

Inhibition of *Helicobacter pylori* Binding to Gastrointestinal Epithelial Cells by Sialic Acid-Containing Oligosaccharides

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Helicobacter pylori, the ulcer pathogen residing in the human stomach, binds to epithelial cells of the gastric antrum. We have examined binding of 13 bacterial isolates to epithelial cell lines by use of a sensitive microtiter plate method in which measurement of bacterial urease activity provides the means for quantitation of bound organisms. Several established human gastrointestinal carcinoma cell lines grown as monolayers were compared for suitability in these assays, and the duodenum-derived cell line HuTu-80 was selected for testing bacterial binding inhibitors. When bacteria are pretreated with oligosaccharides, glycoproteins, and glycolipids, a complex picture of bacterial-epithelial adherence specificities emerges. Among the monovalent inhibitors tested, 3'-sialyllactose (NeuAc α 2-3Gal β 1-4Glc; 3'SL) was the most active oligosaccharide, inhibiting adherence for recent clinical isolates of *H. pylori* with a millimolar 50% inhibitory concentration (IC₅₀). Its α 2-6 isomer (6'SL) was less active. Most of the recent clinical isolates examined were inhibited by sialyllactose, whereas long-passaged isolates were insensitive. Among the long-passaged bacterial strains whose binding was not inhibited by 3'SL was the strain ATCC 43504, also known as NCTC 11637 and CCUG 17874, in which the proposed sialyllactose adhesin was recently reported to lack surface expression (P. G. O'Toole, L. Janson, P. Doig, J. Huang, M. Kostrzynska, and T. H. Trust, *J. Bacteriol.* 177:6049–6057, 1995). Pretreatment of the epithelial monolayer with neuraminidase reduced the extent of binding by those bacteria that are sensitive to inhibition by 3'SL. Other potent inhibitors of bacterial binding are the glycoproteins α ₁-acid glycoprotein, fetuin, porcine gastric and bovine submaxillary mucins, and the glycolipid sulfatide, all of which present multivalent sialylated and/or sulfated galactosyl residues under the conditions of the binding assay. Consistent with this pattern, a multivalent neoglycoconjugate containing 20 mol of 3'SL per mol of human serum albumin inhibited bacterial binding with micromolar IC₅₀. The *H. pylori* isolate most sensitive to inhibition by 3'SL was least sensitive to inhibition by sulfatide, gastric mucin, and other sulfated oligosaccharides. Bacteria that have been allowed to bind epithelial cells are also effectively detached by 3'SL. These results describe a heterogeneous adherence repertoire for these bacteria, but they also confirm the critical role of the 3'SL structure on human gastric epithelial cells as an adherence ligand for recent isolates of *H. pylori*.

Helicobacter pylori is an etiologic agent in the development of gastritis and gastroduodenal ulcers (36, 52), gastric cancer (48), and mucosa-associated lymphoid tumors (26, 55). Histologically, bacteria are observed closely associated with gastric epithelial cells and in the mucus layer. Bacteria are generally most numerous in the antrum but are also found bound to patches of gastric metaplasia in the duodenum and the esophagus (5). The localized adherence of *H. pylori* to gastric epithelial cells as well as their apparent selectivity for the antrum (30) suggests that the organism specifically recognizes epithelial cell surface constituents. Based on hemagglutination and cell binding experiments, *H. pylori* was proposed to recognize 3'-sialyllactose (NeuAc α 2-3Gal β 1-4Glc; 3'SL) (13, 15). An adhesin for sialoglycoconjugates was subsequently identified and cloned (16).

Adherence of *H. pylori* to sulfated oligosaccharides expressed on glycoproteins (42, 50) and glycolipids (27, 44, 45), to phosphatidylethanolamine (34), and to the fucosylated blood group oligosaccharide Lewis^b (4, 17) has also been reported. In addition, binding to basement membrane constituents such as laminin and collagen (49), to heparan sulfate (1, 6), and to unspecified neuraminidase-insensitive ligands (18) has been described.

Investigations into the specificity and mechanisms of bacterial binding have employed a variety of techniques. These include bacterial attachment to biomolecules immobilized on thin-layer chromatography plates (22) and onto plastic surfaces (31), cross-linking of erythrocytes (hemagglutination) (21, 24), and binding to live cells (12, 37) or histological sections (4, 17). We have adapted a binding assay that quantitates via bacterial urease the binding of unaltered living bacteria to intact living epithelial cell monolayers. The urease is considered to be a colonization factor (10, 41, 51) which *H. pylori* produces in abundance (18). This enzyme, in the presence of urea, generates ammonia and carbonate ions.

Using this method in a microtiter plate format, we have surveyed a range of bacterial isolates in early or advanced passage. Based on the ability of the test compounds to inhibit the binding of live bacteria to several types of epithelial cells, we describe a range of binding specificities, especially for sialyllactose and related structures. We compare the binding inhibitory potencies of monovalent and multivalent compounds as well as the abilities of these molecules to detach bacteria prebound to epithelial monolayers.

