (3, 5, 12, 14, 18, 30, 36, 40). *C. pylori* resides within the mucus layer and also appears to be chemotactically attracted to epithelial crypts and to intracellular junctions, where organisms can be seen attached to host cells (5, 14, 28, 36). *C. pylori* adheres very closely to epithelial cells, frequently resulting in pedestal formation, which reveals that the organism changes the morphology of its host cell (14, 36). Numerous bacterial pathogens which colonize epithelial cells are known to possess surface-associated antigens, termed adhesins or CFAs, which facilitate colonization of the host (2, 4, 7, 9, 15, 20, 23). These adhesins are produced in vitro when grown under appropriate culture conditions and are characteristically detectable as hemagglutinins since erythrocyte sialoglycoproteins include many of the sialoglycoconjugates found on epithelial cell surfaces. We found that clinical isolates of *C. pylori* possess strong hemagglutinating activity and that HA of human erythrocytes is independent of erythrocyte phenotype. Also, every species of erythrocyte tested produced HA with *C. pylori*. Examination of *C. pylori*-erythrocyte agglutinates by TEM revealed very close contact between the bacterial cells and erythrocytes, with apparent deformation of the latter. This indicated to us that the *C. pylori* hemagglutinin may be a nonfimbrial CFA since attachment mediated by fimbriae produces a distinctively different spatial arrangement (15, 22–24). Interestingly, enteropathogenic *E. coli*, which attach very closely to intestinal epithelial cells and which also cause pedestal formation, do so via a fibrillar adhesin (15, 24).

Sensitivity of the erythrocyte receptor for the *C. pylori* hemagglutinin to *C. perfringens* neuraminidase, but not to proteinases, provided evidence that this receptor is a sialic acid. Furthermore, the receptor proved to be relatively insensitive to *A. ureafaciens* neuraminidase, indicating that the *C. pylori* hemagglutinin binds to the NeuAc(2-3)Gal isomer of NeuAc-lactose rather than to the NeuAc(2-6)Gal isomer of NeuAc-lactose. HAI assays using NeuAc-lactose from two different sources confirmed that only the bovine compound, consisting primarily of the NeuAc(2-3)Gal isomer, blocks HA at nanogram concentrations. One feature of the NeuAc(2-3)Gal structure is its widespread occurrence in sialoglycoproteins, including serum proteins such as fetuin and α_2-macroglobulin, and sialic acid-containing erythrocyte antigens such as those on human glycophorin A. These three proteins, but not asialofetuin or asialoglycophorin A, also blocked HA by the *C. pylori* hemagglutinin.

The presence of receptor-containing sialoglycoproteins in human serum could explain the observation that washed erythrocytes, but not those in whole blood, readily agglutinate with *C. pylori*. We have not attempted to identify such serum proteins, but we have demonstrated that this effect is probably not due to antihemagglutinin antibody because similar results occur with blood from donors both with and without antihemagglutinin antibody.

It is remarkable that the receptor specificity of the *C. pylori* hemagglutinin places this hemagglutinin in the same class as the S, or sialylgalactoside-binding, fimbriae of uropathogenic *E. coli* and *E. coli* associated with neonatal meningitis (21, 25, 29, 34). Furthermore, Smit et al. (38) have identified the same isomeric form of NeuAc-lactose as the equine erythrocyte receptor for the K99 fimbrial adhesin of enterotoxigenic *E. coli*. The basis for this coincidence remains to be explained but should become more evident as biochemical structures of the target epithelial cell receptors for these various CFAs are elucidated in finer detail.

