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NOTES

Hemagglutination Activity of *Campylobacter pylori*

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Forty-five strains of *Campylobacter pylori* isolated from gastric biopsy specimens showed distinct hemagglutination activity. The activity was partially decreased by treatment with heat, trypsin, or an alkylating agent and was inhibited by porcine gastric mucin but not by various compounds, including D-mannose.

Campylobacter pylori, first isolated from human gastric mucosa in 1983 (19), is a microaerophilic S-shaped bacterium with flagella at one end. Evidence which suggests that the organism is an etiologic agent of human gastritis and possibly peptic ulcer is accumulating (2, 3, 6, 9, 10, 15, 18).

In biopsy specimens, *C. pylori* is found in the mucous layer and gastric pits (6, 14, 17) and is associated with gastric epithelial surfaces. Such close proximity to the mucosal surface must be important as the initial step in mucoidal colonization and infection by the bacterium, as is the case for many pathogenic bacteria (1). Ability of bacteria to attach to animal cells is usually assessed by hemagglutination. In this study we determined the hemagglutination activity of *C. pylori* as the first step to elucidate the relationship between this bacterium and pathogenesis of gastritis and peptic ulcer.

Strains of *C. pylori* were isolated from gastric biopsy specimens of patients undergoing upper gastrointestinal endoscopy at Yamaguchi University Hospital. Samples were taken from the site of pyloric glands, fundic glands, and the intermediary zone; homogenized in phosphate-buffered saline; incubated on sheep blood agar (Nissui) and on modified Skirrow medium (Nissui); and incubated at 37°C for 3 to 5 days under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2). Curved gram-negative rods showing typical colony morphology of *C. pylori* were purified and given CPY (*Campylobacter pylori* culture collection of Yamaguchi University) numbers after identification on the basis of positive reactions for oxidase, catalase, urease; weak H_2S production; and negative reactions for nitrate reduction and hippurate hydrolysis. *C. pylori* NCTC 11637 isolated by B. Marshall (Royal Perth Hospital, Perth, Australia) was supplied by H. Inoue (Hyogo College of Medicine, Nishinomiya, Japan). Clinical isolates of *Campylobacter jejuni* and *Campylobacter coli* were provided by H. Tsuneoka (Nagato General Hospital, Nagato, Japan).

Bacteria were grown on triple-extract peptone agar (Eiken Chemical Co., Tokyo, Japan) supplemented with 6% sheep blood at 37°C for 2 to 3 days in a GasPak jar with an anaerobic gas generation kit (BBL Microbiology Systems, Cockeysville, Md.) without a catalyst. A 25-μl sample of bacterial suspensions which was titrated in twofold steps in phosphate-buffered saline was incubated with a 25-μl portion of a 0.25% erythrocyte suspension in 1% gelatin for 30 min at 37°C and stored at 4°C for 16 h. The endpoint was defined as the last dilution showing complete agglutination. Titers were expressed as reciprocals of endpoint dilutions. Titrations were carried out in duplicate, and the results were reproducible within one well.

Hemagglutination activity for human erythrocytes was determined with 45 strains of *C. pylori* and 18 strains of *C. jejuni* suspended in phosphate-buffered saline at a concentration of approximately 5×10^8 cells per ml. All the *C. pylori* strains tested had hemagglutination activity, and the hemagglutination titers were 1, 16, and 128 for the lowest, the average, and the highest values, respectively. On the other hand, only half of the *C. jejuni* strains showed slight hemagglutination, with the highest titer being 8. *C. pylori* NCTC 11637 and CPY0041-1 had hemagglutination titers of 128 and 16, respectively, and the latter was used for further studies. A strain of *C. coli* did not show hemagglutination. In addition to human erythrocytes, erythrocytes of other animals were agglutinated by the bacteria (Table 1).