MATERIALS AND METHODS

Bacteria. The bacterial strains used in this study and their sources and characteristics are described in Table 1. Bacteria were grown on 5% sheep or human blood-agar plates (Remel) at 37°C and under 12% CO₂ and harvested at 48 h (in the late logarithmic phase) into Dulbecco's phosphate-buffered saline (PBS). For suspension cultures, bacteria were grown in brain heart infusion broth containing 10% fetal bovine serum (FBS) or in brucella broth. The morphology of bacteria

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TABLE 1. *H. pylori* isolates used in the current study

Isolate(s)	Source	Characteristics
43504 ^a , 43526	American Type Culture Collection, Gaithersburg, Md.	Clinical isolates, extensively passaged
1832, 1351, 78, 1971, 1814	B. Marshall, University of Virginia, Charlottesville	Clinical isolates (<10 passages)
1080, 1512	B. Reichwein, Christiana Hospital, Wilmington, Del.	Clinical isolates (<10 passages)
CP22	I. Nachamkin, University of Pennsylvania, Philadelphia	Clinical isolate (<10 passages)
26695	S. Krakowka, Ohio State University, Columbus	Human isolate used to infect piglets (9, 10)
93230	S. Krakowka, Ohio State University, Columbus	Recovered from piglet originally infected with 26695
WV99	S. Krakowka, Ohio State University, Columbus	Human isolate used to infect piglets

^a This strain has also been referred to as CCUG 17874 (38) and NCTC 11637.

grown on agar is mixed, with both coccoid and rod forms, while bacteria grown in suspension culture are predominantly rods. Bacteria were washed in PBS, counted by microscopy with a Petroff-Hausser chamber, and kept at 4°C until experimental use (not more than 24 h). *H. pylori* isolates are cryopreserved in defibrinated horse blood and stored at -70°C. Bacterial urease activity was quantitated by measuring the optical density at 550 or 595 nm following the addition of 50 µl of saline containing 2% urea-0.03% phenol red (UPR), by a modification of the method of Fauchère and Blaser (18).

Cells. Epithelial cell lines were obtained from the American Type Culture Collection (ATCC). The cell line HuTu-80 (ATCC HTB-40) was derived from a human duodenal adenocarcinoma, AGS (ATCC CRL-1739) was from a human gastric adenocarcinoma, and HEp-2 (ATCC CCL-23) was from a human laryngeal adenocarcinoma. The cell lines HT-29 and CaCo-2 were derived from human colon adenocarcinomas, and Y-1 was derived from a murine adrenal carcinoma. HuTu-80, HEp-2, and Y-1 cells were maintained in tissue culture flasks as adherent monolayers in complete culture medium consisting of basal medium with Eagle's salts (Gibco, Grand Island, N.Y.), 10% FBS (Upstate Biotechnology, Inc., Lake Placid, N.Y.), and penicillin (100 µg/ml) and streptomycin (100 U/ml). HT-29 and CaCo-2 were maintained in RPMI 1640 medium supplemented with 10% FBS. For replating, monolayers were detached by the use of trypsin (0.05%) and EDTA (0.53 mM; Gibco). After 10 min at 37°C, trypsin was neutralized by the addition of 10% FBS, and detached cells were washed twice in complete medium. For binding studies, cells were plated in flat-bottom 96-well microtiter plates (Falcon and Costar) in 100 µl of complete culture medium at approximately 5 × 10⁴ cells/ml. They reached confluence in 48 h and were used within 48 h of confluence.

Compounds. 3'SL and its NeuGα2-3 (*N*-glycolylneuraminyllactose) congener as well as NeuAα2-6Galβ1-4Glc (6'-sialyllactose [6'SL]) and NeuAα2-6Galβ1-4GlcNAc (6'-sialyllactosamine [6'SL_N]) were isolated from bovine colostrum by a modification of the method of Parkkinen and Finne (39). Briefly, bovine colostrum was defatted by centrifugation and protein was precipitated with 50% ethanol. The supernatant was applied to a Dowex 1-X8 column. Lactose was eluted with 2 mM pyridine acetate (PyrAc), and monosialylated oligosaccharides were eluted with 50 mM PyrAc. The latter eluate was concentrated by rotary evaporation, redissolved in 2 mM PyrAc, and applied to a Dowex 1-X2 column. The column was eluted with 100 mM PyrAc, and the fractions were analyzed by high-performance liquid chromatography with pulsed amperometric detection (Dionex). Pooled fractions containing purified oligosaccharides were concentrated by rotary evaporation, lyophilized, redissolved in water, titrated to pH 7.2 with 1 N NaOH, and lyophilized again. The final products were characterized by anthrone (total hexose) and resorcinol (sialic acid) reactions, proton nuclear magnetic resonance, methylation analysis with gas chromatography-mass spectrometry, and high-performance liquid chromatography. The purity of all compounds was at least 95%.

Fetuin, asialofetuin, porcine gastric and bovine submaxillary mucin, α₁-acid glycoprotein (AGP), transferrin, sulfatide, galactosyl ceramide, sodium chlorate, dextran (~9,000 and 70,000 Da), dextran sulfate (~8,000 and 70,000 Da), porcine intestinal heparin (high and low molecular weight), heparan sulfate, chondroitin sulfate (A, B, and C), chondroitin disaccharides (2-, 4-, and 6-sulfate), and fucoidan were purchased from Sigma Chemical Co. (St. Louis, Mo.). Neuraminidases from *Clostridium perfringens* and *Arthrobacter ureafaciens* were purchased from Oxford GlycoSystems (Rosedale, N.Y.).

Multivalent neoglycoproteins were synthesized by coupling phenethylamine-isothiocyanate derivatives of oligosaccharides to human serum albumin (HSA) by the method of Zopf et al. (58). By the resorcinol method (47), the sialic acid content was 20 sialic acid residues per albumin molecule.

Epithelial monolayer binding. Preliminary bacterial titration experiments were performed daily by the UPR method to determine the extent of binding. The concentration of bacteria required to produce binding near the top of the linear range was selected for subsequent inhibition assays. Typically, 1 × 10⁷ to 5 × 10⁷ bacteria/ml were added to microtiter wells in 25 µl. Binding is linear over a range of approximately 1.5 logs of bacterial concentration. Binding occurred with gentle agitation for 20 min at ambient temperature. Microtiter plates were washed three times with 100 µl of saline containing 0.03% phenol red to remove

unbound bacteria. Inhibition of bacterial binding was measured by preincubating the bacteria with serial dilutions of the test compounds in a separate round-bottom microtiter plate for 15 min at ambient temperature with gentle agitation, after which the mixture was added to drained epithelial monolayers. The inhibitory concentration was calculated from the optical density readings by the following formula: % inhibition = [(OD_{experimental} - OD_{negative})/(OD_{positive} - OD_{negative})] × 100, where negative wells contain no bacteria, only epithelial monolayers without bacteria, positive wells contain bacteria and monolayer without added inhibitor, and OD is optical density. Each determination was performed in duplicate wells, and all experiments were repeated at least once on separate days.