Bacterial fimbriae and fibrillae vary considerably with respect to their susceptibility to disaggregation, or solubilization, with reagents such as sodium dodecyl sulfate, Triton, urea, and NOG (21, 31, 43). Many of these structures have

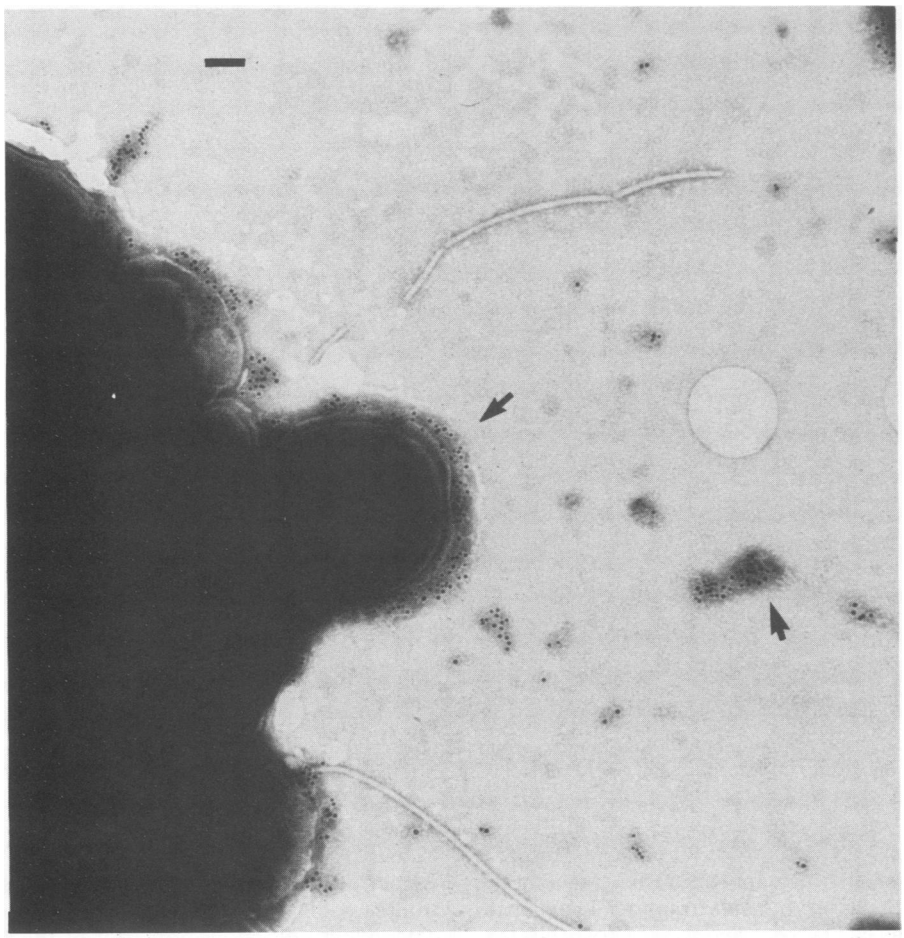

FIG. 10. Electron micrograph of *C. pylori* cells stained by the immunogold method employing antihemagglutinin antibody labeled with 10-nm gold particles. Note reaction with surface-associated material and aggregates of disrupted cell-free material (arrows) but not with the flagella. Bar, 0.1 μm.

been found to reaggregate into their native form upon removal of the solubilizing reagent (17, 21, 43). Also, cell-free preparations of most fimbriae and fibrillae do not exhibit HA activity, even if removed from the cells by homogenization or by heat treatment; this is in part because the cell-free structures are often monovalent, as in the case of CFA/I of the enterotoxigenic *E. coli* (8) and the digalactoside-binding fimbriae of uropathogenic *E. coli* (25). We were successful in obtaining the *C. pylori* hemagglutinin in cell-free form by extraction of the bacterial cells with NOG but have not yet identified the molecular weight of the protein subunit. The results obtained by agarose chromatography in the presence of 0.5% NOG indicate that subunit aggregates are present even under these conditions; as shown in this report, at least four different molecular states were demonstrated, with the lower-molecular-weight species appearing only after considerable purification. On the other hand, this behavior facilitated the preparation of reaggregated hemagglutinin visible by TEM, and the size and shape of the reaggregated structures appear to be in good agreement with the fibrillar structures seen in cell-free homogenates, which were visualized by immunoprecipitation with specific antiserum. Results obtained with the immunogold method show that the fibrillar antigen probably covers the entire cell surface in the form of a capsulelike network. The antihemagglutinin antibody did not react with *C. pylori* flagella. All of these results are consistent with those obtained with fibrillar antigens of other bacterial pathogens (11, 33, 44).