Suspensions of CPY0041-1 (ca. 2×10^9 cells per ml, 250 μl) were incubated at various temperatures, and the remaining hemagglutination activity was determined (Table 2). The activity for guinea pig erythrocytes was stable at 37°C but inactivated rapidly at 56 and 100°C. The bacteria were also incubated with trypsin (type III; Sigma Chemical Co., St. Louis, Mo.) at 37°C and then incubated further for 10 min with trypsin inhibitor (type II-O; Sigma) (Table 2). The hemagglutination activity decreased rapidly but not com-

TABLE 1. Agglutination of erythrocytes from various animals by *C. pylori*

Source of erythrocytes	Hemagglutination titer
Mouse (fresh)	256
Rabbit (fresh)	32
Guinea pig (stored)	32
Human (fresh)	16
Human (stored)	16
Horse (stored)	8
Sheep (stored)	8

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TABLE 2. Effects of various treatments of *C. pylori* on guinea pig erythrocyte hemagglutination activity

Treatment	Time	Hemagglutination activity (%)
None		100
Heat (56°C)	5 min	25
	50 min	12.5
Heat (100°C)	5 min	12.5
	50 min	12.5
Trypsin (50 μg/ml)	5 min	50
	60 min	12.5
Trypsin (250 μg/ml)	5 min	6.3
	60 min	6.3
Formaldehyde (5%)	22 h	6.3

pletely. Essentially the same results were obtained for the agglutination of sheep erythrocytes.

To determine whether the heat-sensitive factors were also trypsin sensitive, bacteria were first treated with heat and then with trypsin. The hemagglutination titer to guinea pig erythrocytes decreased to 12.5% with incubation at 100°C for 30 min, and the titer decreased further to 6% after incubation with either 50, 250, or 500 μg of trypsin per ml. Alkylation of *C. pylori* with formaldehyde at 37°C also affected the hemagglutination activity (Table 2).

Previous studies have demonstrated that bacteria adhere to the surface of epithelial cells and that erythrocytes can be blocked by the compounds which mimic the receptors concerned (4, 5, 8, 11, 12, 16. 20). To detect specific inhibitors for *C. pylori* hemagglutination, the bacteria were preincubated with various compounds at 37°C for 30 min. None of the following compounds showed inhibitory effects on the agglutination of sheep and guinea pig erythrocytes: 250 mM D-mannose, 100 mM methyl-α-D-mannopyranoside, 100 mM L-fucose, 100 mM *N*-acetyl-D-galactosamine, 100 mM *N*-acetyl-D-glucosamine, 10 mM *p*-nitrophenyl-β-D-galactopyranoside, 1 mM *p*-nitrophenol, 1 mM 4-methylumbelliferone, 100 mM L-arginine, 100 mM L-serine, 5 mM $CaCl_2$, 5 mM EDTA, and 20 mg of bovine serum albumin per ml. In contrast, porcine gastric mucin (Nacalai Tesque Inc., Kyoto, Japan) showed a strong inhibitory effect, while bovine submaxillary mucin (type I-S; Sigma) showed a slight inhibition (Table 3). When the bacteria were heat treated, the residual activity was not affected by gastric or submaxillary mucin. These results suggest that gastric mucin has some role in the process of colonization by *C. pylori* on gastric epithelial-cell surfaces.

Neuraminidase treatment of erythrocytes is reported to abolish hemagglutination of certain strains of uropathogenic

TABLE 3. Inhibition of hemagglutination activity of *C. pylori* by porcine gastric and bovine submaxillary mucin

Mucin (mg/ml)	Hemagglutination activity (%) of:	
	Sheep erythrocytes	Guinea pig erythrocytes
None	100	100
Gastric (0.01)	50	50
Gastric (0.1)	12.5	25
Gastric (1.0)	6.3	ND[a]
Submaxillary (0.1)	100	50
Submaxillary (1.0)	25	50

[a] ND, Not determined because of hemolysis of guinea pig erythrocytes by gastric mucin.

Escherichia coli (12). Incubation of sheep or guinea pig erythrocytes (10%, vol/vol) with *Clostridium perfringens* neuraminidase (5 U/ml; type V; Sigma) or trypsin (10 mg/ml) at 37°C for 60 min did not affect the hemagglutination activity of *C. pylori*.

The structure of the cells of *C. pylori* was examined by transmission electron microscopy after uranyl acetate staining and chromium shadowing. Several sheathed flagella (7) were seen at the end of the organism, but other filamentous structures such as pili or fimbriae were not observed. Therefore, a surface-associated molecule, possibly a protein, appears to be involved in the hemagglutination. Perez-Perez and Blaser showed common proteins in whole-cell and outer membrane preparations of *C. pylori* (13). Analysis of such proteins in relation to hemagglutination activity should be useful for characterization of adhesin molecules of *C. pylori*.

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