Neuraminidase treatment. Cell monolayers were treated with 1 U of neuraminidase per ml in a buffer supplied by the vendor consisting of 50 mM sodium acetate, 4 mM CaCl₂, and 0.1 mg of bovine serum albumin (pH 5.5) per ml for 1 h at 37°C, after which the monolayers were washed twice in PBS. The neuraminidases used were from *C. perfringens*, which cleaves, at decreasing rates, sialic acid linked α2-3, α2-6, and α2-8, and from *A. ureafaciens*, which preferentially hydrolyzes sialic acids bound α2-6.

Detachment. To measure detachment, bacteria were first allowed to bind epithelial monolayers for 15 min. Test compounds were included only in the wash buffer, which was used for the removal of unbound bacteria. Detachment was determined by comparing the remaining bacteria in test wells with that in control (buffer-only) wells.

Microscopic examination of bacterial adherence. HuTu-80 cells were grown in nonconfluent monolayers over plastic chamber slides (LabTek). Bacterial binding was conducted under the same conditions of incubation as those described above for the microtiter plate method. Slides with bacteria bound to monolayers were developed by use of the modified Steiner-Steiner silver stain as described in the manufacturer's instructions (Accustain; Sigma).

RESULTS

Epithelial cell line monolayers. Several human gastrointestinal carcinoma cell lines (HuTu-80, AGS, HEp-2, CaCo-2, and HT-29) as well as the murine adrenal carcinoma Y-1 were tested as binding targets for *H. pylori*. Of these, the duodenal carcinoma HuTu-80 and the laryngeal carcinoma HEp-2 formed monolayers with the fewest gaps. Because of their greater sensitivity to bacterial binding inhibitors (see below), HuTu-80 cells were selected as the principal test monolayer.

Effect of bacterial growth conditions. The levels of binding to HuTu-80 monolayers of *H. pylori* grown on blood agar plates and those grown in brain heart infusion broth were compared. Approximately twice as many agar-grown bacteria than liquid culture-grown bacteria bound to epithelial monolayers. In addition, agar-grown bacteria were more sensitive to antiadhesive compounds (data not shown).

Bacterial binding inhibitors. (i) Sialyllactose. Sensitivities to 3'SL inhibition differed among the isolates, with 50% inhibitory concentration (IC₅₀) values ranging from 2.3 to 9.1 mg/ml (3.5 to 13.9 mM; the maximum concentration tested was 10 mg/ml or 15.2 mM) (Tables 2 and 3). Binding was inhibitable by 3'SL in seven of the eight clinical isolates tested on HuTu-80 monolayers (Table 2) and five of the eight isolates on HEp-2 monolayers (Table 3). Interestingly, the adherence of isolates 43504 and 43526, which have been passaged in the lab extensively, could not be inhibited by sialyllactoses. Gradual

TABLE 2. Inhibition of *H. pylori* binding to HuTu-80 monolayers

<i>H. pylori</i> isolate	IC ₅₀ (mg/ml) ^a		
	3'SL	6'SL	6'SL _n
1832	2.3 ± 2.2 (25) ^b	7.2 ± 3.1 (6)	8.8 ± 1.8 (6)
CP22	3.8 ± 3.8 (17)	7.8 ± 3.9 (4)	>10.0 (4)
1351	3.9 ± 2.9 (38)	7.3 ± 3.5 (18)	9.0 ± 2.5 (10)
1512	4.4 ± 3.6 (7)	8.3 ± 2.7 (6)	>10.0 (5)
78	5.8 ± 4.2 (12)	8.6 ± 2.9 (8)	9.6 ± 1.0 (7)
1080	7.8 ± 3.9 (4)	>10.0 (4)	9.8 (1)
1971	8.8 ± 2.2 (4)	>10.0 (5)	9.9 ± 0.2 (5)
1814	>10.0 (8)	9.2 ± 1.2 (8)	7.9 ± 2.0 (8)
43504	>10.0 (7)	9.4 ± 1.3 (5)	>10.0 (5)
43526	>10.0 (3)	>10.0 (3)	>10.0 (3)
93230	7.3 ± 3.6 (11)	6.0 ± 4.1 (11)	8.3 ± 3.2 (7)
WV99	6.9 ± 3.8 (5)	6.4 ± 3.5 (5)	>10.0 (3)
26695	7.5 ± 3.5 (3)	6.2 ± 3.1 (3)	7.1 ± 2.8 (3)

^a IC₅₀ is the concentration required to reduce the number of organisms bound to 50% of the control value (i.e., absence of inhibitor). Compounds are tested in serial dilutions beginning with a concentration of 10 mg/ml. If binding inhibition fails to reach 50% at the maximum concentrations of inhibitor tested (10 mg/ml), this value is used in calculating the mean. Values are the means ± standard deviations. The oligosaccharides Lac, LacNAc, and NeuAc were not inhibitory.

^b Numbers in parentheses indicate the number of determinations.

loss of sensitivity to 3'SL inhibition after multiple passages was also demonstrated with *H. pylori* 1832, which had an IC₅₀ of 1.4 mg/ml in passage 40 compared with 0.6 mg/ml in passage 9.