Finally, the finding that the erythrocyte receptor for the putative CFA of *C. pylori* described here is a structure which occurs widely in nature does not detract from the possibility that this same, or a very similar, structure might serve as the epithelial cell receptor for this organism. Similar relationships have been documented for a large number of bacterial pathogens possessing CFA-type fimbriae, fibrillae, and afibrillar surface antigens, many of which bind to various types of erythrocytes and mammalian cells in tissue culture. This apparent paradox is resolved when the microecology of bacterial pathogens is considered. *C. pylori*, for example, belongs to a class of organism well adapted to survival in the stomach and thus suffers little competition from other organisms. Interestingly, resistance of the *C. pylori* hemagglutinin to the gastric digestive enzyme pepsin and to the intestinal (pancreatic) enzyme trypsin could be an important factor in the ability of this organism to colonize the gastric epithelium. The same factors which account for the compatibility of *C. pylori* with its gastric environment could prevent this organism from successfully colonizing other epithelial surfaces. Thus, more relevant to *C. pylori* is the proportion of receptor-containing sialoglycoproteins on the gastric epithelial cell surface, as opposed to the proportion (or relative concentration) in the immediately surrounding mucus layer.

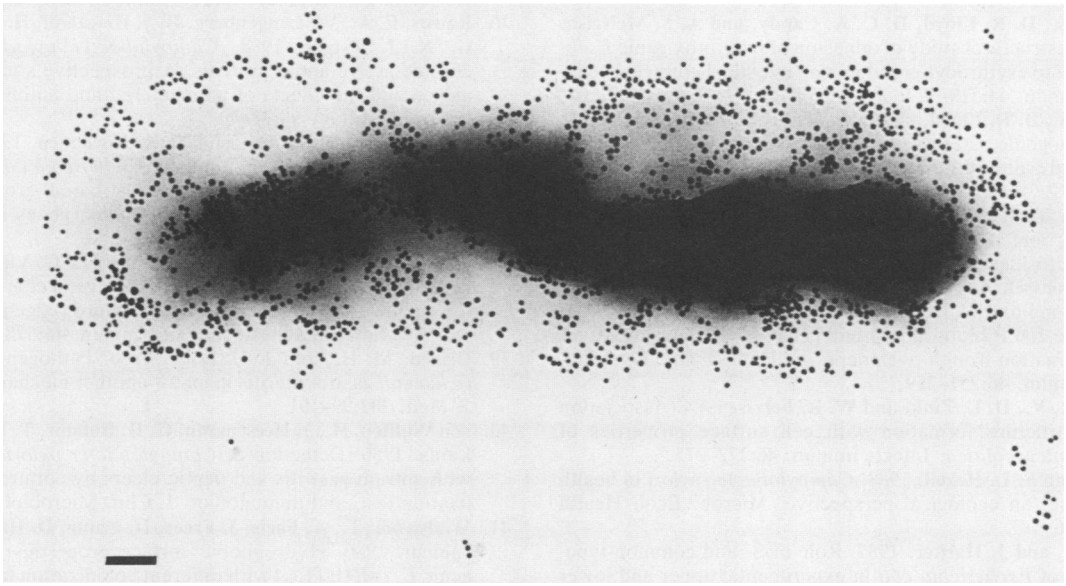

FIG. 11. Electron micrograph of an isolated *C. pylori* cell, prepared as described for Fig. 10. Note labeling of entire cell surface and apparent thickness of the fibrillar (hemagglutinin) coat, giving the appearance of a capsule. Bar, 0.1 μm.

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