Binding inhibition was specific for the sialic acid isomer, 3'SL being a consistently stronger inhibitor than 6'SL. This structural preference occurred with most of the recent clinical isolates on both HuTu-80 and HEP-2 cell monolayers. Microscopic examination of bacterial binding to HuTu-80 monolayers confirmed this ranking of inhibitory sialyllactoses (Fig. 1).

Three *H. pylori* isolates used to infect piglets (93230, WV99, and 26695) exhibited a reduced inhibition by 3'SL and a slightly enhanced inhibition by 6'SL (Tables 2 and 3). In addition, binding inhibition was specific for the NeuAc form of 3'SL as opposed to the NeuGc form (Table 4).

(ii) **Glycoproteins.** The glycoproteins fetuin, porcine gastric and bovine submaxillary mucin, and AGP inhibited *H. pylori* binding to epithelial monolayers, whereas transferrin, which has oligosaccharides terminating only in α2-6-linked NeuAc (20), consistently failed to inhibit binding (Table 5). Among the fresh human isolates that are most sensitive to binding inhibition by 3'SL (1832 and CP22), there was also increased inhibition by fetuin and, to a lesser extent, by AGP. These bacteria were less inhibited by asialofetuin.

Effects of neuraminidases. HuTu-80 cells were treated with neuraminidases prior to the binding of *H. pylori*. Neuraminidase obtained from *C. perfringens*, which cleaves the α2-3 bond preferentially, had a greater inhibitory effect on binding of *H. pylori* 1832 to epithelial cells than the enzyme from *A. ureafaciens*, which preferentially cleaved the α2-6 bond (Table 6). Conversely, isolate 43504, a strain that has been passaged extensively in vitro and is insensitive to 3'SL (Tables 2 and 3), exhibited increased binding to neuraminidase-treated monolayers (Table 6).

Sulfated glycoconjugates. Sulfated compounds were tested for their ability to inhibit *H. pylori* binding to epithelial monolayers. The results depicted in Table 7 indicate that the glycolipid sulfatide is a potent adherence inhibitor of some isolates but not of isolate 1832. And, as seen earlier (Table 5), porcine gastric mucin, a heavily sulfated macromolecule, is also a relatively weak inhibitor of *H. pylori* 1832 adherence. Other sulfated oligosaccharides and polysaccharides tested that did not

block bacterial adherence (IC₅₀, >10 mg/ml) include dextran sulfate (molecular weights, 8,000 and 70,000) and unsulfated dextran (molecular weights, 9,000 and 70,000) included for comparison, fucoidan, a sulfated fucan, heparin, heparan sulfate (high and low molecular weight), chondroitin sulfate (A, B, and C), and chondroitin sulfate disaccharides (2-, 4-, and 6-sulfate). The binding of *H. pylori* CP22 to HuTu-80 monolayers is sensitive to sulfatide inhibition (Table 6). The addition of 2 mg of sulfatide per ml inhibited CP22 binding to HuTu-80 cells by 60%, but sulfatide inhibition was reduced to 36% if the monolayer was pretreated with 10 mM sodium chlorate, a treatment that inhibits biosynthetic sulfation of glycoproteins and glycolipids (23). The binding of isolate 1832, which is not sensitive to inhibition by sulfatide (see Table 7), was not affected by sodium chlorate (data not shown).

Detachment. The ability of 3'SL to detach previously bound bacteria from epithelial monolayers was tested. Following a 15-min incubation with bacteria, monolayers were washed three times with buffer containing 2 mg of 3'SL, porcine gastric mucin, or both per ml. The residual bound bacteria were quantitated by the UPR method and compared with that in wells washed with buffer alone. The results depicted in Fig. 2 indicate that a large fraction of the bacteria was detached from the monolayers by this process. The detachment was inversely proportional to the number of bacteria bound to the monolayers (Fig. 2A) and directly proportional to the concentration of 3'SL (Fig. 2B). The extent of *H. pylori* detachment from HuTu-80 cells was augmented by addition of porcine gastric mucin to 3'SL.

Monovalent or multivalent binding blockers. Multivalent albumin conjugates of 3'SL inhibit bacterial adherence to epithelial monolayers more effectively than monovalent 3'SL (Fig. 3). On the basis of moles of 3'SL, the multivalent form was approximately 1,000-fold more potent as a binding blocker than the monovalent form.

DISCUSSION

Adhesion of bacteria to host epithelial surfaces, in addition to being a requisite first step in the colonization process, can also serve to induce the expression of virulence factors (56). The localization of *H. pylori* to its unique niche in the human stomach may be mediated by a number of specific adhesion ligands, which have been identified in a variety of experimental systems (4, 15, 17, 34, 42, 49). From among these, the antral

TABLE 3. Inhibition of *H. pylori* binding to HEP-2 monolayers

<i>H. pylori</i> isolate	IC ₅₀ (mg/ml) ^a		
	3'SL	6'SL	6'SL _n
1832	6.2 ± 2.3 (5)	9.0 ± 1.7 (4)	9.3 ± 1.3 (5)
CP22	4.8 ± 4.2 (4)	7.3 ± 2.1 (3)	8.9 ± 1.1 (4)
1351	3.1 ± 3.0 (10)	9.5 ± 0.7 (7)	9.1 ± 1.8 (9)
1512	5.4 ± 4.6 (6)	9.1 ± 1.7 (5)	9.9 ± 0.2 (5)
78	>10.0 (5)	>10.0 (5)	9.9 ± 0.3 (5)
1080	>10.0 (2)	9.9 ± 0.2 (2)	8.7 ± 1.4 (2)
1971	9.1 ± 1.1 (5)	>10.0 (5)	8.0 ± 3.0 (5)
1814	>10.0 (6)	9.0 ± 0.8 (6)	7.3 ± 1.1 (6)
43504	>10.0 (4)	9.8 ± 0.4 (4)	>10.0 (4)
43526	>10.0 (5)	9.2 ± 1.1 (5)	8.8 ± 1.3 (5)
93230	>10.0 (5)	9.5 ± 0.9 (4)	6.7 ± 3.2 (5)
WV99	>10.0 (3)	8.8 ± 1.7 (3)	8.2 ± 2.5 (3)
26695	>10.0 (3)	8.1 ± 2.6 (3)	>10.0 (3)

^a See Table 2, footnotes a and b, for details. The oligosaccharides Lac, LacNAc, and NeuAc were not inhibitory.

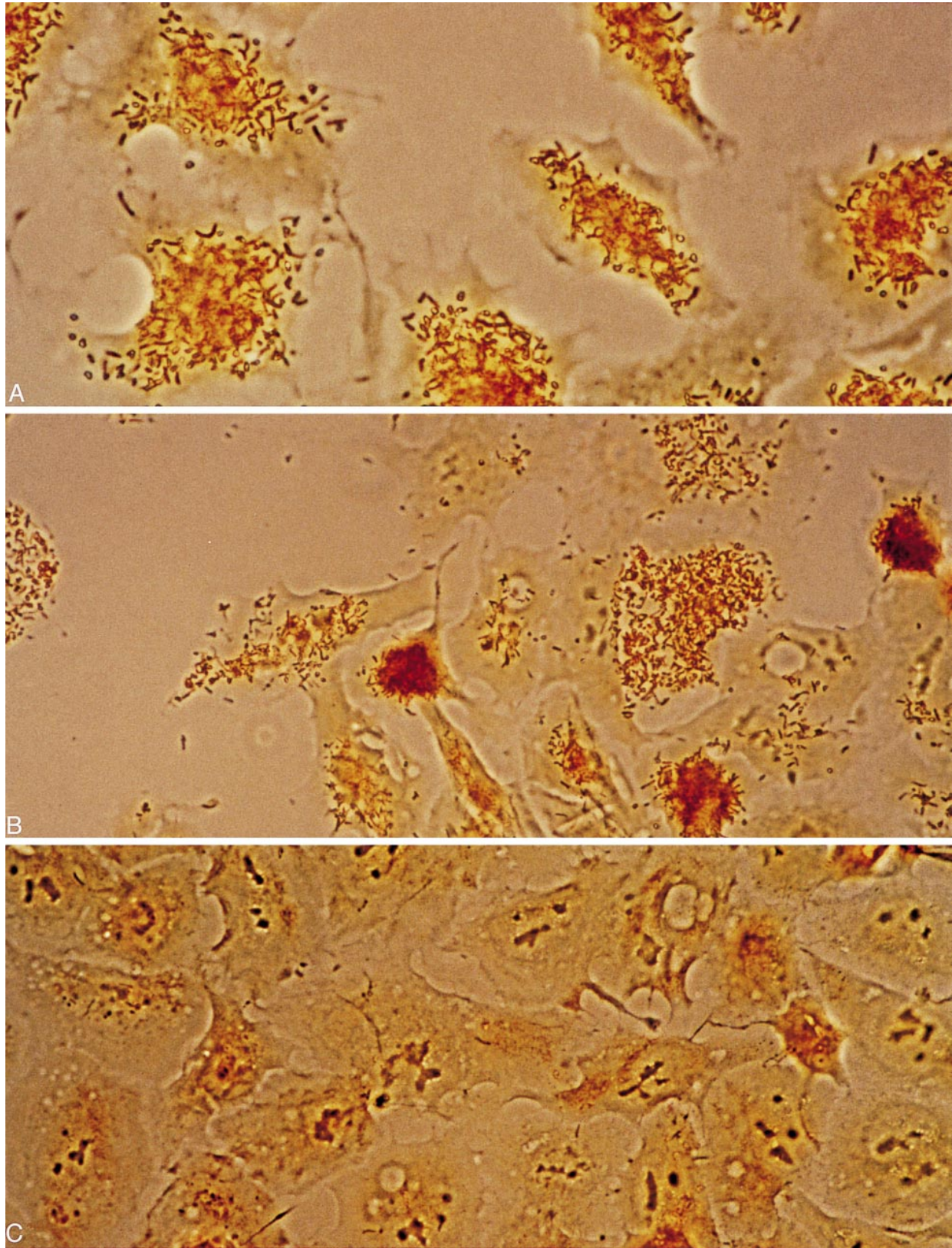


FIG. 1. Visualization of sialyllactose-inhibitible adherence of *H. pylori* to HuTu-80 epithelial cells. Cells were grown overnight in eight-well chamber slides (LabTek). Strain 1832 bacteria were coincubated with oligosaccharides for 15 min and added to the epithelial cells at a density of 10^9 /ml for 20 min, after which nonadherent bacteria were washed away. (A) Lactose, 10 mg/ml; (B) 6'SL, 10 mg/ml; (C) 3'SL, 10 mg/ml. Slides were stained with the Steiner-Steiner silver stain (Sigma) and photographed at $\times 630$ magnification.

TABLE 4. Inhibition of *H. pylori* binding to HuTu-80 monolayers by *N*-acetyl-sialyllactose and *N*-glycolyl-sialyllactose

Inhibitor	IC ₅₀ (mg/ml) ^a		
	CP22	1832	1351
NeuAcα2-3Lac	1.8	2.3	7.0
NeuGcα2-3Lac	9.0	7.2	>10.0
NeuAc	>10.0	>10.0	>10.0
Lac	>10.0	>10.0	>10.0

^a See Table 2, footnote *a*, for details, but the values shown are the averages of two experiments.

epithelium is enriched in the glycolipids sulfatide (I³ SO₃-GalCer) and GM₃ (II³ NeuAc-LacCer) (27, 44). The latter is the ceramide glycolipid that displays the oligosaccharide 3'SL. Both of these structures have been reported by other investigators (34, 45) to mediate bacterial adherence, findings that are consistent with our results.

Evans et al. first described *H. pylori*-mediated hemagglutination that was inhibitable by bovine milk-derived sialyllactose, which contains 85% 3'SL and 15% 6'SL by weight (15). They extended this observation to bacterial binding to the murine adrenal cell line Y-1, which was diminished after neuraminidase treatment of the monolayer, and also by preincubation of the bacteria with fetuin (13). Subsequently, this team cloned a gene for a 20-kDa bacterial adhesin specific for sialoglycoconjugates (16). The gene contains a lysine-rich sequence that has similarity to the sequence of the sialic acid-binding motif of the SfaS adhesin of S-fimbriated *Escherichia coli* (40), the colonization factor antigen I (CFA/I) of enterotoxigenic *E. coli* (28), and the sialylated ganglioside GM₁-binding motif of *Vibrio cholerae* toxin B subunit (19).

We show that the epithelial adherence of the majority of the fresh clinical isolates is selectively inhibited by purified sialyllactoses (Tables 2 and 3) and that 3'SL is the preferred inhibitory isomer. The genetic polymorphism that has been reported for the *H. pylori* sialyllactose adhesin HpaA (14, 25) is reflected in our studies by the diverse susceptibility to adhesion inhibitors by different bacterial strains (Tables 2, 3, and 5). Long-passaged isolates 43504 and 43526 bind epithelial monolayers but are not sensitive to inhibition by 3'SL or 6'SL. Also, the sensitivity of strain 1832 to 3'SL inhibition diminished with increasing passage number, and, unlike other strains, strain 1832 was altogether insensitive to binding inhibition by sulfated compounds (Table 7). This polymorphism may arise from a genotypic diversity, such as that described for genes

responsible for lipopolysaccharide structure, the CagA protein, vacuolating cytotoxin, and neutrophil activation (3). Genetic flexibility of this type may be facilitated by a high level of mutation and the residence of this organism in a highly restricted niche that is relatively devoid of competition (32).

O'Toole et al. (38) reported recently that the gene product for the *H. pylori* HpaA hemagglutinin, originally reported by Evans et al. (15), is, in fact, a cytoplasmic lipoprotein. These investigators reported further that antibodies raised against the presumed sialic acid-binding region of HpaA did not bind to the surface of the bacteria. This study, however, was conducted with strain ATCC 43504 (also known as NCTC 11637 and CCUG 17874). These results, therefore, provide molecular support for our findings that strain 43504 binding is not inhibited by 3'SL (Tables 2 and 3) but raise concern about the use of this strain as representative of clinical *H. pylori* isolates.

Two series of experiments serve to highlight the importance of sialylated groups for bacterial binding. First, pretreatment of epithelial monolayers with neuraminidases altered bacterial binding. As reported by other investigators (15), an enzyme from *C. perfringens* specific for sialic acid linked α2-3 to galactose caused reduced binding of bacterial isolates that make primary use of this group for attachment (e.g., strain 1832 [Table 2]), whereas enzyme from *A. ureafaciens*, which selectively cleaves sialic acid linked α2-6 to galactose, has a lesser effect. Interestingly, bacterial isolates that lack sialyllactose-inhibitable adhesion (e.g., strain 43504) exhibit enhanced binding to neuraminidase-treated monolayers (Table 6), which suggests that these bacteria may rely on other adhesins, perhaps recognizing ligands newly exposed by desialylation. Slight enhancement of *H. pylori* binding following neuraminidase treatment of gastric epithelial cells has also been observed by Dunn et al. (7). One other factor that needs further study is the effect of bacterial neuraminidase production (8), which may affect the interaction of *H. pylori* with sialoglycoconjugates.

The second set of results that establishes the importance of sialosides as adherence ligands arises from binding inhibition experiments using fetuin and its desialylated form. As described by others in hemagglutination experiments (15), we found that the binding of bacterial isolates that are most sensitive to inhibition by 3'SL is comparably susceptible to inhibition by fetuin but considerably less sensitive to asialofetuin (Table 5). The structures of these glycoproteins are complex, offering multivalent presentation of their multiple oligosaccharides. In addition, commercial preparations of these serum glycoproteins contain impurities which may be responsible for residual inhibitory effects after desialylation.

TABLE 5. Inhibition of *H. pylori* binding by glycoproteins to HuTu-80 and HEp-2 monolayers

Monolayers	<i>H. pylori</i> isolate	IC ₅₀ (mg/ml) ^a					
		Fetuin	Asialofetuin	AGP	Mucin		Transferrin
					Gastric	Submaxillary	
HuTu-80	1832	2.3 ± 1.7 (28)	7.2 ± 3.9 (6)	1.8 ± 2.0 (43)	5.3 ± 4.3 (9)	3.5 ± 3.2 (3)	>10.0 (3)
	CP22	1.1 ± 0.9 (19)	2.6 ± 3.0 (7)	4.1 ± 3.7 (19)	1.3 ± 1.6 (33)	1.7 ± 0.6 (2)	>10.0 (1)
	1971	6.5 ± 3.7 (5)	2.8 ± 3.7 (6)	7.1 ± 3.5 (4)	1.3 ± 0 (3)		>10.0 (1)
	43504	4.4 ± 3.7 (14)	4.4 ± 3.2 (9)	3.0 ± 3.4 (6)	0.7 ± 0.4 (3)	0.4 (1)	>10.0 (1)
HEp-2	1832	7.3 ± 2.6 (5)	8.0 ± 3.5 (5)	5.7 ± 3.6 (3)	4.4 ± 2.4 (3)	2.0 (1)	>10.0 (1)
	CP22	3.7 ± 1.7 (3)	7.4 ± 3.7 (3)	9.3 ± 0.9 (3)	3.8 ± 2.3 (2)	0.5 (1)	>10.0 (1)
	1971	>10.0 (4)	9.3 ± 1.4 (5)	2.2 ± 0.4 (2)	7.3 (1)	0.1 (1)	>10.0 (1)
	43504	>10.0 (5)	9.8 ± 0.4 (4)	>10.0 (2)	1.7 (1)	2.9 (1)	>10.0 (1)

^a See Table 2, footnotes *a* and *b*, for details.

TABLE 6. Neuraminidase treatment of HuTu-80 monolayers alters the extent of *H. pylori* binding

<i>H. pylori</i> isolate	% Binding ^a		
	None ^b	<i>C. perfringens</i> ^c	<i>A. ureafaciens</i> ^d
1832	100	35.2 ± 2.6	60.0 ± 1.9
43504	100	151.3 ± 29.7	165.5 ± 27.7

^a Values are the means ± standard deviations of four determinations.

^b HuTu-80 monolayers were either untreated (None) or treated with neuraminidase from *C. perfringens* or *A. ureafaciens*.

^c Cleaves Neu5Acα2-3 bonds preferentially.

^d Cleaves Neu5Acα2-6 bonds preferentially.

The inability of some investigators to detect *H. pylori* binding to sialylated ligands (4, 18) can be attributed to technical features of the experiments or could arise from using isolates that have been extensively passed (as is the case for strains 43504 and 43526). Falk et al. (17) bound fluorescein isothiocyanate (FITC)-derivatized bacteria to gastric tissue sections that had been processed for histology. The *H. pylori* sialyl-adhesin contains key lysine residues in its presumed ligand-binding domain (16), a feature shared with other sialic acid-binding cellular lectins (11). Chemical derivatization of the bacteria with FITC could alter the adhesin's active-site lysine residues and desensitize bacteria to sialylated ligands. Bacterial binding could also be impeded by formalin fixation of gastric sections. Indeed, we have observed strikingly reduced binding by using FITC-labeled bacteria and/or by chemical fixation of epithelial monolayers (unpublished observations). Also, the repeated treatment of tissue sections with aqueous and organic solvents in the course of histological processing could result in the extraction of membrane glycolipids and glycoproteins, including those required for sialyllactose-mediated adherence.

H. pylori is reported to bind a variety of sulfated glycoconjugates, among them the glycolipid sulfatide (27, 44), heparinoids (1, 6), and mucins (42). Our results confirm previous reports (27, 44, 45) that sulfatide is a potent inhibitor of binding for many bacterial isolates (Table 7), although its tendency to form multivalent aggregates, which might increase its blocking potential (33), renders imprecise the determination of its molar inhibitory potency. Curiously, *H. pylori* isolate 1832, which, among the strains we examined, was most sensitive to binding inhibition by 3'SL, was the least sensitive to inhibition by mucin and sulfatide (Tables 5 and 7). The heparinoids, whose direct binding to *H. pylori* was measured by Ascencio et al. (1), were inactive as inhibitors of adhesion in our experiments.

The participation of sulfated structures in bacterial binding is further illustrated by the use of sodium chlorate, an inhibitor of cellular sulfotransferases, which causes the expression of membrane constituents lacking sulfate groups (23). Bacterial strains (e.g., CP22) whose adhesion to epithelial cells is competitively inhibited by sulfatide exhibited reduced sensitivity to sulfatide inhibition when exposed to monolayers pretreated

TABLE 7. Inhibition of *H. pylori* strain adherence to HuTu-80 monolayers by sulfated compounds

Inhibitor	IC ₅₀ (mg/ml) ^a		
	1832	CP22	93230
Sulfatides	>10.0	0.16 ± 0.10	1.15 ± 0.50
Fucoidan	>10.0	9.10 ± 5.50	3.25 ± 0.35

^a See Table 2, footnote a, for details.

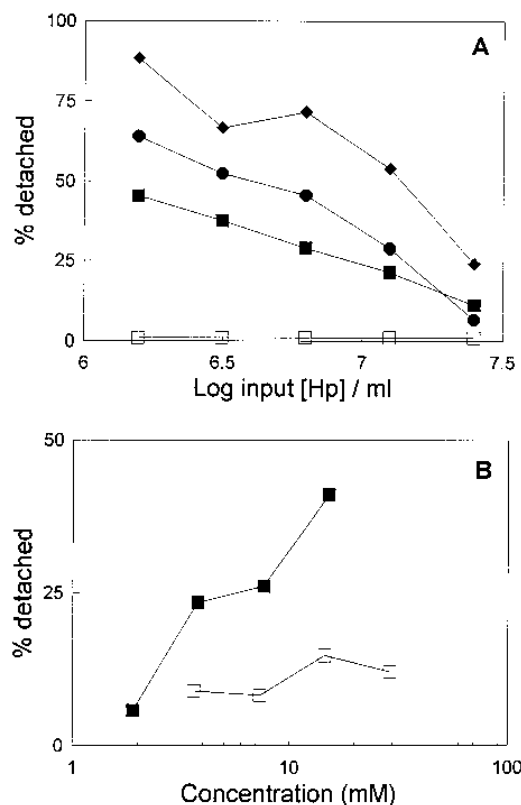


FIG. 2. Bound bacteria can be detached by 3'SL. *H. pylori* CP22 were incubated with HuTu-80 monolayers for 15 min, and unbound bacteria were removed by three washes of buffer alone or buffer containing the indicated compounds. (A) Symbols: ■, 2 mg of 3'SL per ml (3 mM); □, 2 mg of lactose per ml (5.8 mM); ●, 2 mg of gastric mucin per ml; ◆, 2 mg each of 3'SL and gastric mucin per ml. The greatest detachment was obtained at lower bacterial loads. (B) The detachment effect of 3'SL (■) is concentration dependent, while lactose (□) has no effect (bacterial input, 3×10^7 /ml).

with 10 mM sodium chlorate. This effect was not seen with strain 1832, which does not utilize sulfated ligands for attachment.

The parallel occurrence of sialic acid and sulfate group specificities has been observed with other lectins. For example, the vascular adhesion family of selectins exhibits specificity for the blood group oligosaccharides sialyl-Lewis^x and sialyl-Lewis^a bearing either a sialic acid linked α2-3 or a sulfate group on carbon 3 of the nonreducing terminal galactose (53). Another example is the natural killer cell lectin NKR-P1, which binds α2-3-linked sialic acid- or sulfate-modified galactosyl determinants on tumor cell membrane constituents, with greater affinity for the latter (2). However, a similar cross-reactive recognition of the α2-3-sialylated galactose group of 3'SL and the 3-sulfated galactose of sulfatide glycolipids by a single *H. pylori* surface adhesin seems unlikely for two reasons. First, the ability of the bacterial adhesin to distinguish between the *N*-acetyl- and the *N*-glycolyl-neuraminyl forms of sialic acid (Table 4) suggests that recognition extends beyond simple charge interactions with the anionic group on the third carbon of the galactosyl moiety. Second, the ability of isolate 1832 to diverge from other isolates such as CP22 with regard to susceptibility to 3'SL and sulfatide inhibition suggests the existence of discrete adhesins for sialo- and sulfo-conjugates.

Bacterial-epithelial adherence is comprised of multiple non-covalent contact points, each in dynamic equilibrium. In prin-

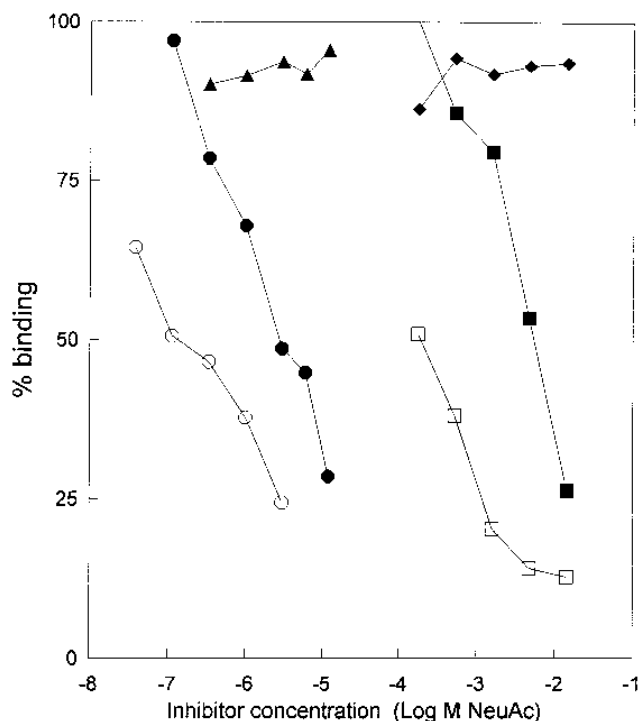


FIG. 3. Multivalent 3'SL is a more potent inhibitor of bacterial binding. *H. pylori* bacteria were bound to epithelial monolayers following preincubation with monovalent and multivalent oligosaccharides. Results for *H. pylori* 1351 and HEp-2 monolayers (closed symbols) and 1832 and HuTu-80 monolayers (open symbols) are shown. Bacteria were treated with HSA derivatized with 20 mol of 3'SL (●, ○), monovalent 3'SL (■, □), unconjugated albumin (▲), and 6'SL (◆). Underivatized albumin is plotted to correspond to the molar concentration of HSA in the 3'SL-HSA construct.

ciple, therefore, molecules capable of competitively inhibiting adhesin-ligand interaction will not only prevent binding but also disrupt established adherence. To test whether adhesin-binding oligosaccharides can detach adherent bacteria, we allowed *H. pylori* to attach to HuTu-80 monolayers and then included 3'SL, gastric mucin, or both in three successive buffer washes. Dose-dependent removal of prebound bacteria from the epithelial monolayers occurred (Fig. 2). Because stomach surface epithelium exfoliates with a half-life of 24 h (35), generations of *H. pylori* must form new attachments to the lining epithelium to resist clearance via the peristaltic flow of gastric contents. The in vitro demonstration that 3'SL can prevent de novo adherence as well as promote detachment of *H. pylori* indicates a possible therapeutic utility for this oligosaccharide.

The presentation of oligosaccharides that serve as attachment ligands for *H. pylori* in multivalent form renders them considerably more potent as adherence blockers. For example, we observed (Fig. 3) that on a molar basis, 3'SL was 2 to 3 orders of magnitude more potent when conjugated to human serum albumin (~20 oligosaccharides per mol of carrier protein) than when it was not. Such dramatic increases in potency are the result of enhanced avidity of the multivalent complex due to cooperativity among ligand-adhesin pairs interacting in tandem. Similar effects by multivalent ligand constructs have been reported in numerous other experimental systems (4, 29, 33, 43, 46, 54). We are studying multivalent molecules by using alternate polymeric supports, which, unlike albumin, will withstand the proteolytic conditions of the stomach.

Bacterial adhesin-inhibitory oligosaccharides provide a nov-

el pharmacologic approach to the management of infectious diseases (57). In view of the growing problem of bacterial resistance to conventional antibiotics, such compounds may afford added therapeutic and prophylactic options to clinicians and their patients.

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ADDENDUM IN PROOF

3'SL administered orally for 28 days to *H. pylori*-infected human subjects caused a significant reduction in the gastric bacterial load, as determined by the [¹³C]urea breath test (D. Zopf, P. M. Simon, M. Hurley, E. McGuire, and S. Roth. Submitted for publication).

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